ARTICLE
Journal of Cellular Biochemistry 113:911–922 (2012)

Journal of Cellular Biochemistry

Calcineurin Serves in the Circadian Output Pathway to Regulate the Daily Rhythm of L-type Voltage-Gated Calcium Channels in the Retina

Cathy Chia-Yu Huang, Michael L. Ko, Darya I. Vernikovskaya, and Gladys Y.-P. Ko*

Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas 77843-4458

ABSTRACT

The L-type voltage-gated calcium channels (L-VGCCs) in avian retinal cone photoreceptors are under circadian control, in which the protein expression of the α1 subunits and the current density are greater at night than during the day. Both Ras-mitogen-activated protein kinase (MAPK) and Ras-phosphatidylionositol 3 kinase-protein kinase B (PI3K-AKT) signaling pathways are part of the circadian output that regulate the L-VGCC rhythm, while cAMP-dependent signaling is further upstream of Ras to regulate the circadian outputs in photoreceptors. However, there are missing links between cAMP-dependent signaling and Ras in the circadian output regulation of L-VGCCs. In this study, we report that calcineurin, a Ca^{2+}/calmodulin-dependent serine (ser)/threonine (thr) phosphatase, participates in the circadian output pathway to regulate L-VGCCs through modulating both Ras-MAPK and Ras-PI3K-AKT signaling. The activity of calcineurin, but not its protein expression, was under circadian regulation. Application of a calcineurin inhibitor, FK-506 or cyclosporine A, reduced the L-VGCC current density at night with a corresponding decrease in L-VGCCα1D protein expression, but the circadian rhythm of L-VGCCα1D mRNA levels were not affected. Inhibition of calcineurin further reduced the phosphorylation of ERK and AKT (at thr 308) and inhibited the activation of Ras, but inhibitors of MAPK or PI3K signaling did not affect the circadian rhythm of calcineurin activity. However, inhibition of adenylate cyclase significantly dampened the circadian rhythm of calcineurin activity. These results suggest that calcineurin is upstream of MAPK and PI3K-AKT but downstream of cAMP in the circadian regulation of L-VGCCs. J. Cell. Biochem. 113: 911–922, 2012.

KEY WORDS: CIRCADIAN RHYTHM; CALCINEURIN; SIGNALING; CALCIUM CHANNEL

Circadian oscillators regulate functional and physiological activities in vertebrates [Eastman et al., 1984; Cohen and Albers, 1991; Boden et al., 1996], and those in retinal photoreceptors control the daily oscillation in their physiological and morphological changes [LaVail, 1980; Pierce and Besharse, 1985; Korenbrot and Fernald, 1989; Pierce et al., 1993; Adly et al., 1999; Burnside, 2001; Ko et al., 2001; Haque et al., 2002; Ko et al., 2007]. In photoreceptors, the continuous release of neurotransmitters is an L-type voltage-gated calcium channel (L-VGCC) dependent process [Barnes and Kelly, 2002], and cone L-VGCCs are under circadian control [Ko et al., 2007]. The circadian regulation of L-VGCCs is mediated through two parallel signaling pathways, Ras-mitogen-activated protein kinase (MAPK) and Ras-phosphatidylionositol 3 kinase-protein kinase B (PI3K-AKT), and both are downstream of cAMP signaling [Ko et al., 2007, 2009].

Calcineurin, also known as protein phosphatase 2B (PP2B), is a Ca^{2+}/calmodulin-dependent ser/thr phosphatase, which often dephosphorylates the targets of Ca^{2+}/calmodulin-dependent kinase II [CaMKII; Wang and Kelly, 1996; Ghetti and Heinemann, 2000; Wen et al., 2004; Gerges et al., 2005]. Calcineurin contains a 58–64 kDa calmodulin-binding catalytic subunit and a 19 kDa Ca^{2+}-binding regulatory subunit [Klee et al., 1979, 1988]. In the retina, calcineurin is expressed in various neurons including photoreceptors [Cooper et al., 1985; Nakazawa et al., 2001]. Glutamate induces hyperpolarization of postsynaptic ON bipolar cells by binding to metabotropic glutamate receptors (mGluR6), and this depression of postsynaptic response is mediated through calcineurin [Snellman and Nawy, 2002]. However, little is known about the role of calcineurin in the retina, or whether it participates in the visual process. Therefore, in this study, we set forth to investigate the role of calcineurin in the regulation of retinal photoreceptors.

Both FK-506 and cyclosporine A are widely used as immunosuppressants in post-organ transplantation and autoimmune

Grant sponsor: National Eye Institute of NIH; Grant number: RO1EY017452.

*Correspondence to: Dr. Gladys Y.-P. Ko, PhD, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, 4458 TAMU, College Station, Texas 77843-4458.
E-mail: gko@cvm.tamu.edu

Received 14 September 2011; Accepted 14 October 2011 • DOI 10.1002/jcb.23419 • © 2011 Wiley Periodicals, Inc.
Published online 22 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

diseases, partially through their actions as calcineurin inhibitors [Emmel et al., 1989; Tocci et al., 1989; Shapiro et al., 1991; Fruman et al., 1992]. FK-506 and cyclosporine A inhibit calcineurin through interactions with FK-506 binding protein 12 (FKBP12) and cyclophilin A, respectively [Liu et al., 1991]. Inhibition of calcineurin causes circadian phase shifts in mammals, which indicates that calcineurin is involved in the circadian input pathway to reset or entrain the circadian clock in the suprachiasmatic nucleus [SCN; Ding et al., 1998; Katz et al., 2008]. In addition, other ser/thr phosphatase families, such as PP2A and PP1, are known to directly regulate the circadian clock mechanism in *Drosophila* and *Neurospora* [Sathyanarayanan et al., 2004; Yang et al., 2004; Schafmeier et al., 2005; Fang et al., 2007]. Therefore, it is possible that calcineruin may have circadian phase-dependent actions in vertebrates. Here, we report that calcineurin served as part of the circadian output pathway, downstream from cAMP but upstream of Ras, to regulate photoreceptor L-VGCCs. These results suggest that in addition to phase shifting as previously reported, calcineurin also serves in the circadian output pathway to regulate downstream targets.

MATERIALS AND METHODS

CELL CULTURES AND CIRCADIAN ENTRAINMENT

Fertilized eggs (*Gallus gallus*) were obtained from the Poultry Science Department, Texas A&M University (College Station, TX). Chicken retinas were dissociated at embryonic day 12 (E12) and cultured for 6 days as described previously [Ko et al., 2007, 2009]. Cultures were prepared in the presence of 20 ng/ml ciliary neurotrophic factor (CNTF; R&D Systems, Minneapolis, MN), which yields cultures highly enriched with cone photoreceptors [Adler et al., 1984; Adler and Hatlee, 1989; Belecky-Adams et al., 1996] and 10% heat-inactivated horse serum. Cell culture incubators (maintained at 39°C and 5% CO_2) were equipped with lights and timers, which allowed for the entrainment of retinal circadian oscillators to 12 h:12 h light–dark (LD) cycles in vitro. Zeitgeber time zero (ZT 0) was designated as the time when the lights turned on and ZT 12 was the time when the lights went off. For in ovo entrainment, intact eggs at E10 were exposed to LD 12 h:12 h for 7 days. Retina cells were then dissociated, cultured, kept in constant darkness (DD), and used for biochemical and molecular biological assays on the second day of DD. In some experiments, after in ovo LD entrainment for 6 days, eggs were kept in DD for another day. On the second day of DD, retinas were collected at different circadian time (CT) points throughout a day for biochemical assays [Ko et al., 2007, 2009]. The reason for using chick embryos from E12 + 6 for in vitro entrainment or E18 for in ovo entrainment is that more than 90% of the retina photoreceptors express functionally mature VGCC currents by E18 [Gleason et al., 1992].

IMMUNOBLOT ANALYSIS

Samples were collected and prepared as described previously [Ko et al., 2007, 2009]. Briefly, intact retinas were homogenized in Tris lysis buffer including (in mM): 50 Tris,1 EGTA, 150 NaCl, 1% Triton X-100, 1% β-mercaptoethanol, 50 NaF, 1 Na_3VO_4; pH 7.5. Samples were separated on 10% sodium dodecyl sulfate–polyacrylamide gels by electrophoresis and transferred to nitrocellulose membranes. The primary antibodies used in this study were anti-pan calcineurin A (Cell Signaling Technology, Danvers, MA), an antibody specific for di-phospho-ERK (pERK; Sigma, St. Louis, MO), an antibody insensitive to the phosphorylation state of ERK (total ERK, used for loading control; Santa Cruz Biochemicals, Santa Cruz, CA), anti-VGCCα1D subunit (Alomone, Jerusalem, Israel), and anti-Ras (Millipore, Temecula, CA). Blots were visualized using appropriate secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) and an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL). Relative protein expressions for all proteins involved in this study are reported as a ratio to total ERK, since total ERK remains constant throughout the day. Band intensities were quantified by densitometry using Scion Image (NIH, Bethesda, MD). All measurements were repeated at least three times.

CALCINEURIN ACTIVITY ASSAY

Retina samples were lysed in a phosphatase lysis buffer including (in mM): 50 Tris, pH 7.5, 1 EGTA, 150 NaCl, 1%Triton X-100, and 1% β-mercaptoethanol. Calcineurin activities were assayed using a commercially available ser/thr phosphatase assay kit (Promega, Madison, WI). This kit can distinguish between tyrosine (tyr) and ser/thr phosphatases by using a synthetic polypeptide, RRA(pT)VA, that is compatible with ser/thr phosphatases but is structurally incompatible for tyr phosphatases. To differentiate between PP2A, 2B, and 2C, the reaction buffer is made to favor one over the others since this class of enzymes has a diverse range of optimum conditions. For calcineurin (PP2B), the reaction buffer contained 250 mM imidazole (pH 7.2), 1 mM EGTA, 50 mM $MgCl_2$, 5 mM $NiCl_2$, 250 ug/ml calmodulin, and 0.1% β-mercaptoethanol, as described in the manufacturer's protocol. Free cytoplasmic phosphate was first removed from the samples then dephosphorylation of the kit's calcineurin substrate proceeded for 30 min at room temperature (RT). This system determines the amount of free phosphate generated in a reaction by measuring the absorbance (600 nm) of a molybdate/malachite green/phosphate complex.

ELECTROPHYSIOLOGY

Whole cell patch-clamp configuration of L-VGCC current recordings was carried out using mechanically ruptured patches. For retinal photoreceptors, the external solution was (in mM): 110 NaCl, 10 $BaCl_2$, 0.4 $MgCl_2$, 5.3 KCl, 20 TEA-Cl, 10 HEPES, and 5.6 glucose, pH 7.35 with NaOH. The pipette solution was (in mM): 135 Cs acetate, 10 CsCl, 1 NaCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, and 10 HEPES, pH 7.3 adjusted with CsOH. Recordings were made only from cells with elongated cell bodies with one or more prominent oil droplets (hallmark of avian cone photoreceptors). Currents were recorded at RT (23°C) using an Axopatch 200B (Axon Instruments/Molecular Devices, Union City, CA) or A-M 2400 amplifier (A-M Systems Inc., Carlsborg, WA). Signals were low-pass filtered at 2 kHz and digitized at 5 kHz with Digidata 1440A interface and pCLAMP 10.0 software (Molecular Devices). After Gigaohm seals were formed, the electrode capacitance was compensated. Cells were held at −65mV, and ramp voltage commands from −80 to +60 mV in 500 ms were used to evoke Ba^{2+} currents. Current–voltage (I–V) relations were also elicited from a holding potential of −65 mV in

200 ms steps (5 s between steps) to test potentials over a range of −80 to +60 mV in 10 mV increments. The maximal currents were obtained when the steps depolarized to 0 ∼Y+10YmV. The membrane capacitance, series resistance, and input resistance of the recorded photoreceptors were measured by applying a 5 mV (100 ms) depolarizing voltage step from a holding potential of −65 mV. Cells with an input resistance smaller than 1 GΩ were discarded. The membrane capacitance reading was used as the value for whole cell capacitance. The current densities (pA/pF) were obtained by dividing current amplitudes by membrane capacitances. FK-506 and cyclosporine A were obtained from A.G. Scientific (San Diego, CA). The concentrations of FK-506 [Wilson et al., 2001; Okazawa et al., 2009; Mukherjee et al., 2010] and cyclosporine A [McDonald et al., 1996; Bambrick et al., 2006; Chen et al., 2009; Rana et al., 2009; Tan et al., 2011] used in this report were based on previous studies using these inhibitors in various neuronal tissue or cell preparations.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION (RT) POLYMERASE CHAIN REACTION (Q-PCR)

The method used for Q-PCR analysis was described previously [Ko et al., 2004, 2007]. Total RNA was isolated using a commercially available kit (Qiagen, Valencia, CA). Three hundred nanogram of total RNA was used to quantify *VGCCα1D* and *β-actin* (loading control) mRNA by Q-PCR using the Taqman one-step RT-PCR kit and an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). All primers and probes were purchased from Applied Biosystems and sequences were listed previously [Ko et al., 2004, 2007]. All measurements were repeated six times.

CYCLIC AMP ASSAY

The amount of cAMP in retina samples was determined by a commercially available immunoassay kit (Arbor Assays, Ann Arbor, MI). Whole retina (for time point analysis) or cultured retina cells (for FK-506 treatment) were lysed with a small portion being saved for protein concentration determination (Bradford method; Bio-Rad, Hercules, CA). Samples were incubated at RT for 30 min in the microplate wells provided. Reactions were then stopped, and the optical density of each well was determined at 450 nm. Cyclic AMP amount was calculated by comparing sample absorbance readings to a standard curve; n = 4–5.

RAS ACTIVATION ASSAY

Ras activity was determined by a commercially available kit (Millipore), and the procedure was outlined previously [Ko et al., 2004]. The procedure takes advantage of the fact that only activated Ras binds to the Ras binding domain of Raf-1 (Raf-1 RBD). The Raf-1 RBD is a GST (glutathione S-transferase) fusion protein bound to glutathione agarose. Cultured retina cells (control and FK-506 treated) were lysed in a Mg^{2+} lysis buffer. A small portion (20 μl) of the supernatant was saved for total ERK (loading control) analysis by western blotting. The remaining supernatant was incubated with Raf-1 RBD agarose for 45 min at 4°C. Subsequently, the agarose beads were pelleted, washed, and boiled in 2× Lamelli buffer (20 μl). Samples were then subjected to western immunoanalysis as described above; n = 4.

STATISTICAL ANALYSIS

All data are presented as mean ± SEM (standard error of mean). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test for unbalanced n was used for statistical analyses. Throughout, $^{*}P < 0.05$ was regarded as significant. Any defined rhythmic expression had to exhibit at least a 1.5-fold change in rhythmic amplitude [Karaganis et al., 2008].

RESULTS

CALCINEURIN ACTIVITY, BUT NOT ITS PROTEIN EXPRESSION, IS UNDER CIRCADIAN CONTROL

Calcineurin is known to be involved in circadian phase shifting in mammals [Katz et al., 2008], but it is not clear whether calcineurin itself is expressed in circadian oscillations. We first examined whether the protein expression or the activity of calcineurin was under circadian control. Chick embryos were entrained to LD cycles for 7 days in ovo then kept in DD. On the second day of DD, retinas were collected at six different CT points for immunoblotting or calcineurin activity assay. Previously, we showed that the total amount of ERK protein is constant throughout the day, while the phosphorylation status of ERK (phosphorylated ERK) is under circadian control [Ko et al., 2001, 2007]. Therefore, in this study, we used total ERK as the loading control. We found that calcineurin protein expression was constant throughout the day (Fig. 1A), but its activity was under circadian control with peak activity during the middle of the subjective night (CT16) with a threefold difference between apex and trough values (Fig. 1B). Hence, there was a circadian regulation of calcineurin activity at the post-translational level in the chick retina.

THERE IS A CIRCADIAN PHASE-DEPENDENT MODULATION OF L-VGCCs BY CALCINEURIN

We previously found that L-VGCCs are under circadian control in cone photoreceptors [Ko et al., 2007, 2009], with maximal current density elicited at 0 mV significantly larger when cells are recorded during the subjective night than during the subjective day [Fig. 2; Ko et al., 2007, 2009]. The underlying mechanism of the L-VGCC circadian rhythm is in part attributed to the circadian regulation of both mRNA and protein expression of the L-VGCCα1 subunit [Ko et al., 2007], as well as α1 subunit trafficking and insertion/retention into the plasma membrane [Ko et al., 2007, 2009; Shi et al., 2009]. Various signaling pathways are involved in the circadian regulation of L-VGCCs, including CaMKII, Ras-MAPK, and Ras-PI3K-AKT [Ko et al., 2007, 2009]. Since CaMKII is involved in the circadian regulation of L-VGCCs, we hypothesized that calcineurin, a Ca^{2+}-calmodulin dependent phosphatase that often dephosphorylates the same targets of CaMKII, might also participate in the circadian regulation of L-VGCCs.

We examined whether inhibitors of calcineurin might have a circadian phase-dependent effect on L-VGCC currents. Whole-cell patch recordings were performed from cultured cone photoreceptors at either ZT (or CT) 4–8 or 16–20. We observed that calcineurin inhibitors evoked a circadian phase-dependent modulation of L-VGCCs. Application of the calcineurin inhibitor FK- 506 (10 μM)

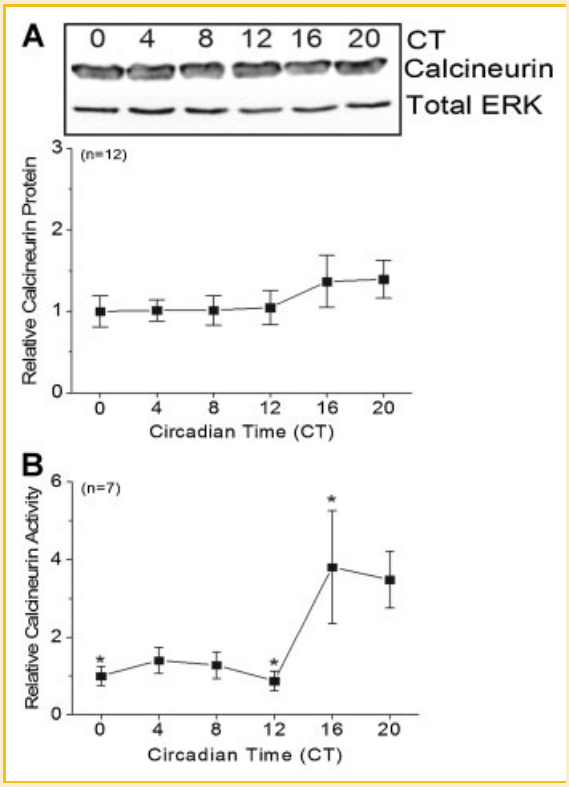

Fig. 1. Calcineurin activity was under circadian control. On the second day of DD after entrainment to 12 h LD cycles for 7 days in ovo, intact retinas were collected at six different circadian time points (CT 0, 4, 8, 12, 16, and 20) for the following assays. A: There was no significant change in the protein expression of calcineurin across six circadian times by western immunoblotting; n = 12 for each time point. B: There was a circadian regulation of calcineurin activity. * indicates that the calcineurin activity is significantly higher at CT 16 compared to CT 0 and CT 12; n = 7 for each time point. *$P < 0.05$.

for 2 h prior to recordings decreased L-VGCC current density when cells were recorded at night (ZT 16–20), but did not affect L-VGCC recordings during the day (ZT 4–8; Fig. 2B,C). Similarly, application of a structurally unrelated calcineurin inhibitor, cyclosporine A (2 μM), for 2 h prior to recordings also caused a significant decrease in photoreceptor L-VGCC current densities when recorded during the subjective night (CT16–20; Fig. 2D). Hence, treatment with a calcineurin inhibitor decreased L-VGCC currents at night under both LD (ZT 16–20) and DD (constant darkness, CT 16–20) conditions.

Since calcineurin is known to cause circadian phase shifting in mammals [Katz et al., 2008], it is possible that the circadian phase-dependent regulation of L-VGCCs by calcineurin could be due to phase-shifting, in which L-VGCCα1 subunit mRNA would be affected after calcineurin inhibitor treatments. However, we found that while FK-506 dampened the circadian rhythm of L-VGCCα1D protein expression (Fig. 3A), it had no effect on the circadian rhythm of L-VGCCα1D mRNA levels (Fig. 3B). Hence, the circadian phase-dependent action of calcineurin was not as a circadian input to shift the circadian phase of L-VGCCs, since affecting the circadian input pathway or the molecular clock itself would alter the circadian rhythm/phase of L-VGCCα1D mRNA levels. Thus, we set forth to examine the role of calcineurin as part of the circadian output to regulate L-VGCCs.

CALCINEURIN IS UPSTREAM OF MAPK AND PI3K-AKT SIGNALING IN THE CIRCADIAN OUTPUT REGULATION OF L-VGCCs

Both MAPK and PI3K-AKT signaling pathways are known to regulate ion channel trafficking and insertion into the plasma membrane [Lhuillier and Dryer, 2000, 2002; Le Blanc et al., 2004; Keifer et al., 2007]. We showed that both MAPK and PI3K-AKT signaling serve as parallel circadian outputs to regulate L-VGCC trafficking and membrane insertion in photoreceptors [Ko et al., 2007, 2009]. Since the circadian phase-dependent action of calcineurin on L-VGCCs was through post-translational modulation, we next examined whether calcineurin interacted with MAPK and/or PI3K-AKT signaling pathways. Chick embryos were entrained in LD cycles, and on the last day of LD, retinal cells were cultured and kept in DD. On the second of DD, cells were treated with the calcineurin inhibitor FK-506 (10 uM) or cyclosporine A (2 uM) for 2 h prior to harvest at CT 4 and CT 16 for western blotting or PP2B activity assay. As shown previously [Ko et al., 2007, 2009] both phosphorylated ERK (pERK) and pAKT at thr308 ($pAKT_{thr308}$) are under circadian control and significantly higher during the subjective night than during the subjective day (pERK rhythm, Fig. 4A,C; $pAKT_{thr308}$ rhythm, Fig. 4B,D). Treatment with FK-506 or cyclosporine A dampened the circadian rhythm of pERK (Fig. 4A,C) and $pAKT_{thr308}$ (Fig. 4B,D). However, inhibition of MAPK signaling using a MEK1 inhibitor PD98059 (50 μM) or the PI3K-AKT pathway with a PI3K inhibitor LY294002 (50 μM) did not alter the circadian rhythm of calcineurin activity (Fig. 5A,B). Hence, calcineurin was upstream of both MAPK and PI3K-AKT signaling as part of the circadian output pathway to regulate L-VGCCs.

CALCINEURIN IS DOWNSTREAM OF cAMP BUT UPSTREAM OF RAS IN THE CIRCADIAN OUTPUT REGULATION OF L-VGCCs

In the retina, the activity of Ras is under circadian control with higher activity at night [Ko et al., 2004]. Both MAPK and PI3K-AKT signaling pathways are downstream of Ras, since inhibition of Ras abolishes the circadian rhythm of pERK and pAKT [Ko et al., 2009], while inhibition of cAMP signaling dampens the circadian rhythm of Ras activity [Ko et al., 2004]. Since calcineurin was also upstream of both MAPK and PI3K-AKT signaling, we next investigated the potential interaction among calcineurin, Ras, and cAMP signaling. Using a commercially available cAMP assay kit, we found that cAMP content in the chick retina was rhythmic with its peak during the subjective night (Fig. 6A). This result was similar to previous reports [Ivanova and Iuvone, 2003; Nikaido and Takahashi, 1998]. Treatment with the calcineurin inhibitor FK-506 did not alter the circadian rhythm of cAMP (Fig. 6B), but the adenylate cyclase inhibitor MDL-12330A (50 μM) significantly dampened the circadian rhythm of calcienurin activity (Fig. 6C). Furthermore, inhibition of calcineurin with FK-506 abolished the circadian rhythm of Ras activity (Fig. 6D). Therefore, calcineurin was downstream of cAMP but upstream of Ras to serve as a circadian output to regulate L-VGCCs.

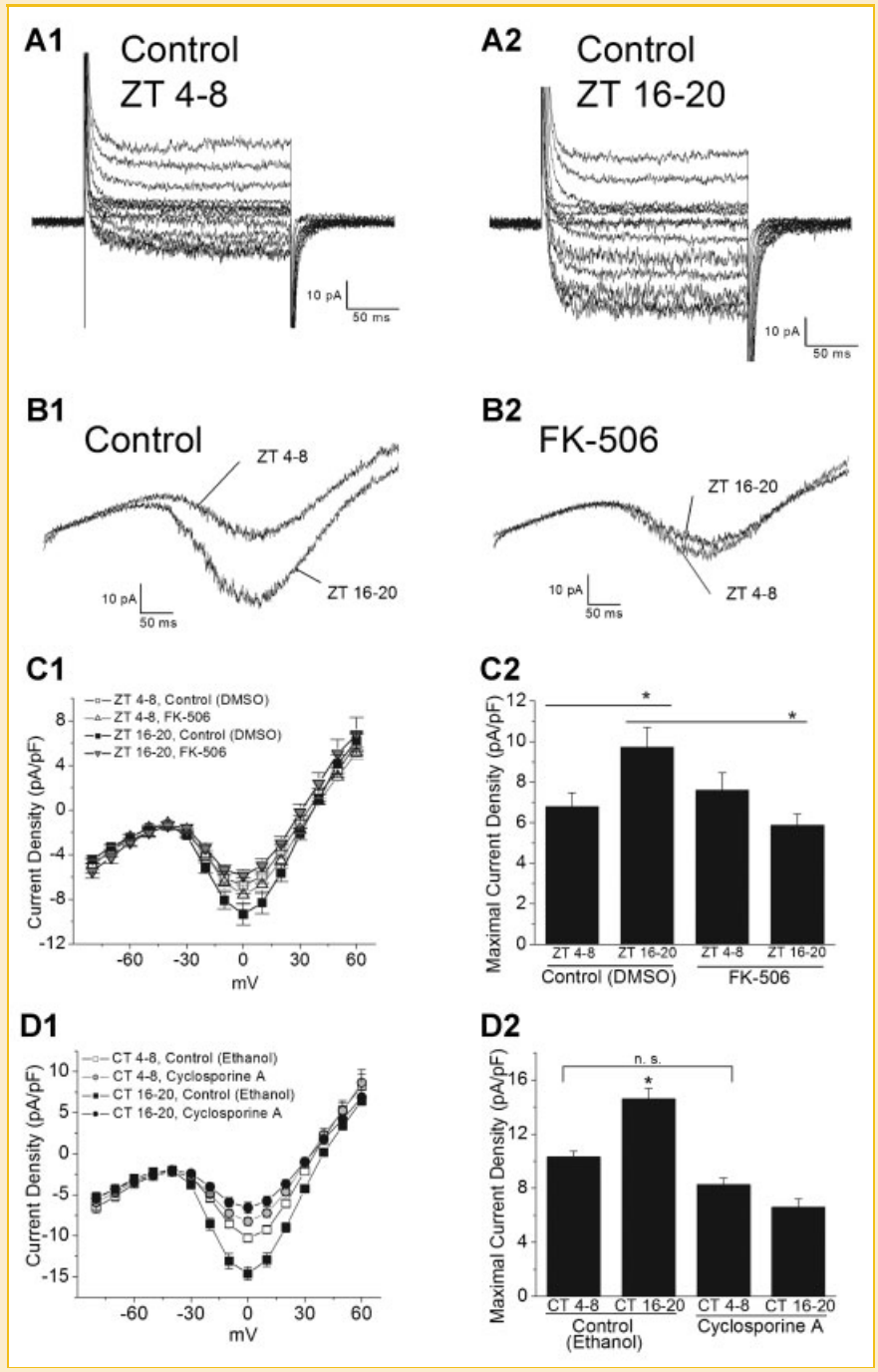

Fig. 2. There was a circadian phase-dependent modulation of L-VGCCs by calcineurin. L-VGCCs were recorded from cultured chick cone photoreceptors on the sixth day of LD entrainment during the day (ZT 4–8) or at night (ZT 16–20). Some cells were recorded on the second day of DD after LD entrainment during the subjective day (CT 4–8) or the subjective night (CT 16–20). Two representative L-VGCC current traces from cells treated with 0.1% DMSO (control) using step commands recorded during the (A1) day (ZT 4–8) and (A2) at night (ZT 16–20). B1: Two representative L-VGCC traces were recorded under a ramp command during the day (ZT 4–8) or at night (ZT 16–20) under the control condition (0.1% DMSO). B2: Two representative traces (ramp command) after treatment with FK-506 (10 μM), a calcineurin inhibitor, for 2 h prior to recordings. C1: The average current-voltage (I-V) relationships in current density (pA/pF) and step-voltage (mV). C2: Maximal current densities were elicited at 0 mV of the step command. Treatment with FK-506 significantly dampened the rhythm of maximal L-VGCC current densities. * indicates that the L-VGCC current density recorded at night (ZT 16–20; n = 12) is significantly higher than those recorded during the day (control, ZT 4–8; n = 11) and FK-506 treated cells recorded at night (FK-506, ZT 16–20; n = 8). There was no statistical difference in the values of maximal L-VGCC current densities recorded from cells treated with FK-506 during the day (ZT 4–8; n = 9) or at night compared to the control recorded during the day (ZT 4–8). (D1 and D2) A similar effect was produced by treatment with another calcineurin inhibitor cyclosporine A (2 μM in 0.1% ethanol) for 2 h prior to recordings. Cells treated with cyclosporine A for 2 h were recorded at CT 4–8 and CT 16–20 on the second day of DD. There was no difference in the circadian rhythm of L-VGCC current densities in control groups treated with 0.1% ethanol for 2 h. Control (ethanol), CT 4–8 and CT 16–20, n = 9; cyclosporine A, CT 4–8, n = 17; cyclosporine A, CT 16–20, n = 14. $^{*}P < 0.05$.

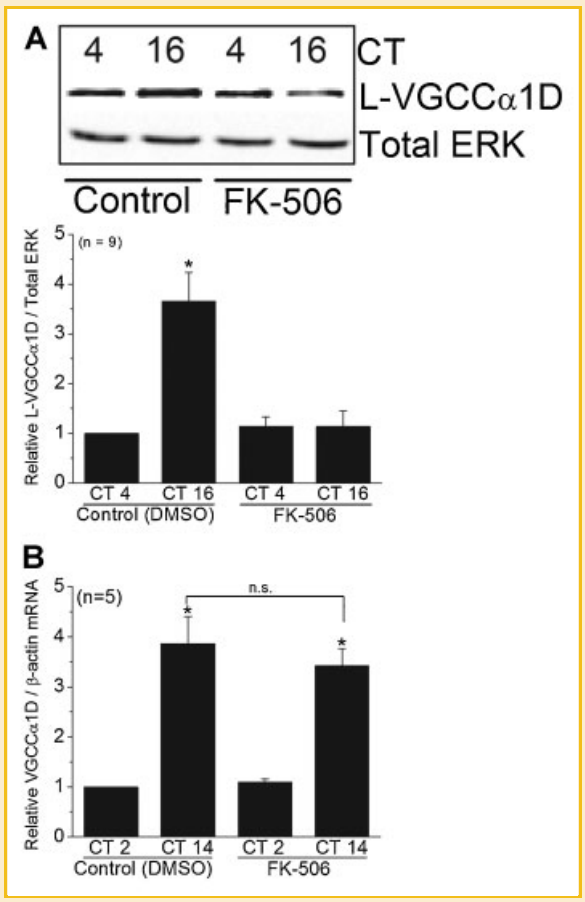

Fig. 3. Inhibition of calcineurin dampens the circadian rhythm of L-VGCCα1D protein expression. On the last day of LD entrainment in ovo, retinae were dissociated, cultured, and kept in DD for one day. On the second day of DD, cultures were treated with 0.1% DMSO (control) or 10 μM FK-506 for 2 h prior to harvest at CT 4 or CT 16 for western immnoblotting (A) or at CT 2 and CT 14 for Q-PCR (B) to measure the protein expression (A) or the mRNA level (B) of the L-VGCCα1D subunit. A: FK-506 dampened the protein expression circadian rhythm of the L-VGCCα1D subunit. The protein level of L-VGCCα1D in control cells harvested at CT 16 was significantly higher than control cells harvested at CT 4, as well as cells treated with FK-506. n = 9 for each group. B: Inhibition of calcineurin with FK-506 had no effect on the mRNA level of L-VGCCα1D. Cells harvested from the control and FK-506 treated groups at CT 14 had significantly higher L-VGCCα1D mRNA compared to cells harvested at CT 2 from both the control and FK-506 treated groups; n = 5 for each group. $^{*}P < 0.05$.

DISCUSSION

Calcineurin is involved in diverse biological processes, including regulating nuclear factor of activated T cells (NFAT) transcriptional activation [O'Keefe et al., 1992; Jain et al., 1993] and apoptosis [Yazdanbakhsh et al., 1995; Wang et al., 1999]. In neurons, calcineruin participates in the modulation of synaptic plasticity [Mulkey et al., 1994; Wang and Kelly, 1997; Zhuo et al., 1999], neurotransmitter release [Halpain et al., 1990; Renstrom et al., 1996; Nishi et al., 1997], and gating of ion channels [Chen et al., 1995; Marrion, 1996; Marcaida et al., 1996; Oliveria et al., 2007]. Since the visual system must anticipate large daily changes in ambient illumination, circadian oscillators in the retina provide a mechanism for the visual system to initiate more sustained adaptive changes throughout the course of a day [Cahill and Besharse, 1995; Green and Besharse, 2004]. In this study, we demonstrated that calcineurin was involved in the circadian phase-dependent modulation of L-VGCCs in the retina. We focus on the circadian regulation of the L-VGCCα1D subunit since in the avian retina, α1D is distributed mainly on the cell bodies of photoreceptors, bipolar cells, and ganglion cells, while L-VGCCα1C is abundant in Müller glia cells [Firth et al., 2001; Ko et al., 2007]. In addition, L-VGCCα1D is present in the cell bodies and terminals of rodent photoreceptors, while VGCCα1C is not observed in rat photoreceptors [Xu et al., 2002; Morgans et al., 2005]. Hence, α1D, but not α1C, is the dominant L-VGCCα1 subunit in retinal photoreceptors across several vertebrate species.

We found that calcineurin activity was under circadian control (significantly higher during the subjective night than the subjective day), but its protein expression remained constant throughout the day (Fig. 1). Application of a calcineurin inhibitor, FK-506 or cyclosporine A, for 2 h at night decreased L-VGCC current density in cone photoreceptors (Fig. 2) corresponding with a decrease in the protein expression of the L-VGCCα1D subunit in cultured retinal cells, but the circadian rhythm of L-VGCCα1D mRNA was not affected (Fig. 3). Therefore, the circadian phase-dependent action of calcineurin was not due to circadian phase shifting of L-VGCCs. Instead, calcineurin served in the circadian output pathway to regulate L-VGCCs. We previously demonstrated that the circadian rhythm of L-VGCCs is in part through both Ras-ERK and Ras-PI3K-AKT signaling, both of which are involved in the protein trafficking and membrane insertion of L-VGCCα1 subunits [Ko et al., 2007, 2009]. Here, we showed that calcineurin regulated L-VGCCs in a circadian-phase dependent manner through modulation of ERK and PI3K-AKT signaling, since calcineurin inhibitors dampened the circadian rhythms of phosphorylated ERK and AKT $_{thr308}$ (Fig. 4), while inhibition of either signaling pathway did not alter calcineurin activity (Fig. 5).

In the chick retina, cAMP content is under circadian control as previously shown by others [Nikaido and Takahashi, 1998; Ivanova and Iuvone, 2003; Chaurasia et al., 2006] and in this study (Fig. 6A), and the activity of Ras is also under circadian control and is downstream of cAMP signaling [Ko et al., 2004]. We found that the adenylate cyclase inhibitor MDL-12230A dampened the circadian rhythm of calcineurin activity, but the calcineurin inhibitor FK-506, while having the ability to inhibit the Ras rhythm, had no effect on the circadian rhythm of retinal cAMP content (Fig. 6). Hence, calcineurin acted downstream of cAMP and upstream of Ras to regulate the circadian rhythm of L-VGCCs through regulating ion channel trafficking and membrane insertion (Fig. 7).

Photoreceptors are non-spiking neurons, and its neurotransmitter release is continuous in the dark through voltage-dependent activation of L-VGCCs [Barnes and Kelly, 2002]. The circadian regulation of L-VGCCs has been shown in gold fish retinal bipolar cells [Hull et al., 2006] and avian cone photoreceptors [Ko et al., 2007]. In each case, the L-VGCC current density is greater at night than during the day. The mechanism of this circadian rhythm is in

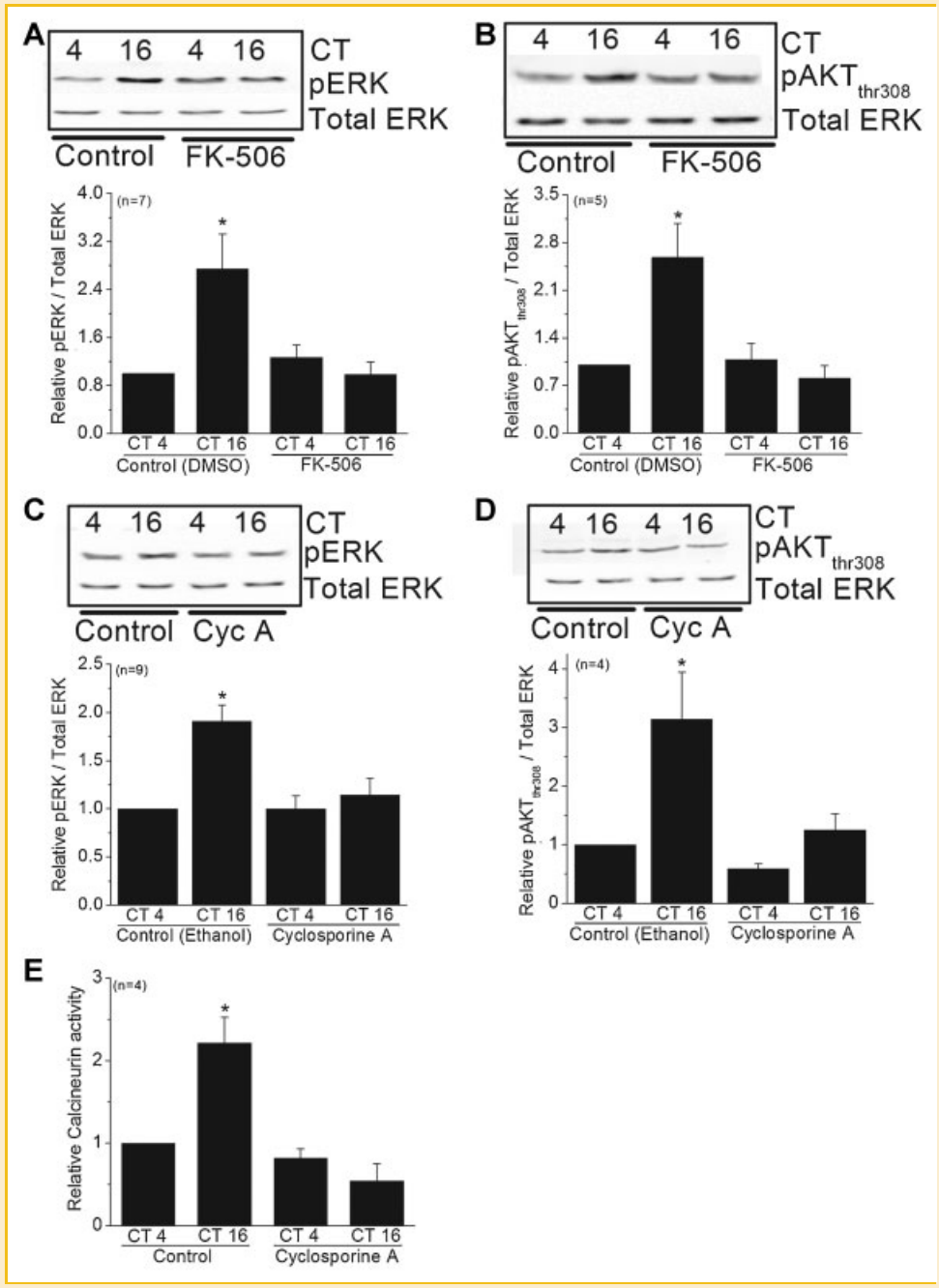

Fig. 4. Inhibition of calcineurin dampens the circadian rhythm of pERK and pAKT. A: ERK phosphorylation (pERK) was higher during the subjective night (CT 16) than the subjective day (CT 4) in control retinal cells (0.1% DMSO). Treatment with FK-506 (10 μM) decreased pERK during the subjective night (CT 16); n = 7 for each group. B: Phosphorylation of AKT at thr308 ($pAKT_{thr308}$) was higher during the subjective night (CT 16) than the subjective day (CT 4) in control cells (0.1% DMSO). Treatment with FK-506 decreased $pAKT_{thr308}$ during the subjective night. n = 5 for each group. C and D: Treatment with cyclosporine A (2 μM) caused similar effects and decreased pERK during the subjective night (C) and diminished $pAKT_{thr308}$ during both subjective day and night (D). C: n = 9 for each group. D: n = 4 for each group. E: Serving as an internal control, treatment with cyclosporine A decreased calcineurin activities during both subjective day and night; n = 4 for each group. * indicates that the levels of pERK, $pAKT_{thr308}$, or calcineurin activity from control cells harvested at CT 16 are significantly higher than other groups. $^{*}P < 0.05$.

part through the circadian regulation of mRNA and protein expression of the L-VGCCα1 subunits [Ko et al., 2007], as well as channel subunit trafficking and insertion/retention into the plasma membrane [Ko et al., 2007, 2009; Shi et al., 2009]. There are two parallel signaling pathways that take part in the circadian regulation of L-VGCCs, Ras-MAPK, and Ras-PI3K-AKT [Ko et al., 2007, 2009], and both pathways are known to regulate ion channel trafficking [Lhuillier and Dryer, 2000, 2002; Le Blanc et al., 2004; Keifer et al., 2007]. Since calcineurin was upstream of Ras, we concluded that the circadian phase-dependent regulation of L-VGCCs by calcineurin was also through the regulation of channel trafficking and insertion of VGCCα1.

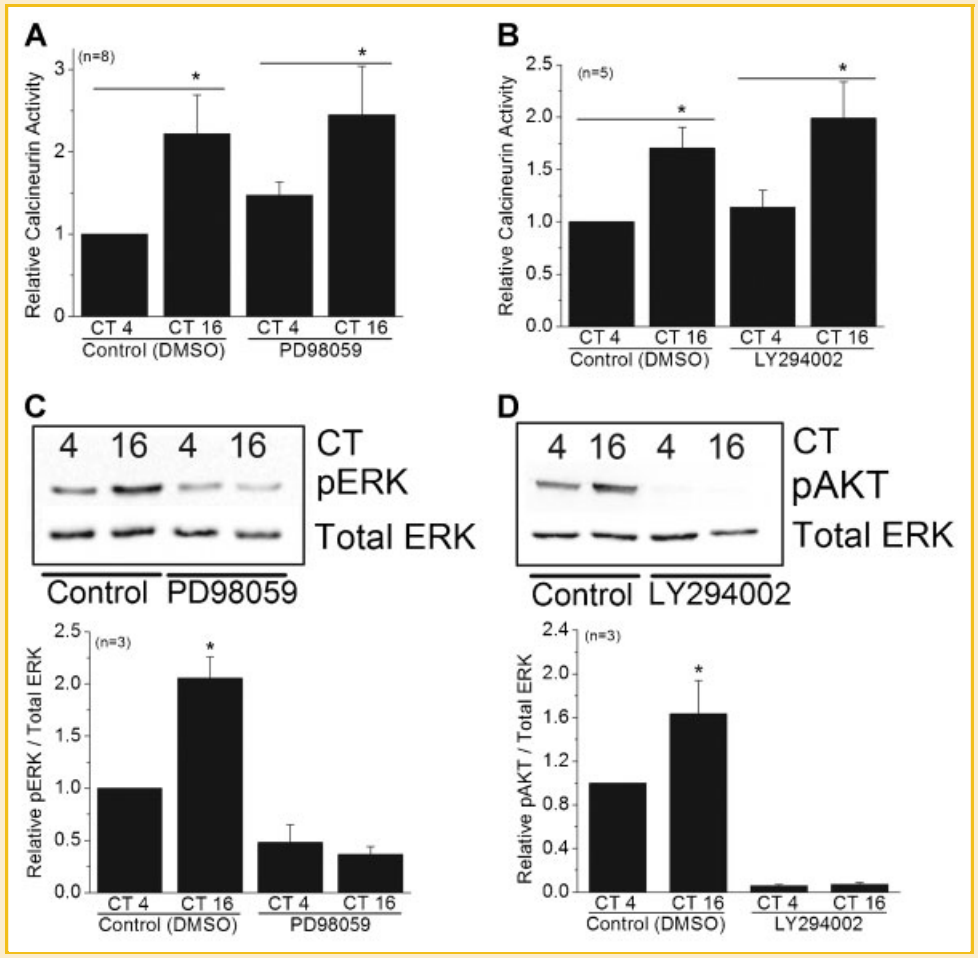

Fig. 5. Inhibition of MAPK or PI3K-AKT signaling does not affect the circadian rhythm of calcineurin activity. A: Calcineurin activity of control cells (0.1% DMSO) was higher during the subjective night (CT 16) than the subjective day (CT 4). Treatment with MEK1 inhibitor PD 98059 (50 μM) had no effect on the circadian rhythm of calcineurin activity; n = 8 for each group. B: Similarly, treatment with PI3K inhibitor LY294002 (50 μM) did not affect the circadian rhythm of calcineurin activity: n = 5 for each group. C and D: As internal controls, treatment with PD98059 inhibits pERK (C), while treatment with LY294002 inhibits pAKT $_{thr308}$ (D); n = 3 for each group. $^{*}P < 0.05$.

Calcineurin is also involved in circadian phase shifting in mammals, since in vivo administration of calcineurin inhibitors blocks circadian responses to light at night, produces circadian phase advances when applied during the subjective day, and disrupts circadian locomotor behavior rhythms when applied chronically in hamsters [Katz et al., 2008]. These effects of calcineurin inhibitors on circadian phase shifting are in part attributed to their interference with intracellular Ca^{2+} storage and release in SCN neurons [Ding et al., 1998]. In addition, calcineurin is an important regulator of casein kinase-I (CKI) and glycogen synthase kinase 3β [GSK3β; Cegielska et al., 1998; Lowrey et al., 2000; Liu et al., 2002; Kim et al., 2009]. CKI and GSK3β are able to regulate the circadian core oscillator by phosphorylating circadian clock proteins [Vielhaber et al., 2000; Eide and Virshup, 2001; Iitaka et al., 2005; Yin et al., 2006]. Therefore, calcineurin may well be involved in the circadian core oscillator mechanism through dephosphorylation of CKI and GSK3β in the mammalian SCN. Since we did not observe any changes in the circadian rhythm of L-VGCCα1D mRNA levels after inhibition of calcineurin, we concluded that calcineurin is part of the circadian output pathway to regulate L-VGCCs post-translationally. However, we cannot rule out the possibility that calcineurin might also serve in the circadian input pathway of retinal circadian oscillators, which will require further investigation.

In addition, there is a circadian oscillation of calcineurin activity in the mouse heart, with a gradual increase throughout the night when these animals are active and decrease when these animals are at rest [Sachan et al., 2011]. We also observed a circadian rhythm of calcineurin activity in the retina when its protein level remained constant. Hence, it is possible that while calciuneurin participates in the modulation of the circadian core oscillator, calcineurin activity is also subject to circadian control. Other examples, such as MAPK, CaMKII, and L-VGCCs, are all under circadian regulation [Sanada et al., 2000; Ko et al., 2001, 2007, 2009; Pennartz et al., 2002; Hull et al., 2006], and yet they all can shift the circadian phase [Obrietan et al., 1998; Butcher et al., 2002; Nahm et al., 2005]. This phenomenon seems to reinforce the model proposed by Roenneberg and Merrow [1999]: Pathways that lead to entrainment of the core oscillator (the circadian inputs) can themselves be regulated by the oscillator and serve as components of the physiologically relevant

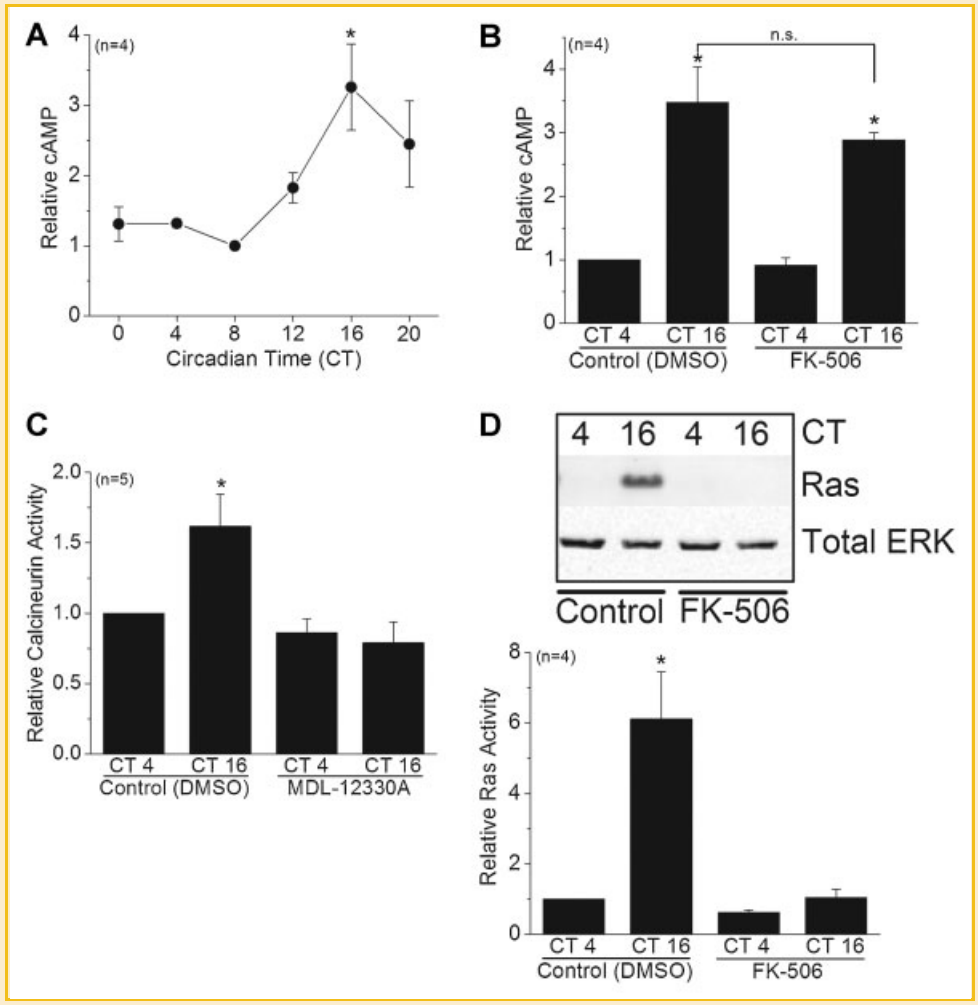

Fig. 6. Calcineurin is downstream of cAMP signaling. A: The cAMP levels in the retina are under circadian control. The cAMP levels were measured in intact retinas taken on the second day of DD after several days of in ovo LD entrainment. The cAMP level peaked during the middle of the subjective night; n = 4 for each circadian time point. * indicates that CT 16 was significantly different from CT 4 and 8. B: Treatment with FK-506 did not affect the circadian rhythm of cAMP levels in cultured retina cells. * indicates that the cAMP levels of the control cells (0.1% DMSO) as well as FK-506 treated cells at CT 16 were significantly higher than the cells harvested at CT 4 from both treatments. There was no statistical difference (n.s.) between control cells and FK-506 treated cells at CT 16; n = 4 for each group. C: Treatment with an adenylate cyclase inhibitor MDL-12330A (50 μM) significantly dampened the circadian rhythm of calcineurin activity. * indicates that the calcineurin activity of the control cells (0.1% DMSO) was significantly higher at CT 16 than all other groups; n = 5 for each group. $^{*}P < 0.05$. D: Treatment with FK-506 significantly dampened the circadian rhythm of Ras activity. * indicates that Ras activity of the control cells (0.1% DMSO) was significantly higher at CT 16 than all other groups; n = 4 for each group. $^{*}P < 0.05$.

circadian output pathways. Hence, these additional feedback loops (the output components feeding back to the inputs) can markedly enhance the stability of the overall oscillator system at the cellular level [Roenneberg and Merrow, 1999].

Calcineurin is also an important regulator of L-VGCCs in various cell types. However, the effect of calcineurin on L-VGCCs varies, as both inhibition [Chad and Eckert, 1986; Armstrong, 1989; Victor et al., 1997; Schuhmann et al., 1997] and enhancement [Norris et al., 2002; Tandan et al., 2009] have been observed. The L-VGCCα1C (Cav1.2) subunit can form macromolecular signaling complexes that comprise the β-adrenergic receptor, G(s) protein, adenylate cyclase, protein kinase A (PKA), as well as PP2A and calcineurin in the heart and brain [Xu et al., 2010]. Calcineurin is capable of binding the C-terminus of the cardiac L-VGCCα1C [Xu et al., 2010]. In the present study, we found that inhibition of calcineurin decreased L-VGCC currents in cone photoreceptors only at night (Fig. 2), and this inhibition was due to decreased L-VGCCα1D protein expression (Fig. 3A). Therefore, we rule out direct dephosphorylation as the circadian phase-dependent action of calcineurin on L-VGCCs in cone photoreceptors. Even though we demonstrated that calcineurin was downstream of cAMP signaling and upstream of Ras, it is still not known which molecule is the direct target of calcineurin. Missing links remain between cAMP signaling and calcineurin, as well as between calcineurin and Ras, and the complexity of the signaling network in the circadian regulation of L-VGCCs are not completely understood (Fig. 7). Thus far, we have shown that at the post-translational level, the trafficking and membrane insertion/retention of L-VGCCs are under circadian control [Ko et al., 2007, 2009], in which calcineurin was part of the output pathway as shown in this study. However, we do not know whether the internalization

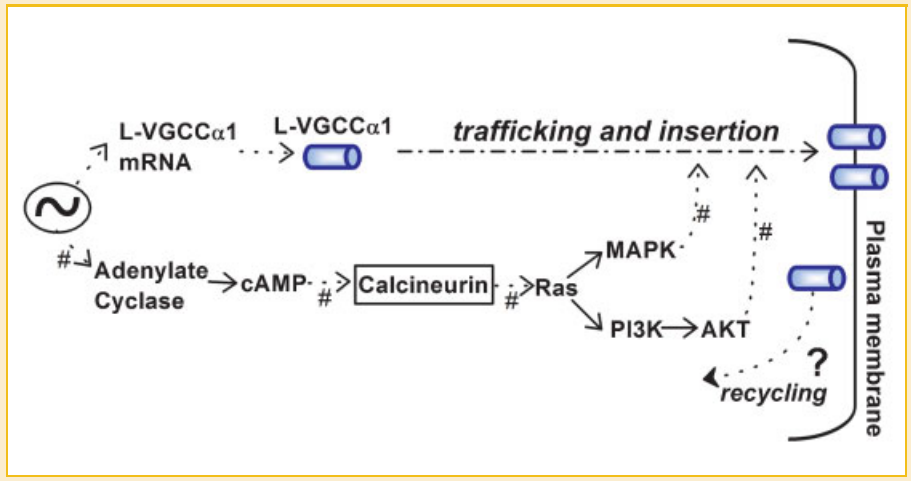

Fig. 7. A schematic model of the circadian output regulation of L-VGCCs. The calcineurin activity rhythm is driven by the circadian oscillator in photoreceptors. This calcineurin activity rhythm is downstream of the rhythmic oscillation of cAMP, while it is upstream of both MAPK and PI3K–AKT signaling that lead to the circadian regulation of L-VGCCα1 subunit trafficking and membrane insertion. There are still numerous unknown steps (marked by #) that intervene between the various signaling components. Whether the sequestering and recycling of the L-VGCC subunits are under circadian control will need further investigation (marked by ?). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

and recycling of the channel subunits are also under circadian control. The circadian rhythm of L-VGCCs could be the focal point between channel protein expression, insertion/retention into the plasma membrane, and sequestering/recycling of the channel subunits. In conclusion, our present study provides new insight on the mechanism underlying the circadian regulation of L-VGCCs in chick cone photoreceptors.

ACKNOWLEDGMENTS

The authors thank fruitful comments and discussion from Drs. Kirill Grushin and Liheng Shi. This project is supported by NIHRO1 EY017452 to G. K.

REFERENCES

Adler R, Hatlee M. 1989. Plasticity and differentiation of embryonic retinal cells after terminal mitosis. Science 243(4889):391–393.

Adler R, Lindsey JD, Elsner CL. 1984. Expression of cone-like properties by chick embryo neural retina cells in glial-free monolayer cultures. J Cell Biol 99(3):1173–1178.

Adly MA, Spiwoks-Becker I, Vollrath L. 1999. Ultrastructural changes of photoreceptor synaptic ribbons in relation to time of day and illumination. Invest Ophthalmol Vis Sci 40(10):2165–2172.

Armstrong DL. 1989. Calcium channel regulation by calcineurin, a Ca2+-activated phosphatase in mammalian brain. Trends Neurosci 12(3):117–122.

Bambrick LL, Chandrasekaran K, Mehrabian Z, Wright C, Krueger BK, Fiskum G. 2006. Cyclosporin A increases mitochondrial calcium uptake capacity in cortical astrocytes but not cerebellar granule neurons. J Bioenerg Biomembr 38(1):43–47.

Barnes S, Kelly ME. 2002. Calcium channels at the photoreceptor synapse. Adv Exp Med Biol 514:465–476.

Belecky-Adams T, Cook B, Adler R. 1996. Correlations between terminal mitosis and differentiated fate of retinal precursor cells in vivo and in vitro: analysis with the "window-labeling" technique. Dev Biol 178(2):304–315.

Boden G, Ruiz J, Urbain JL, Chen X. 1996. Evidence for a circadian rhythm of insulin secretion. Am J Physiol 271(2 Pt 1):E246–252.

Burnside B. 2001. Light and circadian regulation of retinomotor movement. Prog Brain Res 131:477–485.

Butcher GQ, Doner J, Dziema H, Collamore M, Burgoon PW, Obrietan K. 2002. The p42/44 mitogen-activated protein kinase pathway couples photic input to circadian clock entrainment. J Biol Chem 277(33):29519–29525.

Cahill GM, Besharse JC. 1995. Circadian rhythmicity in vertebrate retinas - Regulation by a photoreceptor oscillator. Prog Ret Eye Res 14(1):267–291.

Cegielska A, Gietzen KF, Rivers A, Virshup DM. 1998. Autoinhibition of casein kinase I epsilon (CKI epsilon) is relieved by protein phosphatases and limited proteolysis. J Biol Chem 273(3):1357–1364.

Chad JE, Eckert R. 1986. An enzymatic mechanism for calcium current inactivation in dialysed Helix neurones. J Physiol 378:31–51.

Chaurasia SS, Haque R, Pozdeyev N, Jackson CR, Iuvone PM. 2006. Temporal coupling of cyclic AMP and Ca/calmodulin-stimulated adenylyl cyclase to the circadian clock in chick retinal photoreceptor cells. J Neurochem 99(4):1142–1150.

Chen PC, Lao CL, Chen JC. 2009. The D(3) dopamine receptor inhibits dopamine release in PC-12/hD3cells by autoreceptor signaling via PP-2B, CK1, and Cdk-5. J Neurochem 110(4):1180–1190.

Chen TC, Law B, Kondratyuk T, Rossie S. 1995. Identification of soluble protein phosphatases that dephosphorylate voltage-sensitive sodium channels in rat brain. J Biol Chem 270(13):7750–7756.

Cohen RA, Albers HE. 1991. Disruption of human circadian and cognitive regulation following a discrete hypothalamic lesion: A case study. Neurology 41(5):726–729.

Cooper NG, McLaughlin BJ, Tallant EA, Cheung WY. 1985. Calmodulin-dependent protein phosphatase: immunocytochemical localization in chick retina. J Cell Biol 101(4):1212–1218.

Ding JM, Buchanan GF, Tischkau SA, Chen D, Kuriashkina L, Faiman LE, Alster JM, McPherson PS, Campbell KP, Gillette MU. 1998. A neuronal ryanodine receptor mediates light-induced phase delays of the circadian clock. Nature 394(6691):381–384.

Eastman CI, Mistlberger RE, Rechtschaffen A. 1984. Suprachiasmatic nuclei lesions eliminate circadian temperature and sleep rhythms in the rat. Physiol Behav 32(3):357–368.

Eide EJ, Virshup DM. 2001. Casein kinase I: Another cog in the circadian clockworks. Chronobiol Int 18(3):389–398.

Emmel EA, Verweij CL, Durand DB, Higgins KM, Lacy E, Crabtree GR. 1989. Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. Science 246(4937):1617–1620.

Fang Y, Sathyanarayanan S, Sehgal A. 2007. Post-translational regulation of the Drosophila circadian clock requires protein phosphatase 1 (PP1). Genes Dev 21(12):1506–1518.

Firth SI, Morgan IG, Boelen MK, Morgans CW. 2001. Localization of voltage-sensitive L-type calcium channels in the chicken retina. Clin Experiment Ophthalmol 29(3):183–187.

Fruman DA, Klee CB, Bierer BE, Burakoff SJ. 1992. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proc Natl Acad Sci USA 89(9):3686–3690.

Gerges NZ, Alzoubi KH, Alkadhi KA. 2005. Role of phosphorylated CaMKII and calcineurin in the differential effect of hypothyroidism on LTP of CA1 and dentate gyrus. Hippocampus 15(4):480–490.

Ghetti A, Heinemann SF. 2000. NMDA-Dependent modulation of hippocampal kainate receptors by calcineurin and Ca(2+)/calmodulin-dependent protein kinase. J Neurosci 20(8):2766–2773.

Gleason E, Mobbs P, Nuccitelli R, Wilson M. 1992. Development of functional calcium channels in cultured avian photoreceptors. Vis Neurosci 8(4):315–327.

Green CB, Besharse JC. 2004. Retinal circadian clocks and control of retinal physiology. J Biol Rhythms 19(2):91–102.

Halpain S, Girault JA, Greengard P. 1990. Activation of NMDA receptors induces dephosphorylation of DARPP-32 in rat striatal slices. Nature 343(6256):369–372.

Haque R, Chaurasia SS, Wessel JH III, Iuvone PM. 2002. Dual regulation of cryptochrome 1 mRNA expression in chicken retina by light and circadian oscillators. Neuroreport 13(17):2247–2251.

Hull C, Studholme K, Yazulla S, von Gersdorff H. 2006. Diurnal changes in exocytosis and the number of synaptic ribbons at active zones of an ON-type bipolar cell terminal. J Neurophysiol 96(4):2025–2033.

Iitaka C, Miyazaki K, Akaike T, Ishida N. 2005. A role for glycogen synthase kinase-3beta in the mammalian circadian clock. J Biol Chem 280(33):29397–29402.

Ivanova TN, Iuvone PM. 2003. Circadian rhythm and photic control of cAMP level in chick retinal cell cultures: a mechanism for coupling the circadian oscillator to the melatonin-synthesizing enzyme, arylalkylamine N-acetyltransferase, in photoreceptor cells. Brain Res 991(1–2):96–103.

Jain J, McCaffrey PG, Miner Z, Kerppola TK, Lambert JN, Verdine GL, Curran T, Rao A. 1993. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature 365(6444):352–355.

Karaganis SP, Kumar V, Beremand PD, Bailey MJ, Thomas TL, Cassone VM. 2008. Circadian genomics of the chick pineal gland in vitro. BMC Genomics 9:206.

Katz ME, Simonetta SH, Ralph MR, Golombek DA. 2008. Immunosuppressant calcineurin inhibitors phase shift circadian rhythms and inhibit circadian responses to light. Pharmacol Biochem Behav 90(4):763–768.

Keifer J, Zheng ZQ, Zhu D. 2007. MAPK signaling pathways mediate AMPA receptor trafficking in an in vitro model of classical conditioning. J Neurophysiol 97(3):2067–2074.

Kim Y, Lee YI, Seo M, Kim SY, Lee JE, Youn HD, Kim YS, Juhnn YS. 2009. Calcineurin dephosphorylates glycogen synthase kinase-3 beta at serine-9 in neuroblast-derived cells. J Neurochem 111(2):344–354.

Klee CB, Crouch TH, Krinks MH. 1979. Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc Natl Acad Sci USA 76(12):6270–6273.

Klee CB, Draetta GF, Hubbard MJ. 1988. Calcineurin. Adv Enzymol Relat Areas Mol Biol 61:149–200.

Ko GY, Ko ML, Dryer SE. 2001. Circadian regulation of cGMP-gated cationic channels of chick retinal cones. Erk MAP Kinase and Ca2+/calmodulin-dependent protein kinase II. Neuron 29(1):255–266.

Ko GY, Ko ML, Dryer SE. 2004. Circadian regulation of cGMP-gated channels of vertebrate cone photoreceptors: Role of cAMP and Ras. J Neurosci 24(6):1296–1304.

Ko ML, Jian K, Shi L, Ko GY. 2009. Phosphatidylinositol 3 kinase-Akt signaling serves as a circadian output in the retina. J Neurochem 108(6): 1607–1620.

Ko ML, Liu Y, Dryer SE, Ko GY. 2007. The expression of L-type voltage-gated calcium channels in retinal photoreceptors is under circadian control. J Neurochem 103(2):784–792.

Korenbrot JI, Fernald RD. 1989. Circadian rhythm and light regulate opsin mRNA in rod photoreceptors. Nature 337(6206):454–457.

LaVail MM. 1980. Circadian nature of rod outer segment disc shedding in the rat. Invest Ophthalmol Vis Sci 19(4):407–411.

Le Blanc C, Mironneau C, Barbot C, Henaff M, Bondeva T, Wetzker R, Macrez N. 2004. Regulation of vascular L-type Ca2+ channels by phosphatidylinositol 3,4,5-trisphosphate. Circ Res 95(3):300–307.

Lhuillier L, Dryer SE. 2000. Developmental regulation of neuronal KCa channels by TGFbeta 1: Transcriptional and posttranscriptional effects mediated by Erk MAP kinase. J Neurosci 20(15):5616–5622.

Lhuillier L, Dryer SE. 2002. Developmental regulation of neuronal K(Ca) channels by TGFbeta1: An essential role for PI3 kinase signaling and membrane insertion. J Neurophysiol 88(2):954–964.

Liu F, Virshup DM, Nairn AC, Greengard P. 2002. Mechanism of regulation of casein kinase I activity by group I metabotropic glutamate receptors. J Biol Chem 277(47):45393–45399.

Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL. 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66(4):807–815.

Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M, Takahashi JS. 2000. Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science 288(5465):483–492.

Marcaida G, Kosenko E, Minana MD, Grisolia S, Felipo V. 1996. Glutamate induces a calcineurin-mediated dephosphorylation of Na+,K(+)-ATPase that results in its activation in cerebellar neurons in culture. J Neurochem 66(1):99–104.

Marrion NV. 1996. Calcineurin regulates M channel modal gating in sympathetic neurons. Neuron 16(1):163–173.

McDonald JW, Goldberg MP, Gwag BJ, Chi SI, Choi DW. 1996. Cyclosporine induces neuronal apoptosis and selective oligodendrocyte death in cortical cultures. Ann Neurol 40(5):750–758.

Morgans CW, Bayley PR, Oesch NW, Ren G, Akileswaran L, Taylor WR. 2005. Photoreceptor calcium channels: Insight from night blindness. Vis Neurosci 22(5):561–568.

Mukherjee A, Morales-Scheihing D, Gonzalez-Romero D, Green K, Taglialatela G, Soto C. 2010. Calcineurin inhibition at the clinical phase of prion disease reduces neurodegeneration, improves behavioral alterations and increases animal survival. PLoS Pathog 6(10):e1001138 (1–12).

Mulkey RM, Endo S, Shenolikar S, Malenka RC. 1994. Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature 369(6480):486–488.

Nahm SS, Farnell YZ, Griffith W, Earnest DJ. 2005. Circadian regulation and function of voltage-dependent calcium channels in the suprachiasmatic nucleus. J Neurosci 25(40):9304–9308.

Nakazawa A, Usuda N, Matsui T, Hanai T, Matsushita S, Arai H, Sasaki H, Higuchi S. 2001. Localization of calcineurin in the mature and developing retina. J Histochem Cytochem 49(2):187–195.

Nikaido SS, Takahashi JS. 1998. Day/night differences in the stimulation of adenylate cyclase activity by calcium/calmodulin in chick pineal cell cultures: Evidence for circadian regulation of cyclic AMP. J Biol Rhythms 13(6):479–493.

Nishi A, Snyder GL, Greengard P. 1997. Bidirectional regulation of DARPP-32 phosphorylation by dopamine. J Neurosci 17(21):8147–8155.

Norris CM, Blalock EM, Chen KC, Porter NM, Landfield PW. 2002. Calcineurin enhances L-type Ca(2+) channel activity in hippocampal neurons: Increased effect with age in culture. Neuroscience 110(2):213–225.

O'Keefe SJ, Tamura J, Kincaid RL, Tocci MJ, O'Neill EA. 1992. FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature 357(6380):692–694.

Obrietan K, Impey S, Storm DR. 1998. Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. Nat Neurosci 1(8):693–700.

Okazawa M, Abe H, Katsukawa M, Iijima K, Kiwada T, Nakanishi S. 2009. Role of calcineurin signaling in membrane potential-regulated maturation of cerebellar granule cells. J Neurosci 29(9):2938–2947.

Oliveria SF, Dell'Acqua ML, Sather WA. 2007. AKAP79/150 anchoring of calcineurin controls neuronal L-type Ca2+ channel activity and nuclear signaling. Neuron 55(2):261–275.

Pennartz CM, de Jeu MT, Bos NP, Schaap J, Geurtsen AM. 2002. Diurnal modulation of pacemaker potentials and calcium current in the mammalian circadian clock. Nature 416(6878):286–290.

Pierce ME, Besharse JC. 1985. Circadian regulation of retinomotor movements. I. Interaction of melatonin and dopamine in the control of cone length. J Gen Physiol 86(5):671–689.

Pierce ME, Sheshberadaran H, Zhang Z, Fox LE, Applebury ML, Takahashi JS. 1993. Circadian regulation of iodopsin gene expression in embryonic photoreceptors in retinal cell culture. Neuron 10(4):579–584.

Rana OR, Saygili E, Meyer C, Gemein C, Kruttgen A, Andrzejewski MG, Ludwig A, Schotten U, Schwinger RH, Weber C, Weis J, Mischke K, Rassaf T, Kelm M, Schauerte P. 2009. Regulation of nerve growth factor in the heart: the role of the calcineurin-NFAT pathway. J Mol Cell Cardiol 46(4):568–578.

Renstrom E, Ding WG, Bokvist K, Rorsman P. 1996. Neurotransmitter-induced inhibition of exocytosis in insulin-secreting beta cells by activation of calcineurin. Neuron 17(3):513–522.

Roenneberg T, Merrow M. 1999. Circadian systems and metabolism. J Biol Rhythms 14(6):449–459.

Sachan N, Dey A, Rotter D, Grinsfelder DB, Battiprolu PK, Sikder D, Copeland V, Oh M, Bush E, Shelton JM, Bibb JA, Hill JA, Rothermel BA. 2011. Sustained hemodynamic stress disrupts normal circadian rhythms in calcineurin-dependent signaling and protein phosphorylation in the heart. Circ Res 108(4):437–445.

Sanada K, Hayashi Y, Harada Y, Okano T, Fukada Y. 2000. Role of circadian activation of mitogen-activated protein kinase in chick pineal clock oscillation. J Neurosci 20(3):986–991.

Sathyanarayanan S, Zheng X, Xiao R, Sehgal A. 2004. Posttranslational regulation of Drosophila PERIOD protein by protein phosphatase 2A. Cell 116(4):603–615.

Schafmeier T, Haase A, Kaldi K, Scholz J, Fuchs M, Brunner M. 2005. Transcriptional feedback of Neurospora circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. Cell 122(2):235–246.

Schuhmann K, Romanin C, Baumgartner W, Groschner K. 1997. Intracellular Ca2+ inhibits smooth muscle L-type Ca2+ channels by activation of protein phosphatase type 2B and by direct interaction with the channel. J Gen Physiol 110(5):503–513.

Shapiro R, Jordan M, Fung J, McCauley J, Johnston J, Iwaki Y, Tzakis A, Hakala T, Todo S, Starzl TE. 1991. Kidney transplantation under FK 506 immunosuppression. Transplant Proc 23(1 Pt 2):920–923.

Shi L, Jian K, Ko ML, Trump D, Ko GY. 2009. Retinoschisin, a new binding partner for L-type voltage-gated calcium channels in the retina. J Biol Chem 284(6):3966–3975.

Snellman J, Nawy S. 2002. Regulation of the retinal bipolar cell mGluR6 pathway by calcineurin. J Neurophysiol 88(3):1088–1096.

Tan AR, Cai AY, Deheshi S, Rintoul GL. 2011. Elevated intracellular calcium causes distinct mitochondrial remodelling and calcineurin-dependent fission in astrocytes. Cell Calcium 49(2):108–114.

Tandan S, Wang Y, Wang TT, Jiang N, Hall DD, Hell JW, Luo X, Rothermel BA, Hill JA. 2009. Physical and functional interaction between calcineurin and the cardiac L-type Ca2+ channel. Circ Res 105(1):51–60.

Tocci MJ, Matkovich DA, Collier KA, Kwok P, Dumont F, Lin S, Degudicibus S, Siekierka JJ, Chin J, Hutchinson NI. 1989. The immunosuppressant FK506 selectively inhibits expression of early T cell activation genes. J Immunol 143(2):718–726.

Victor RG, Rusnak F, Sikkink R, Marban E, O'Rourke B. 1997. Mechanism of Ca(2+)-dependent inactivation of L-type Ca2+ channels in GH3cells: Direct evidence against dephosphorylation by calcineurin. J Membr Biol 156(1):53–61.

Vielhaber E, Eide E, Rivers A, Gao ZH, Virshup DM. 2000. Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I epsilon. Mol Cell Biol 20(13):4888–4899.

Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF, Reed JC. 1999. Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science 284(5412):339–343.

Wang JH, Kelly PT. 1996. The balance between postsynaptic Ca(2+)-dependent protein kinase and phosphatase activities controlling synaptic strength. Learn Mem 3(2–3):170–181.

Wang JH, Kelly PT. 1997. Postsynaptic calcineurin activity downregulates synaptic transmission by weakening intracellular Ca2+ signaling mechanisms in hippocampal CA1 neurons. J Neurosci 17(12):4600–4611.

Wen Z, Guirland C, Ming GL, Zheng JQ. 2004. A CaMKII/calcineurin switch controls the direction of Ca(2+)-dependent growth cone guidance. Neuron 43(6):835–846.

Wilson RI, Kunos G, Nicoll RA. 2001. Presynaptic specificity of endocannabinoid signaling in the hippocampus. Neuron 31(3):453–462.

Xu H, Ginsburg KS, Hall DD, Zimmermann M, Stein IS, Zhang M, Tandan S, Hill JA, Horne MC, Bers D, Hell JW. 2010. Targeting of protein phosphatases PP2A and PP2B to the C-terminus of the L-type calcium channel Ca v1.2. Biochemistry 49(48):10298–10307.

Xu HP, Zhao JW, Yang XL. 2002. Expression of voltage-dependent calcium channel subunits in the rat retina. Neurosci Lett 329(3):297–300.

Yang Y, He Q, Cheng P, Wrage P, Yarden O, Liu Y. 2004. Distinct roles for PP1 and PP2A in the Neurospora circadian clock. Genes Dev 18(3): 255–260.

Yazdanbakhsh K, Choi JW, Li Y, Lau LF, Choi Y. 1995. Cyclosporin A blocks apoptosis by inhibiting the DNA binding activity of the transcription factor Nur77. Proc Natl Acad Sci USA 92(2):437–441.

Yin L, Wang J, Klein PS, Lazar MA. 2006. Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock. Science 311(5763):1002–1005.

Zhuo M, Zhang W, Son H, Mansuy I, Sobel RA, Seidman J, Kandel ER. 1999. A selective role of calcineurin aalpha in synaptic depotentiation in hippocampus. Proc Natl Acad Sci USA 96(8):4650–4655.