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Phosphatidylinositol 3 kinase—Akt signaling serves as a circadian output in the retina

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Abstract

The daily rhythm of L-type voltage-gated calcium channels (L-VGCCs) is part of the cellular mechanism underlying the circadian regulation of retina physiology and function. However, it is not completely understood how the circadian clock regulates L-VGCC current amplitudes without affecting channel gating properties. The phosphatidylinositol 3 kinase—protein kinase B (PI3K—Akt) signaling pathway has been implicated in many vital cellular functions especially in trophic factor-induced ion channel trafficking and membrane insertion. Here, we report that PI3K—Akt signaling participates in the circadian phase-dependent modulation of L-VGCCs. We found that there was a circadian regulation of Akt phosphorylation on Thr308 that peaked at night. Inhibition of PI3K or Akt significantly decreased L-VGCC current amplitudes and the expression of membrane-bound L-VGCC α 1D subunit only at night but not during the subjective day. Photoreceptors transfected with a dominant negative Ras had significantly less expression of phosphorylated Akt and L-VGCC α 1D subunit compared with non-transfected photoreceptors. Interestingly, both PI3K—Akt and extracellular signal-related kinase were downstream of Ras, and they appeared to be parallel and equally important pathways to regulate L-VGCC rhythms. Inhibition of either pathway abolished the L-VGCC rhythm indicating that there were multiple mechanisms involved in the circadian regulation of L-VGCC rhythms in retina photoreceptors.

Keywords

circadian; L-type voltage-gated calcium channel; photoreceptor; protein trafficking; signal transduction

The phosphatidylinositol 3 kinase—protein kinase B (PI3K—Akt) signaling pathway regulates a vast array of cellular processes and biological functions (Shaw and Cantley 2006; Yoon *et al.* 2008) from post-translational modulation of proteins, protein and vesicle trafficking, cytoskeletal remodeling, to controlling cell cycles, proliferation, and survival (Blair and Marshall 1997; Lhuillier and Dryer 2002, 2003; Roth *et al.* 2004; Viard *et al.* 2004). The Akt family consists of three major isoforms that are encoded by three separate genes but share a conserved structure that includes an N-terminal pleckstrin homology domain, a kinase domain,

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Supporting information

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

Fig. S1 Cultures without CNTF did not affect circadian rhythmicity of L-VGCCs.

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and a C-terminal regulatory domain containing the hydrophobic motif phosphorylation site (Fayard *et al.* 2005; Manning and Cantley 2007). All three isoforms are expressed in retina photoreceptors (Reiter *et al.* 2003; Li *et al.* 2007). Activation of Akt requires a multi-step process that includes phosphorylation of Thr308 in the kinase domain and Ser473 within the regulatory domain (Fayard *et al.* 2005). Various ion channel activities are augmented following the activation of PI3K—Akt signaling in the presence of extracellular stimulators like insulin (Blair *et al.* 1999), endothelin-1 (Kawanabe *et al.* 2002), norepinephrine (Viard *et al.* 2001), and angiotensin II (Quignard *et al.* 2001). For instance, kidney aquaporin 2 channels (Tajika *et al.* 2004), non-selective cation channels (Kanzaki *et al.* 1999), calcium-dependent potassium channels (Lhuillier and Dryer 2002), and L-type voltage-gated calcium channels (L-VGCCs) (Le Blanc *et al.* 2004) depend on trophic factor-induced PI3K—Akt activation for channel protein trafficking and insertion into the plasma membrane.

Retina photoreceptors are non-spiking neurons, and their continuous release of neurotransmitters is dependent on L-VGCCs (Barnes and Kelly 2002). In humans, a mutation of the VGCC α 1 subunit gene causes incomplete congenital stationary night blindness with a defect in neurotransmission between photoreceptors and second-order neurons (Bech-Hansen *et al.* 1998). We previously showed that the L-VGCCs in chick retina photoreceptors are under circadian control (Ko *et al.* 2007). The protein expression of the L-VGCC α 1 subunit and the maximum current amplitude are greater during the subjective night than during the subjective day. Daily changes in L-VGCC current amplitudes are also observed in fish retina bipolar cells (Hull *et al.* 2006). Therefore, the circadian regulation of L-VGCCs may be a more general phenomenon in the retina that could underlie why several components of electroretinograms recorded from different species, including humans, display circadian rhythms (Manglapus *et al.* 1998; Tuunainen *et al.* 2001; Miranda-Anaya *et al.* 2002). However, the gating properties of L-VGCCs do not change as a function of the time of the day (Hull *et al.* 2006; Ko *et al.* 2007). As L-VGCC α 1D mRNA and protein are rhythmically expressed (Ko *et al.* 2007), it is possible that the circadian regulation of channel trafficking and membrane insertion is part of the mechanism underlying L-VGCC rhythms.

In this study, we show that the PI3K—Akt signaling pathway participates in the circadian phase-dependent modulation of L-VGCCs. Inhibition of PI3K—Akt by applying inhibitors for 2 h in cultured photoreceptors decreased the maximum current amplitude of L-VGCCs during the subjective night but did not alter these currents during the subjective day. Inhibitors of the PI3K—Akt pathway had no effect on the mRNA levels of L-VGCC α 1D subunits, nor did they block the L-VGCCs directly *in vitro*. Cell surface biotinylation assays revealed that PI3K—Akt mainly affected the membrane insertion of VGCC α 1D subunits. To date, no other report has dealt with the circadian rhythmicity of Akt activity. Circadian regulation of Akt activity occurs through phosphorylation of Thr308, but not on Ser473, in intact chick retinæ (*in ovo*). We also found that PI3K—Akt, like extracellular signal-related kinase (Erk), is downstream of Ras but appears to be a separate, yet, equally important pathway to regulate L-VGCCs in cultured photoreceptors. Hence, we provide the first evidence that PI3K—Akt signaling serves as part of the circadian output to regulate L-VGCC trafficking. The Ras—PI3K—Akt and Ras—Erk signaling pathways serve as parallel output pathways to regulate the circadian rhythms of L-VGCCs.

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, USA). Chick retinas were dissociated at embryonic day 12 (E12) and cultured for 5 days in the presence of 20 ng/mL ciliary neurotrophic factor (CNTF; R&D Systems, Minneapolis, MN, USA) and 10% heat-inactivated horse serum as

described previously (Ko *et al.* 2001,2007). Cell culture incubators (maintained at 39°C and 5% CO₂) were equipped with lights and timers, which allowed for the entrainment of retinal circadian oscillators to 12 : 12 h light—dark (LD) cycles *in vitro*. Zeitgeber time 0 was designated as when the lights come on, and Zeitgeber time 12 was the time when the lights turn off. The following experiments were carried out on the second day of constant darkness (dark—dark; DD), after 5 days of prior entrainment to LD cycles without exchange of media as described previously (Ko *et al.* 2001,2007). Cultures without the presence of CNTF did not affect the circadian rhythmicity of L-VGCCs, but photoreceptors appeared fewer in numbers and less healthy compared with the cultures containing CNTF (Fig. S1). For *in ovo* entrainment, retinas were excised from E17 embryos obtained from intact eggs that were exposed to LD 12 : 12 h for 6 days (from E11 to E16). Retina cells were then dissociated, cultured in the dark, and used for biochemical assays on the second day of culture in DD (Ko *et al.* 2007).

Immunoblot analysis and cell-surface biotinylation assays

Samples were collected and prepared as described previously (Ko *et al.* 2001,2007). Briefly, cultured cells were washed in ice-cold phosphate-buffered saline and lysed in radio immunoprecipitation assay buffer. In some experiments, intact retinas were homogenized in radio immunoprecipitation assay buffer. The samples were separated on 10% sodium dodecyl sulfate—polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The primary antibodies used in the studies were anti-VGCC α 1D (Alomone, Jerusalem, Israel), anti-phospho308 Akt (pAktThr308; Cell Signaling Technology, Danvers, MA, USA), anti-phospho473 Akt (pAktSer473; Cell Signaling Technology), a polyclonal antibody insensitive to the phosphorylation state of Akt (total Akt, used for loading control; Cell Signaling Technology), a monoclonal antibody specific for di-phospho-Erk (pErk; Sigma, St Louis, MO, USA), and a polyclonal antibody insensitive to the phosphorylation state of Erk (total Erk, used for internal control and loading control; Santa Cruz Biochemicals, Santa Cruz, CA, USA). Blots were visualized using appropriate secondary antibodies conjugated to horse radish peroxidase (Cell Signaling Technology) and an ECL detection system (Pierce, Rockford, IL, USA). For the cell-surface biotinylation assays, cultured cells were washed and exposed to sulfo-NHS-LC-biotin at 4°C for 30 min using a commercially available cell-surface biotinylation assay kit (Pierce). A portion of the whole-cell lysate was used to measure the VGCC α 1D subunit and total Erk. The rest was used to determine plasma membrane-bound VGCC α 1D subunits by incubating it with streptavidin-linked agarose beads. The beads were collected, and the amount of VGCC α 1D subunit was determined by immunoblotting. The ratio of VGCC α 1D subunit to total Erk, pAktThr308 to total Erk, and pAktSer473 to total Erk in each sample was determined by densitometry using Scion Image (NIH, Bethesda, MD, USA). Previously, we showed that there is a circadian regulation of pErk in chick retinæ (Ko *et al.* 2001,2007). We used the ratio of pErk/total Erk as our internal control of circadian timing (data not shown). All measurements were repeated five to six times. PD 98059 [mitogen-activated kinase kinase (MEK) inhibitor] and manumycin A (small GTPase inhibitor) were from A. G. Scientific (San Diego, CA, USA); LY 294002 (PI3K inhibitor) and 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt inhibitor; Akti) were obtained from Calbiochem/EMD (San Diego, CA, USA).

Quantitative real-time RT-PCR

The method used for quantitative real-time reverse transcription-PCR analysis was described previously (Ko *et al.* 2004,2007). Cone photoreceptors were cultured, LD entrained, and placed in DD as described above. On the second day of DD, cells were treated with an Akti or vehicle for 2 hrs at circadian time (CT) 2 and CT 10. At CT 4 and CT 12, cells were lysed and total RNA was collected using a commercially available kit (Qiagen, Valencia, CA, USA). Three hundred nanograms of total RNA was used to quantify VGCC α 1D and β -actin (loading control) mRNA by quantitative-PCR using the Taqman one-step RT-PCR kit and an ABI Prism 7500

sequence detection system (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers for β -actin and VGCC α 1D were previously listed (Ko *et al.* 2004,2007).

Patch-clamp recordings and statistical analysis

Whole-cell patch-clamp configuration of L-type Ca^{2+} channels (L-VGCCs) was carried out using either β -escin based perforated-patch recording methods (Fan and Palade 1998; Ko *et al.* 2007) or direct whole-cell configuration. For retinal photoreceptors, the external solution was (in mM): 110 NaCl, 10 BaCl₂, 0.4 MgCl₂, 5.3 KCl, 20 TEACl, 10 HEPES, and 5.6 glucose, pH 7.4 with NaOH. The pipette solution was (in mM): 135 Cs acetate, 10 CsCl, 2 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, and 10 HEPES, pH 7.4 adjusted with CsOH (Gleason *et al.* 1992). Beta-escin (Sigma) was prepared as a 25 mM stock solution in water and added to the pipette solution to yield a final concentration of 25 μ M. Recordings were made from cells with elongated cell bodies with one or more prominent oil droplets. Currents were recorded at 24°C using an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Union City, CA, USA). Signals were low-pass filtered at 2 kHz and digitized at 5 kHz with Digidata 1440A interface and pCLAMP 10.0 software (Axon Instruments). Currents were leak subtracted. After G Ω seals formed, the electrode capacitance was compensated. Cells were held at -65 mV, and ramp voltage commands (-80 to +60 mV in 500 ms) were used to evoke Ba²⁺ currents. The Ba²⁺ currents were recorded immediately after the patches were completely perforated (within 3–10 min after G Ω seals formed). Current–voltage relations were elicited from a holding potential of -65 mV using 200 ms steps (5 s between steps) to test potentials over a range of -80 to +60 mV in 10 mV increments. 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 a series resistance > 10% of the input resistance (series resistance > 100 M Ω or input resistance < 1 G Ω) were discarded. The membrane capacitance reading was used as the value for whole-cell capacitance. In our experiments, the average value of membrane capacitance was 2.8 ± 0.1 pF ($n = 58$). The current densities of $I_{\text{Ba}^{2+}}$ (I_{density}) were obtained by dividing the membrane capacitances from current amplitudes. Each group contained 12–17 cells. All of the data were presented as mean $\pm$ SE. One-way ANOVA followed by Tukey's *post hoc* test for unbalanced n was used for statistical analyses. Throughout, $*p < 0.05$ was regarded as significant.

Biolistic transfection and immunocytochemistry

Chick retinas from E12 were dissociated, cultured on coverslips, and entrained under LD cycles for 5 days and then kept in constant darkness (DD) in the presence of 40 ng/mL CNTF and 15% heat-inactivated horse serum (Ko *et al.* 2001,2003,2004,2007). On the first day of DD, cells were co-transfected with plasmids encoding enhanced green fluorescent protein (eGFP; Vitality® hrGFP II-1 Mammalian Expression Vector, #240143; Stratagene, La Jolla, CA, USA) and Ras dominant negative RasS17N (Ras DN17; Upstate Biotechnology, Lake Placid, NY, USA) using a biolistic particle delivery system (Helium Gene Gun; Bio-rad, Hercules, CA, USA). Plasmids were precipitated onto 1 μ M gold microcarriers according to the manufacturer's protocol. The particle delivery system generated a helium shock wave with a pressure gradient of 150 psi to accelerate the coated microcarriers onto cultured cells.

After transfection, cells were cultured under DD for another day, and at CT 17, cells were fixed with Zamboni's fixative in phosphate buffer (PB; 0.1 M, pH 7.4) for 30 min at 24°C followed by three washes in PB containing 0.1% Triton-X, a blocking step with 0.1% Triton-X/PB containing 3% normal goat serum for an hour, and then the cells were incubated with primary antibodies, VGCC α 1D antibody (1 : 250) or pAktThr308 (1 : 250) independently overnight. The cells were washed several times with PB and incubated with fluorescent conjugated secondary antibodies (Alexa 594 nm goat anti-rabbit; Molecular Probes, Carlsbad, CA, USA) in PB containing 1.5% normal goat serum for 2 h in the dark. After several washes in PB, the

coverslips were mounted on a glass slide and stored at 4°C for later observation on a Zeiss (Zeiss, Thornwood, NY, USA) microscope with epi-fluorescence to determine the localization of VGCC α 1D or pAktThr308 in both transfected (with GFP) and non-transfected cells. Green or red fluorescence images were taken under identical settings including exposure time and magnification. The average fluorescence intensities of the outlined structures were analyzed without any modification using the luminosity channel of the histogram function in Adobe Photoshop 6.0 software (San Jose, CA, USA), and the green or red fluorescence intensity (FI) was measured on a scale of 0–255. The background fluorescence intensity was acquired from an adjacent area without any cells (*B*). The relative FI from each group was converted as:

$$FI = [(F1 - B) + (F2 - B) + (F3 - B) + \dots + (Fn - B)] / n,$$

where *n* is the total number of cells. Statistical comparisons were made between transfected and non-transfected photoreceptors using Student's *t*-test, and *p* < 0.05 was regarded as significant. All of the FI analyses were carried out blind.

Results

There is a circadian phase-dependent modulation of L-VGCCs by Akt

Previously, we showed that there is a circadian regulation of L-VGCCs in chick photoreceptors (Ko *et al.* 2007). The mRNA of the VGCC α 1D subunit peaks during the transition from subjective day to subjective night (CT 12), while the protein expression of VGCC α 1D peaks 4 h later (CT 16). The maximal L-VGCC current amplitude is larger during the subjective night than during the subjective day, but channel gating properties do not change throughout the day (Ko *et al.* 2007). On the second day of DD, elongated photoreceptors with oil droplets were recorded using perforated whole-cell recordings with both ramp and step commands. In comparing ramp and step commands (from the same photoreceptor), we found that there was no difference in maximal current amplitudes or channel gating kinetics using either method. The L-VGCC currents were reversibly blocked by 3 mM CoCl₂ as we reported previously (Ko *et al.* 2007); and there was no difference in L-VGCC current amplitudes and gating kinetics between photoreceptors with or without 0.1% dimethylsulfoxide (DMSO) treatment (data not shown). Therefore, we only present the photoreceptors recorded in 0.1% DMSO treatment as our control groups (CT 4–7 and CT 16–19; Fig. 1).

The maximal L-VGCC current amplitude and current density were significantly greater in cells recorded during the subjective night (CT 16–19, maximal current amplitude: 28 ± 4 pA; maximal current density: 9.3 ± 1.7 pA/pF; *n* = 17) than during the subjective day (CT 4–7, maximal current amplitude: 15 ± 3 pA; maximal current density: 5.9 ± 1.1 pA/pF; *n* = 15; Fig. 1a and d). We observed that inhibition of Akt evoked a phase-dependent inhibition of L-VGCCs in chick photoreceptors. In these experiments, an Akti, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (Akti, 10 μ M, in 0.1% DMSO), was applied for 2 h prior to whole-cell recordings from cultured photoreceptors on the second day of DD. Akti had no effect on L-VGCCs in cells recorded during the subjective day (CT 4–7; maximal current amplitude: 13 ± 4 pA; maximal current density: 4.7 ± 1.3 pA/pF; *n* = 12; Fig. 1b and d). However, 2-h exposure to Akti caused a significant (**p* < 0.05) decrease in the maximal L-VGCC current amplitude in cells recorded during the subjective night (CT 16–19; maximal current amplitude: 8 ± 2 pA; maximal current density: 3.8 ± 0.9 pA/pF; *n* = 16; Fig. 1c and d). It is important to note that L-VGCC gating properties were not altered by Akti treatment. Maximum current amplitudes were evoked by identical voltages in control and Akti-treated photoreceptors. Statistical analysis comparing maximum current amplitudes of different groups showed the same pattern and significance as comparing maximum current densities. As a result, we only present the statistical comparisons in current densities (Fig. 1d). Treatment

with 50 μ M LY 294002 (in 0.1% DMSO), a PI3K inhibitor, exhibited a similar result (data not shown). In summary, 2 h of continuous inhibition of PI3K—Akt signaling modulated cone photoreceptor L-VGCCs by lowering the current amplitudes during the subjective night without affecting channel gating kinetics.

As the PI3K—Akt pathway is involved in insulin-like growth factor 1-induced potentiation in the cerebellum through Akt phosphorylation of L-VGCC subunits (Blair *et al.* 1999), we next addressed whether the lower L-VGCC current amplitude caused by Akti during the subjective night was because of inhibition of Akt-mediated phosphorylation of L-VGCCs. In whole-cell voltage-clamp configuration, we found that extracellular perfusion of Akti did not alter the L-VGCC current amplitudes or gating properties during either the subjective day (CT 4–7) or subjective night (CT 16–19; Fig. 2c and d), while superfusion with CoCl_2 , a reversible calcium channel blocker, completely inhibited L-VGCC currents (Fig. 2b). Therefore, Akti did not interfere with the channel pores or phosphorylation sites of L-VGCCs. As we reported previously, the mRNA of L-VGCC α 1D peaks at CT 12, which is significantly more abundant than other CT points, while protein levels reach its maximum at CT 16 (Ko *et al.* 2007). We found that treatment with the Akti for 2 h did not alter L-VGCC α 1D mRNA levels at CT 4 and CT 12 (Fig. 2f). This result revealed that Akt is part of the circadian output pathway to regulate L-VGCC α 1D. As inhibition of Akt caused a circadian phase-dependent decrease of L-VGCC current amplitude without affecting channel gating properties, transcription or protein synthesis, we therefore postulated that the Akti-induced decrease of L-VGCC current amplitudes during the subject night could, at least in part, be attributable to a decrease in channel α subunit trafficking and insertion into the plasma membrane of photoreceptors.

The phosphorylation of Akt on Thr308 is under circadian control

Overall, Akt activity is the result of an equilibrium between its two phosphorylation sites on Thr308 and Ser473 (Beaulieu *et al.* 2007). We performed western blots to investigate the possible circadian regulation of Akt activity of both phosphorylation sites using antibodies against pAktThr308 or pAktSer473. Chick embryos were entrained to LD cycles for 6 days *in ovo* then switched to constant darkness (DD). On the second day of DD, retinæ were harvested at six different CT points for immunoblotting. In chick retinæ, Erk activity (phosphorylated Erk) is under circadian control and is higher at night and lower during the day, while the total amount of Erk protein (both phosphorylated and non-phosphorylated forms) remains constant throughout the day (Ko *et al.* 2001, 2003, 2004, 2007). Here, we chose to use a polyclonal antibody insensitive to the phosphorylation state of Erk (total Erk) in western blots as an internal control as well as loading control. We found that pAktThr308 in chick retinæ were under circadian control with peak activity in the middle of the subjective night (CT 16) with a fivefold difference between the apex and trough values (Fig. 3a). Interestingly, while pAktThr308 was rhythmic, pAktSer473 was not, and total Akt (both phosphorylated and non-phosphorylated), like total Erk, was also constant throughout the day (Fig. 3b). Therefore, there was an apparent differential regulation of circadian rhythmicity between the two phosphorylation sites of Akt. We next further examined the circadian mechanism by which the PI3K—Akt signaling pathway modulated L-VGCCs.

Both PI3K—Akt and Erk signaling contribute to the circadian phase-dependent regulation of L-VGCC α 1 subunit trafficking

To further expound upon our results discussed above, we inhibited each pathway and monitored effects on plasma membrane bound L-VGCCs. Chick embryos were LD entrained *in ovo* and photoreceptors cultured as described above. On the second day of DD, cells were treated with LY 294002 (50 μ M; PI3K inhibitor), Akti (10 μ M), or 0.1% DMSO (control) for 2 h starting at CT 3 or CT 15. At CT 5 or CT 17, cells were washed with ice-cold phosphate-buffered saline and exposed to a membrane-impermeable biotinylation reagent, sulfo-NHS-LC-biotin, for 30

min at 4°C. Cells were lysed after the biotinylation reaction was terminated, and the presence of L-VGCC α 1D subunit in both plasma membrane and cytosolic compartments was determined by immunoblot analysis. We found that in whole-cell lysate preparations (containing both plasma membrane-bound and cytosolic VGCC α 1D), the protein expression of VGCC α 1D was under circadian control, and the protein level was significantly higher in cells harvested during the subjective night (CT 17) than during the subjective day (CT 5; Fig. 4a), which was similar to our previous report (Ko *et al.* 2007). This VGCC α 1D rhythm was dampened by a 2-h treatment with LY 294002 or Akti (Fig. 4b). Further analysis revealed that the level of membrane-bound VGCC α 1D was significantly higher in cells harvested during the subjective night (CT 17) than during the subjective day (CT 5; Fig. 4c), while there was no significant difference of VGCC α 1D in the cytosolic fraction between cells harvested at CT 5 and CT 17 (Fig. 4d). Treatment with LY 294002 or Akti dampened the circadian rhythm of membrane-bound VGCC α 1D with no significant effects on cytosolic VGCC α 1D (Fig. 4c and d). Interestingly, inhibition of Ras—Erk signaling with manumycin A (1 μ M, an inhibitor of the small GTPase Ras) or PD 98059 (MEK inhibitor; MEK phosphorylates and activates Erk) also showed a similar result, where manumycin A and PD 98059 significantly attenuated the circadian rhythm of membrane-bound VGCC α 1D (Fig. 4e and g) with no effects on cytosolic VGCC α 1D (Fig. 4e and h). While the PI3K—Akt pathway is known to regulate protein trafficking and membrane insertion of L-VGCCs and other ion channels (Kanzaki *et al.* 1999; Lhuillier and Dryer 2002; Le Blanc *et al.* 2004; Tajika *et al.* 2004), and Ras—Erk signaling is also known to regulate channel insertion into neuronal membrane (Keifer *et al.* 2007), our results demonstrate that both pathways play an important role as circadian outputs to regulate L-VGCC insertion into the plasma membrane, which is part of the underlying mechanism of circadian regulation of L-VGCC currents in retina photoreceptors.

Circadian rhythms of both Erk and PI3K—Akt signaling pathways are parallel and downstream of Ras

We demonstrated that Ras—Erk—calcium-calmodulin kinase II signaling serves as part of a ‘common’ circadian output pathway to regulate cGMP-gated ion channels (CNGCs; Ko *et al.* 2001, 2004) and L-VGCCs (Ko *et al.* 2007), and retinoschisin, a photoreceptor secreted protein (Ko *et al.* 2008). As the Erk and PI3K—Akt pathways modulate VGCC α 1D in a similar fashion, a question arises as to whether these two pathways lie in series or in parallel. To answer this conundrum, chick embryos were LD entrained *in ovo*, and retinal cells were dissociated and cultured as described above. On the second day of DD, cells were treated with DMSO (0.1%, control), LY 294002 (50 μ M, PI3K inhibitor), Akti (10 μ M), or PD 98059 (50 μ M, MEK inhibitor) for 2 h prior to harvesting at CT 5 and CT 17 for western immunoblotting. We found that PD 98059 blocked the circadian rhythm of pErk, while LY 294002 and Akti had no effect on the pErk rhythm (Fig. 5a and b). Conversely, the circadian rhythm of pAktThr308 was significantly dampened by LY 294002 and Akti, while PD 98059 had no effect (Fig. 5a and c). Treatment with manumycin A (1 μ M) abolished the rhythms of both pErk and pAktThr308 (Fig. 6a and b).

Manumycin A is a farnesyl transferase inhibitor, but the monomeric GTPases of the Ras family are not the only proteins that require farnesylation for normal biological activity. Therefore, we used another approach to determine the role of Ras specifically by over-expression of a selective dominant negative mutant, the S17N mutation of Ras (Ras DN17) using a biolistic transfection procedure. Cultured photoreceptors were circadian entrained as described above. On the first day of DD, cells were co-transfected with eGFP and Ras DN17 and maintained in DD for another day. On the following day at CT 17, cells were fixed and processed for immunostaining with either anti-pAktThr308 or anti-L-VGCC α 1D antibody. The distribution of L-VGCC α 1D in photoreceptors was mainly concentrated in the soma below the oil droplet as described previously (Ko *et al.* 2007), while pAktThr308 was distributed from the cell bodies

to synaptic terminals but not in the outer segments. Photoreceptors transfected with Ras DN17 had significantly lower fluorescence intensities in both pAktThr308 and L-VGCC α 1D compared with non-transfected photoreceptors on the same coverslip (Fig. 7). Transfection with eGFP alone had no effect on the circadian rhythm of either pAktThr308 or L-VGCCs (data not shown). Therefore, our results demonstrate that PI3K—Akt and Erk are parallel pathways downstream of Ras. Both pathways work in conjunction to control the L-VGCC rhythm by regulating L-VGCC α subunit trafficking in photoreceptors.

Discussion

In this study, we show that PI3K—Akt signaling participated in the phase-dependent modulation of L-VGCC rhythms. The mechanism underlying the action of PI3K—Akt signaling in the circadian modulation of L-VGCCs was through the regulation of L-VGCC α 1D subunit trafficking and insertion into the plasma membrane. It is worth noting that for some circadian regulated genes and their proteins, the mRNA levels are advanced a few hours earlier than their protein expressions. As we reported previously, the mRNA of L-VGCC α 1D peaks at CT 12, while its protein level reaches maximum at CT 16 (Ko *et al.* 2007). As inhibition of PI3K—Akt signaling only affected VGCC α 1D trafficking without influencing its mRNA rhythmicity, this signaling pathway only served as part of the circadian output pathway to regulate L-VGCCs. If PI3K—Akt also served in the circadian input entrainment, then inhibition of this pathway would change the mRNA levels of L-VGCCs or other core oscillator genes, to which we did not find any effects on cBmal mRNA rhythmicity in cultured photoreceptors after treatment with Akti for 2 h (data not shown).

Activation of Akt requires multiple steps that includes the generation of the second messenger PtdIns(3,4,5)P₃ from PtdIns(4,5)P₂ by PI3K, translocation of Akt from cytoplasm to plasma membrane involving its pleckstrin homology domain, and phosphorylation of Thr308 in the kinase domain and Ser473 within the regulatory domain (Fayard *et al.* 2005). As a downstream target of PI3K, the overall activity of Akt is the result of an equilibrium between these two phosphorylation sites (Beaulieu *et al.* 2007). Even though most reports show that humoral factors can induce Akt phosphorylation on both phosphorylation sites, there is evidence showing differential expression and chemical-induced phosphorylation between pAktThr308 and pAkt-Ser473 in D₂ and D₃ dopaminergic receptor knockout mice (Beaulieu *et al.* 2007). We found that only one phosphorylation site of Akt was under circadian control: the phosphorylation at Thr308. Akt phosphorylation at Ser473, as well as total Akt, remained constant throughout the day. To date, no other report deals with the circadian regulation of Akt activity or gene/protein expression of Akt, and little is known about the circadian regulation of PI3K—Akt signaling. In *Drosophila*, Susi, a negative regulator of PI3K, is expressed in a circadian fashion (Wittwer *et al.* 2005). In rats, intracerebroventricular infusion of melatonin induces Akt phosphorylation in the hypothalamus (Anhe *et al.* 2004). Here, we are the first to show that there is a circadian regulation of Akt activity both *in ovo* (Fig. 3) and *in vitro* (Fig. 4). However, we cannot exclude the possibility that the circadian regulation of Akt activity might be because of rhythms in endogenous humoral factors in the intact retina (for example, dopamine released from amacrine cells) that act on photoreceptors. In two transgenic mouse models that lead to overall increases in Akt phosphorylation, the free-running period of these mutant mice is lengthened and closer to 24 h than wild type controls, but their circadian entrainment to LD cycles is normal and similar to the wild type pattern (Harrington *et al.* 2007; Ogawa *et al.* 2007). Hence, circadian rhythmicity of Akt phosphorylation may be an important process in the regulation of free-running periods, as well as circadian outputs as indicated in this study.

Various ion channel activities are regulated by PI3K—Akt signaling in the presence of extracellular stimulators (Blair *et al.* 1999; Kanzaki *et al.* 1999; Lhuillier and Dryer 2002; Le

Blanc *et al.* 2004; Tajika *et al.* 2004). In parasympathetic neurons, PI3K—Akt signaling is necessary for trophic factor-induced protein trafficking and channel insertion of large-conductance calcium activated-potassium channels during development (Lhuillier and Dryer 2002). Activation of PI3K—Akt signaling promotes translocation of L-VGCCs to the plasma membrane and therefore enhances the L-VGCC currents in cardiomyocytes, neurons, and COS cells (Viard *et al.* 2001, 2004). We have shown here that inhibition of PI3K—Akt signaling significantly dampened the circadian regulation of L-VGCC current amplitude without affecting channel gating (Fig. 1). Using membrane surface biotinylation assays, we found that inhibition of PI3K or Akt caused a significant decrease in membrane-bound VGCC α 1D subunit without any increase of cytosolic VGCC α 1D (Fig. 4). The mRNA and protein expression of the VGCC α 1D subunit are under circadian control, but mRNA levels peak about 4 h ahead of protein expression (Ko *et al.* 2007). We postulate that after translation, the channel protein is inserted into the plasma membrane within a short period of time. This notion is supported by our results that show peak activity of pAktThr308 (Fig. 3) is concurrent with the peak protein level of L-VGCC α 1D (Ko *et al.* 2007) and the maximum current amplitudes recorded at CT 16–19. Higher levels of phosphorylated Akt lead to more VGCC α 1D subunits inserted into the plasma membrane and greater L-VGCC current amplitudes during the subjective night. Hence, PI3K—Akt signaling is important in the circadian regulation of L-VGCC trafficking and membrane insertion.

It was perplexing that treatment with PI3K/Akti at night decreased the membrane compartment of VGCC α 1D without a corresponding increase of cytosolic VGCC α 1D (Fig. 4), which would seem to indicate that PI3K/Akti might affect VGCC α 1D protein translation and contradict the notion that PI3K—Akt is involved only in channel trafficking and membrane insertion in this case. This view point would be true if VGCC α 1D protein synthesis, trafficking to the membrane, membrane retention, as well as protein recycling and degradation were maintained at the same rate during the day and night, with the only difference being in the amount of VGCC α 1D mRNA levels. If membrane retention of inserted protein and/or protein degradation of non-inserted/recycled protein are at a higher rate at night than during the day, then we would observe more membrane protein at night without a difference in cytosolic protein between day and night. We recently found that after VGCC α 1D is inserted into the plasma membrane, it interacts with an extracellular protein, retinoschisin, that aids in plasma membrane retention of this channel (Shi *et al.* 2009). Retinoschisin is an extracellular protein that is secreted mainly from photoreceptors and bipolar cells, and there is a circadian regulation of mRNA and protein expression of retinoschisin in the chick retina (Ko *et al.* 2008). The physical interaction between VGCC α 1D and retinoschisin in the retina is under circadian control, which is higher at night than during the day, and hence, the membrane retention of VGCC α 1D is higher at night (Shi *et al.* 2009). In mice, there is a diurnal change in protein expression of Rab3A in whole-brain synaptosome preparations (Darna *et al.* 2009). The Rab proteins are small Ras-related GTPases that have emerged as important regulators for endocytic transport, recycling, vesicle sorting, and transportation to lysosomes for degradation (Stein *et al.* 2003). Rab3A has been shown to interact with the sodium-selective amiloride-sensitive epithelial channel, and this interaction mediates channel protein recycling and degradation (Saxena *et al.* 2005). Recent advances have further shown that ubiquitin-mediate proteolysis and protein degradation serves as one of the post-translational mechanisms regulating circadian rhythms (He and Liu 2005; Fujiwara *et al.* 2008; Yang *et al.* 2009). Therefore, there could be a possible circadian regulation of Rab-mediated L-VGCC recycling and protein degradation in the retina yet to be explored.

Even though the Ras—Erk signaling pathway is important as an input pathway in circadian entrainment and phase-shifting (Obrietan *et al.* 1998; Butcher *et al.* 2002), it also serves as part of the output pathway to regulate cellular responses and behaviors (Ko *et al.* 2001, 2004, 2007, 2008; Williams *et al.* 2001). Both Ras and Erk activities are rhythmic in chick retinas (Ko *et al.* 2004), and the L-VGCC current amplitude rhythm (Ko *et al.* 2007) and the apparent

affinity rhythm of CNGCs (Ko *et al.* 2001, 2004) are under the output control of Ras—Erk. In *Drosophila*, mutations of neurofibromatosis-1, an upstream regulator of Ras, do not alter the rhythmicities of oscillator genes, but the locomotor behavior rhythm of 90% of the adult mutants carrying a null mutation in the neurofibromatosis-1 gene by deletion became arrhythmic (Williams *et al.* 2001). Hence, Ras—Erk could serve as part of a ‘universal’ output pathway to regulate circadian rhythms.

The PI3K—Akt and Erk signaling pathways are distinct from each other, but these two pathways share very similar functional roles in regulation of protein translation, cell cycles, protein transport and trafficking, cell-survival, and trophic factors-induced differentiation among others (Yoon *et al.* 2008). Melatonin induces phosphorylation of both Akt and Erk in the hypothalamus (Anhe *et al.* 2004). Both Ras—Erk and PI3K—Akt are necessary for transforming growth factor β 1-induced large-conductance calcium activated-potassium channel expression and membrane insertion in chick ciliary ganglion neurons (Lhuillier and Dryer 2002), as well as insulin growth factor 1-induced chondrogenic differentiation in adult mesenchymal stem cells (McMahon *et al.* 2008). As these two pathways could work synergistically for a particular cellular process (Shankar *et al.* 2008) or respond to the same stimulation independently (Chavarria *et al.* 2007), it is possible that the circadian oscillators can regulate both pathways simultaneously to govern down-stream rhythmic outputs.

Here, we found that Ras is a common upstream regulator for both Erk and PI3K—Akt signaling pathways to regulate L-VGCC rhythms (Fig. 6), where inhibition of Ras abolished the rhythmicity of pErk and pAktThr308 (Fig. 6). In addition, we have shown in this study that PI3K—Akt and Erk signaling are parallel pathways that both regulate the L-VGCC rhythm in photoreceptors. Inhibition of either pathway abolishes the L-VGCC rhythm, but the inhibition of one does not affect the rhythmic activity of the other. Therefore, both pathways are equally important in the circadian regulation of L-VGCC rhythms in retina photoreceptors (Fig. 8). Inhibition of Ras does not cause global perturbations of membrane trafficking in photoreceptors, as we previously showed that Ras—Erk regulates the apparent affinity rhythm of cGMP to CNGCs without affecting channel insertion, and the maximum current amplitude and α subunit expression of CNGCs do not change as a function of time of the day (Ko *et al.* 2001,2004;Chae *et al.* 2007). Treatment with a Ras inhibitor or transfection with a Ras dominant negative gene into photoreceptors does not change the maximum current amplitude of CNGCs (Ko *et al.* 2004). The apparent affinity rhythm of CNGCs is because of the circadian regulation of tyrosine phosphorylation of the CNGC auxiliary subunits (Chae *et al.* 2007). On the other hand, the circadian regulation of VGCC α 1D subunit expression (Ko *et al.* 2007) and its insertion into the plasma membrane as shown in this study are required for the circadian rhythm of L-VGCC current amplitudes (Ko *et al.* 2007). Inhibition of either Ras—Erk or PI3K—Akt prevented membrane insertion of VGCC α 1D as well as dampened L-VGCC maximum current amplitude rhythms. It is possible that these two parallel signaling pathways have different downstream targets that could ultimately lead to the regulation of L-VGCC insertion into the cell membrane, while Ras—Erk signaling participates in additional cellular events related to the circadian regulation of ion channels.

In conclusion, the circadian oscillators in the retina regulate the activity of Akt, and both Ras—PI3K—Akt and Ras—Erk signaling pathways play equally important roles in regulating L-VGCC channel trafficking that leads to the circadian regulation of L-VGCC current amplitudes. The circadian rhythms of L-VGCCs further leads to the circadian control of retinoschisin secretion from photoreceptors that might contribute to the circadian regulation of synaptic plasticity in the retina (Ko *et al.* 2008). Therefore, the circadian regulation of L-VGCCs is essential to the daily rhythms of retina function and physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CNGC, cGMP-gated ion channel
 CNTF, ciliary neurotrophic factor
 CT, circadian time
 DD, constant darkness
 DMSO, dimethylsulfoxide
 E12, embryonic day 12
 eGFP, enhanced green fluorescent protein
 Erk, extracellular signal-related kinase
 FI, fluorescence intensity
 LD cycle, light—dark cycle
 L-VGCC, L-type voltage-gated calcium channel
 MEK, mitogen-activated kinase kinase
 PB, phosphate buffer
 pErk, di-phosphorylated Erk
 PI3K, phosphatidylinositol 3 kinase
 Ras DN17, Ras dominant negative RasS17N

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Fig. 1.

There is a circadian phase-dependent modulation of L-type calcium currents by Akt. The L-type VGCC currents were recorded from cultured chick cone photoreceptors during the subjective day (CT 4–7) and subjective night (CT 16–19) in free-running period after 5 days of LD entrainment. All cells were recorded using a ramp command from -80 to 60 mV in 500 ms, as well as a 200 ms step command with holding potential at -65 mV and steps from -80 to 60 mV at 10 mV increments. Representative current traces under a ramp command are shown in [a(i)], and traces under a step command are shown in [b(i) and c(i)]. Averaged current–voltage relationships were obtained from the step command as in current amplitude [a(ii), b(ii), and c(ii)] and in current density [a(iii), b(iii), and c(iii)]. [a(i–iii)] The L-type VGCC

currents are under circadian control in cone photoreceptors treated with 0.1% DMSO (served as our controls, see Results for details). On the second day of constant darkness (DD), β -escin perforated-patch recordings showed that the maximum current amplitudes [a(ii)] and current densities [a(iii)] were significantly larger during the subjective night (CT 16–19; filled circles) than during the subjective day (CT 4–7; open circles) without changes in channel gating properties. [b(i–iii)] Application of 10 μ M Akt inhibitor (Akti; in 0.1% DMSO) for 2 h prior to whole-cell recordings during the subjective day (CT 4–7, open triangles) has no effect on L-VGCCs. However, 2 h exposure to Akti caused a significant decrease in the maximal L-VGCC current amplitudes and current densities in cells recorded during the subjective night [CT 16–19, closed triangles; c(i–iii)]. (d) Inhibition of Akt with Akti evoked a phase-dependent modulation of L-VGCC currents in chick photoreceptors. The maximum inward current densities were elucidated at 0 mV of the step command. In the control group (DMSO), the average maximum current density obtained during the subjective night (CT 16–19) was significantly larger than during the subjective day (CT 4–7). Treatment with Akti during the subjective night (CT 16–19) significantly decreased the maximum current density. The asterisk ‘*’ indicates statistically significant differences of control CT 16–19, compared with all other groups. Comparisons were made using ANOVA with Tukey’s *post hoc* test for unbalanced n; * $p < 0.05$.

Fig. 2.

The Akt inhibitor, Akti, does not block L-VGCCs or affect L-VGCCα1D mRNA levels. (a–e) All photoreceptors were recorded using a ramp command indicated in (e) from -80 to 60 mV in 500 ms in whole-cell mode. (a) A photoreceptor was recorded under control conditions. Cells on the coverslip were perfused with normal external bath solution. The currents were stable within 2 min after the whole-cell mode was established. #1 indicates the current recorded immediately after the whole-cell was established, and #2 indicates the current recorded 2 min after the whole-cell was formed. There was a slight rundown of the currents after 4 min of recording (indicated as #3). (b) Extracellular superfusion with 3 mM CoCl₂, a reversible voltage-gated calcium channel blocker, completely blocked the inward calcium current (#2). The calcium current recovered 2 min after washing out the CoCl₂ with control extracellular bath solution (#3). #1 indicates the calcium current recorded right after the whole-cell was established. (c–d) Entrained photoreceptors were recorded during the subjective day, CT 4–7 (c) and during the subjective night, CT 16–19 (d). Acute superfusion with 10 μM Akti had no effects on the inward calcium currents (indicated by #2). The currents only showed a slight rundown 2 min after washing out with control extracellular bath solution (#3) that was comparable with the normal rundown (as in a). #1 indicates the calcium current recorded immediately after the whole-cell was established. (f) Treatment with Akti did not affect the

mRNA levels of VGCC α 1D. Entrained cells were treated with 0.1% DMSO (control) or Akti (10 μ M) for 2 h and then harvested at CT 4 (subjective day) and CT 12 (transition to subjective night) for quantitative RT-PCR. Comparisons were made between CT 4 and CT 12 using Student's *t*-test; **p* < 0.05, *n* = 3 in each group.

Fig. 3.

Only pAktThr308, but not pAktSer473, is under circadian regulation. Immunoblot analysis showed differential Akt phosphorylation in whole retinæ harvested at different circadian times on the second day of DD. (a) The protein level of pAktThr308 showed a circadian rhythm with its peak at CT 16 ($n = 6$ for each time point). (b) The phosphorylation level of pAktSer473 did not display circadian rhythmicity ($n = 6$ for each time point). Asterisks “*” denote statistically significant differences at CT 16 compared with CT 0, 4, and 8 using ANOVA with Tukey’s *post hoc* test, $p < 0.05$.

Fig. 4. Circadian phase-dependent regulation of L-VGCC α 1D membrane insertion is mediated by both PI3—Akt and Ras—Erk signaling. The distribution of L-VGCC α 1D subunit in the plasma membrane, cytosol, and whole lysate were analyzed using membrane surface biotinylation assays. Representative blot images from two sets of experiments are shown in (a and e). (b) In whole-cell lysate preparations, the protein expression of L-VGCC α 1D in cultures harvested during the subjective night (CT 17) was significantly higher than during the subjective day (CT 5) in controls (treated with 0.1% DMSO). Treatment with the PI3K inhibitor LY 294002 (50 μ M) or the Akt inhibitor (Akti; 10 μ M) for 2 h abolished the circadian rhythm of L-VGCC α 1D subunits. (c) There was a significantly higher quantity of membrane-bound L-

VGCC α 1D in cultures harvested during the subjective night (CT 17) than during the subjective day (CT 5). Treatment with LY 294002 or Akti for 2 h during the subjective night significantly decreased the membrane-bound L-VGCC α 1D. (d) There was no difference in L-VGCC α 1D present in the cytosolic compartment of the controls and cultures treated with LY 294002 or Akti. (f) Treatment with a Ras inhibitor (manumycin A, 1 μ M) or MEK1 inhibitor (PD 98059, 50 μ M) for 2 h dampened the circadian rhythm of L-VGCC α 1D subunits in the whole cell lysate preparations. (g) Manumycin A and PD 98059 abolished the circadian rhythmicity of membrane-bound L-VGCC α 1D. (h) Treatment with manumycin A or PD 98059 had no significant effect on cytosolic L-VGCC α 1D. Comparisons were made using ANOVA with Tukey's *post hoc* test among controls, LY 294002, and Akti, as well as controls (another set of controls), PD 98059, and manumycin A; * $p < 0.05$.

Fig. 5.

The Erk and PI3K—Akt signaling pathways are parallel in the circadian regulation of VGCCs. On the second day of DD, cells were treated with DMSO (0.1%, controls), LY 294002 (50 μ M, PI3K inhibitor), Akti (10 μ M; Akt inhibitor), or PD 98059 (50 μ M, MEK inhibitor) for 2 h prior to harvest at CT 5 and CT 17 for western immunoblotting of pErk or pAktThr308. One representative blot image is shown in (a). (b) The protein expression of pErk was significantly higher in cells harvested during the subjective night (CT 17) than during the subjective day (CT 5). Treatment with LY 294002 or Akti for 2 h did not have any effect on pErk rhythms, while PD 98059 abolished the circadian rhythm of pErk. (c) The Akt phosphorylation on Thr308 (pAktThr308) was under circadian control, and pAktThr308 was significantly higher in cells harvested at CT 17 than CT 5. Treatment with PD 98059 had no effect on the pAktThr308 rhythm, while LY 294002 and Akti dampened the circadian rhythmicity of pAktThr308. Comparisons were made using ANOVA with Tukey's *post hoc* test; * $p < 0.05$, $n = 6-8$ for each group.

Fig. 6.

Both Erk and PI3K—Akt signaling pathways are downstream of Ras. On the second day of DD, cells were treated with 0.1% DMSO (Con; controls) or 1 μ M manumycin A (Manu) for 2 h prior to harvest at CT 5 and CT 17 for western immunoblotting of pErk or pAktThr308. (a) The protein expression of pErk was significantly higher in cells harvested during the subjective night (CT 17) than during the subjective day (CT 5). Treatment with manumycin A for 2 h abolished the circadian rhythm of pErk. (b) The Akt phosphorylation on Thr308 (pAktThr308) was also under circadian control, and pAktThr308 was significantly higher in cells harvested at CT 17 than CT 5. Treatment with manumycin A dampened the circadian rhythmicity of pAktThr308. Comparisons were made using ANOVA with Tukey's *post hoc* test; * $p < 0.001$; $n = 5-9$ for each group.

Fig. 7.

Transfection with a dominant negative Ras dampens the levels of VGCCα1D and pAkt. Entrained photoreceptors were co-transfected with eGFP and a dominant negative mutant S17N Ras (Ras DN17) using a biolistic technique. Cells were fixed at CT 17 and processed for immunostaining with either anti-VGCCα1D or anti-pAktThr308 antibody. The arrows indicate the cone photoreceptors, double arrows indicate transfected photoreceptors, and arrowheads indicate the gold particles used as DNA microcarriers. (a) The photo in the top panel was taken using a 40x phase contrast objective. The middle panel shows a transfected photoreceptor (green), and the bottom panel shows the distribution of VGCCα1D (red). The bar = 10 μM. (b) The top panel was taken using a 63x objective without phase contrast. The middle panel shows a transfected photoreceptor (green), and the bottom panel shows the distribution of pAktThr308 (red). The bar = 7 μM. (c) Photoreceptors transfected with Ras DN17 had significantly lower VGCCα1D fluorescence intensity compared with non-transfected photoreceptors. Control, $n = 33$; Ras DN17, $n = 10$. (d) Photoreceptors transfected with Ras DN17 had significantly lower pAktThr308 fluorescence intensity. Control, $n = 28$; Ras DN17, $n = 10$. (c and d) Comparisons were made using Student's t -test, $*p < 0.05$.

Fig. 8.

A model illustrates that both Ras—PI3K—Akt and Ras—Erk signaling pathways play equally important roles in regulating L-VGCC channel trafficking and insertion into the plasma membrane, which leads to the circadian regulation of L-VGCC current amplitudes. The pharmacological inhibitors acting on Ras, Erk, PI3K, and Akt are indicated.