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ARTICLE

# A new role for AMP-activated protein kinase in the circadian regulation of L-type voltage-gated calcium channels in late-stage embryonic retinal photoreceptors

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## Abstract

AMP-activated protein kinase (AMPK) is a cellular energy sensor, which is activated when the intracellular ATP production decreases. The activities of AMPK display circadian rhythms in various organs and tissues, indicating that AMPK is involved in the circadian regulation of cellular metabolism. In vertebrate retina, the circadian clocks regulate many aspects of retinal function and physiology, including light/dark adaptation, but whether and how AMPK was involved in the retinal circadian rhythm was not known. We hypothesized that the activation of AMPK (measured as phosphorylated AMPK) in the retina was under circadian control, and AMPK might interact with other intracellular signaling molecules to regulate photoreceptor physiology. We combined ATP assays, western blots, immunostaining, patch-clamp recordings, and pharmacological treatments to decipher the role of AMPK in the circadian regulation of photoreceptor physiology. We found that the overall retinal ATP content displayed a diurnal rhythm

that peaked at early night, which was nearly anti-phase to the diurnal and circadian rhythms of AMPK phosphorylation. AMPK was also involved in the circadian phase-dependent regulation of photoreceptor L-type voltage-gated calcium channels (L-VGCCs), the ion channel essential for sustained neurotransmitter release. The activation of AMPK dampened the L-VGCC currents at night with a corresponding decrease in protein expression of the L-VGCC $\alpha$ 1 pore-forming subunit, while inhibition of AMPK increased the L-VGCC current during the day. AMPK appeared to be upstream of extracellular-signal-regulated kinase and mammalian/mechanistic target of rapamycin complex 1 (mTORC1) but downstream of adenylyl cyclase in regulating the circadian rhythm of L-VGCCs. Hence, as a cellular energy sensor, AMPK integrates into the cell signaling network to regulate the circadian rhythm of photoreceptor physiology.

**Keywords:** circadian, metabolism, photoreceptor, signaling.  
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AMP-activated protein kinase (AMPK) is a cellular energy sensor that is responsible for balancing cellular metabolism. When the intracellular AMP to ATP ratio rises, AMPK is activated and promotes catabolic pathways while inhibiting anabolic pathways so that more ATP is generated (Hardie 2007). AMPK is a heterotrimeric protein kinase with a catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ; Hardie 2007). When the intracellular ATP level is low, AMP or ADP binds to the AMPK  $\gamma$  subunit and leads to AMPK activation through the phosphorylation of threonine 172 (Thr172; Oakhill *et al.* 2011; Xiao *et al.* 2011). In addition to the binding of AMP or ADP, there are kinases and phosphatases that also modulate AMPK activation by

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**Abbreviations used:** AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AKT, protein kinase B; AMPK, AMP-activated protein kinase; CT, circadian time; DAPI, 4',6-diamidino-2-phenylindole; DD, constant darkness; DMSO, Dimethylsulfoxide; ERK, extracellular signal-regulated kinase; LD, light-dark; L-VGCC, L-type voltage-gated calcium channel; MAPK, mitogen-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; ZT, Zeitgeber time.

targeting its Thr172, such as live kinase B1, Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase β (Hawley *et al.* 2003; Woods *et al.* 2005; Mihaylova and Shaw 2011), and protein phosphatase 2A and 2C (Davies *et al.* 1995; Kudo *et al.* 1996; Wu *et al.* 2007).

The activation of AMPK is not only regulated by the cellular energy expenditure and kinases/phosphatases, the activities of AMPK display circadian rhythms in various tissues and organs including the liver, muscles, and heart (Lamia *et al.* 2009; Um *et al.* 2011) indicating that either AMPK is under circadian control, or AMPK is part of the circadian regulation for cellular metabolism in these tissues. The endogenous circadian clocks regulate the physiology and behavior in living organisms and allow organisms to anticipate upcoming daily environmental changes, such as daily temperature fluctuations and cycling ambient illumination (Bell-Pedersen *et al.* 2005). Because the circadian clocks participate in metabolic processes throughout the day, elements of metabolism including metabolites, enzymes, transporters, and receptors all display daily rhythms (Panda *et al.* 2002). The canonical mechanism of circadian clocks is governed by a specific set of ‘clock genes’ and their protein products, which generate self-regulated transcription-translation feedback loops with a period near 24 h (Ko and Takahashi 2006). Post-translational modifications such as phosphorylation, ubiquitination, methylation, along with various cellular signaling pathways further contribute to the regulation of circadian oscillations (Gallego and Virshup 2007). Disruption of the clock genes perturbs the energy homeostasis and eventually leads to the development of various metabolic syndromes such as diabetes (Bass and Takahashi 2010; Froy 2010). While the activities of AMPK display circadian rhythms (Lamia *et al.* 2009; Um *et al.* 2011), stimulation of AMPK destabilizes cryptochrome, one of the specific clock proteins, and alters the circadian rhythms (Lamia *et al.* 2009). Genetic disruption of AMPK alters the circadian wheel running behavior and the expression of circadian genes in rodents (Um *et al.* 2011). Hence, AMPK is not only a cellular energy censor regulated by the circadian clock, it is important in circadian regulation at both cellular and systemic levels.

In the vertebrate retina, the circadian clocks are present in different cell types and involved in many aspects of retinal function and physiology. The circadian clocks in photoreceptors regulate the outer segment disk shedding and renewal (LaVail 1980), gene and protein expressions (Korenbrot and Fernald 1989; Pierce *et al.* 1993; Haque *et al.* 2002; Liu *et al.* 2012), as well as ion channel activities (Ko *et al.* 2001, 2007). The L-type voltage-gated calcium channels (L-VGCCs) located in the inner segment and synaptic terminals of photoreceptors are essential for cellular metabolism, calcium homeostasis, and neurotransmission (Barnes and Kelly 2002). The L-VGCCs are under circadian control in

cone photoreceptors and bipolar cells (Hull *et al.* 2006; Ko *et al.* 2007). Both mRNA and protein expressions of L-VGCCα1D display circadian rhythms in cone photoreceptors (Ko *et al.* 2007). An intricate cell signaling network regulates the trafficking of L-VGCCs from the cytosol to the plasma membrane and is correlated with the circadian rhythms of L-VGCC currents (Ko *et al.* 2007, 2009, 2013; Huang *et al.* 2012, 2013).

Since transportation of ion channels to the plasma membrane requires energy, we postulate that AMPK, the energy sensor, might be required in the circadian regulation of L-VGCCs in photoreceptors, and the activation/phosphorylation of AMPK might also display circadian rhythm in the retina. In addition, because the retinal light sensitivities are under circadian regulation (Cameron and Lucas, 2009; Lu *et al.*, 1995; Manglapus *et al.*, 1998; McGoogan and Cassone, 1999), and the retinal energy consumption is light-dependent (Linton *et al.* 2010; Wei *et al.* 2012), we hypothesized that the overall retinal energy expenditure and production could be under circadian control. In this report, we examined the possibility of AMPK serving in the circadian regulation of L-VGCCs in the retina, which might shed light on the role of AMPK in modulating retinal light sensitivities.

## 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). Chicken retinas were dissociated at embryonic day 12 (E12) and cultured for 6–7 days as described previously (Ko *et al.* 2007, 2009). Cultures were prepared in the presence of 20 ng/mL ciliary neurotrophic factor (R&D Systems, Minneapolis, MN, USA), which yields cultures highly enriched with cone photoreceptors (above 70% of total cells; 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<sub>2</sub>) were equipped with lights and timers for the entrainment of retinal circadian oscillators to 12 : 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 circadian time (CT) experiments, after LD entrainment chick embryos or cultured cells were kept in constant darkness (DD) for a 24 h free-run period. Chick embryos were LD entrained for 7 days if whole retinas were used for experiments. For cultured cell experiments, chick embryos (*in ovo*) were LD entrained for 7–8 days. Retinas were then dissected, cultured, and kept in DD. On the second day of DD, tissues or cultured cells were collected at different CT time points (Ko *et al.* 2007, 2009). In these 2-day cultures, photoreceptors nearly reached 50% of total cells. We used chick embryos from E12+6 for *in vitro* entrainment or E18 for *in ovo* entrainment because more than 95% of the retinal photoreceptors will express functionally mature VGCC currents by E18 (Gleason *et al.* 1992). All experiments were approved by the Texas A&M University.

### Adenosine 5'-triphosphate (ATP) assay

The ATP assay was carried out using the ATP bioluminescent assay kit (Sigma-Aldrich, St Louis, MO, USA). Briefly, retinas harvested from E19 embryos at 6 different time points (ZT 1, 5, 9, 13, 17, and 21) were homogenized with a radioimmunoprecipitation assay buffer supplemented with 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and a protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were centrifuged at 14 000 g for 15 min at 4°C. The supernatants were diluted (1/5) and processed for the ATP assay in a 96 well optiplate (Perkin-Elmer, Waltham, MA, USA). The intensity of luminescence was detected with a luminometer (Synergy 2 Multi-Mode Reader; BioTek, Winooski, VT, USA). Relative ATP values were normalized against ZT 1. Each experimental group contained at least seven different experiments ( $n = 7$  for each group).

### Immunoblot analysis

Samples were collected and prepared as described previously (Huang *et al.* 2012, 2013). 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<sub>3</sub>VO<sub>4</sub>, 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-di-phospho-extracellular signal-regulated kinase (pERK; Sigma-Aldrich), anti-ERK (total ERK, used for loading control; Santa Cruz Biochemicals, Santa Cruz, CA, USA), anti-phospho-AMPK (Thr172; Cell Signaling Technology, Danvers, MA, USA), anti-AMPK (total AMPK; Cell Signaling Technology), anti-phospho-S6 (ser240/244; Cell Signaling Technology), anti-S6 (total S6; Cell Signaling Technology), anti-phospho-protein kinase B (AKT) (Thr308; Cell Signaling Technology). Blots were visualized using appropriate secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) and an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA). Relative expressions for all proteins involved in this study are reported as a ratio to total ERK, which remains constant throughout the day. Band intensities were quantified by densitometry using Scion Image (NIH, Bethesda, MD, USA). Each experimental group contained at least four different experiments ( $n = 4$  for each group).

### Electrophysiology

Whole cell patch-clamp configuration of L-VGCC current recordings were carried out using mechanically ruptured patches. For retinal photoreceptors, the external solution was (in mM): 110 NaCl, 10 BaCl<sub>2</sub>, 0.4 MgCl<sub>2</sub>, 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<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 1.1 EGTA, and 10 HEPES, pH 7.3 adjusted with CsOH. Recordings were only made from cells with elongated cell bodies with one or more prominent oil droplets (hallmark of avian cone photoreceptors; Gleason *et al.* 1992; Ko *et al.* 2001; Pierce *et al.* 1993). Currents were recorded at room temperature (RT, 23°C) using an A-M 2400 amplifier (A-M Systems Inc., Carlsborg, WA, USA). Signals were low-pass filtered at 2 kHz and digitized at 5 kHz with Digidata 1440A interface and pCLAMP 10.0 software (Molecular Devices, Sunnyvale, CA, USA). Electrode capacitance was compensated after gigohm (GΩ) seals were formed. Cells

were held at -65 mV, and ramp voltage commands from -80 to +60 mV in 500 ms were used to evoke Ba<sup>2+</sup> 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 ~ +10 mV. 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. Leak currents were subtracted manually after data acquisition.

5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and compound C were obtained from EMD Millipore (Billerica, Massachusetts, USA) and Sigma-Aldrich, respectively. AICAR was dissolved in water, while Compound C was dissolved in dimethylsulfoxide (DMSO) (the final concentration of DMSO vehicle was 0.1%). The concentration of AICAR (Yang *et al.* 2011) and Compound C (Bain *et al.* 2007) used in this study were based on previous studies using these inhibitors in various neuronal tissue or cell preparations.

### Immunocytochemistry

Samples were collected and prepared as described previously (Huang *et al.* 2013). Briefly, dissociated retinas were cultured on coverslips and entrained under LD cycles. Cells were fixed at CT 4 or CT 16 with Zamboni fixative then permeabilized in 1% Triton-X phosphate buffer. Samples were blocked in 10% goat serum in 0.1% Triton-X/phosphate buffer and incubated with VGCCα1D primary antibody (1 : 100 dilution; Alomone, Jerusalem, Israel) at 4°C overnight. The fluorescent conjugated secondary antibody (1 : 200 dilution, Alexa Fluor® 488 goat anti-rabbit; Molecular Probes, Carlsbad, CA, USA) was applied on the coverslips at room temperature (22–23 °C) for 2 h in the dark. Coverslips were then re-washed and mounted with ProLong® Gold antifade reagents with 4',6-diamidino-2-phenylindole (Invitrogen, Eugene, OR, USA) on a glass slide and stored at 4°C for later observation on a Zeiss Stallion microscope (Carl Zeiss AG, Oberkochen, Germany) with epifluorescence to determine the localization of VGCCα1D and the nucleus (with 4',6-diamidino-2-phenylindole). Green or blue fluorescent images were taken under identical settings, including exposure time and magnification. The fluorescence intensity was measured using Adobe Photoshop 12 software (Adobe Systems, San Jose, CA, USA) as described previously (Ko *et al.* 2007). The fluorescence intensity analyses were done blind. Each experimental group contained at least four different experiments ( $n = 4$  for each group).

### Statistical analysis

All data are presented as mean ± SEM (standard error of mean). One-way analysis of variance 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

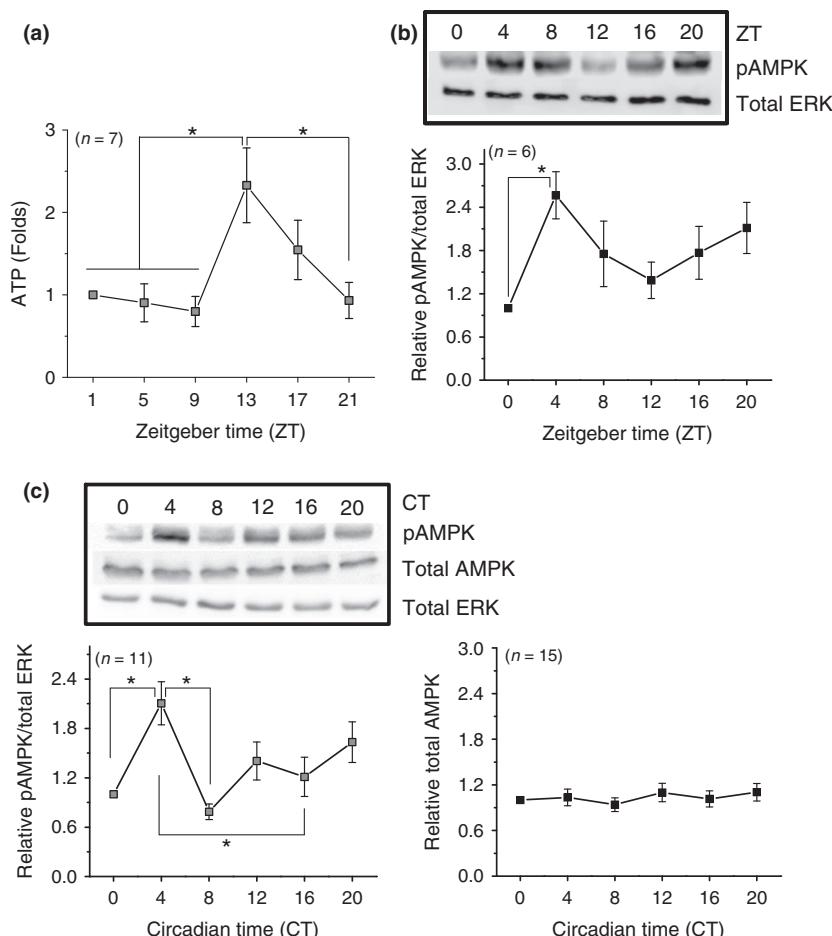
### Retinal ATP content and AMPK activity are under circadian control

We first examined whether the overall retinal energy levels oscillated daily. Embryonic chicken retinal samples were collected at six different time points throughout a day. The overall retinal ATP content was higher at night with a peak at ZT 13 and lower during the day (Fig. 1a). Since AMPK is the cellular energy sensor, we next examined whether the activation/phosphorylation of AMPK in the retina was under circadian control. Because the AMPK $\alpha$ 1 protein, as well as AMPK $\beta$ 2 and AMPK $\gamma$ 1 mRNAs, display circadian rhythms in the liver (Lamia *et al.* 2009), we used total ERK as the loading control because retinal total ERK remains constant throughout the day (Ko *et al.* 2001). We found that phosphorylated AMPK (pAMPK) at Thr172, the major site of AMPK phosphorylation and activation, peaked at ZT 4 when the chicken embryos were entrained under LD cycles (Fig. 1b). The diurnal rhythm of pAMPK was nearly anti-phase with the retinal ATP rhythm. To further confirm that the rhythmicity of AMPK activities (measured as pAMPK) was indeed governed by the retinal circadian clock, retinas

were collected (every 4 h) on the second day of DD after embryos were LD entrained. The pAMPK showed a circadian rhythm with a peak during the subjective day at CT 4 (Fig. 1c). Different from the liver (Lamia *et al.* 2009), the total amount of retinal AMPK (total AMPK) remained constant throughout the day (Fig. 1c). Hence, the retinal ATP content displayed a diurnal rhythm, which revealed the summation of overall ATP production and consumption in the retina. The activity of AMPK was under circadian regulation in the retina, which might reflect the overall retinal energy expenditure throughout a day.

### AMPK is involved in the circadian regulation of L-VGCC currents

There is a circadian rhythm of L-VGCCs in chick cone photoreceptors, with peak maximal currents elicited at 0 mV in the middle of the night (ZT 16–19) and trough during the middle of the day (ZT 4–7; Fig. 2a; Ko *et al.* 2007). To investigate the role of AMPK in modulating the circadian rhythm of L-VGCCs, we applied the AMPK activator, AICAR, an analog of AMP, to cultured photoreceptors for 2 h prior to patch-clamp recordings. Treatments with AICAR



**Fig. 1** The retinal ATP content and AMP-activated protein kinase (AMPK) activity are under circadian control. The retinas were collected at six different time points throughout a day for ATP assays or immunoblotting analyses after entrainment to 12 : 12 h light-dark (LD) cycles for 8 days *in ovo*. (a) The overall retinal ATP content displays a diurnal rhythm with a peak at Zeitgeber time (ZT) 13. \*indicates that ZT 13 is significantly different from ZT 1, 5, 9, and 21. (b) The phosphorylation of AMPK at Thr172 (pAMPK) shows a diurnal rhythm with a peak at ZT 4. \*indicates that ZT 4 is significantly different from ZT 0. (c) After entrainment to 12 : 12 h LD cycles for 7 days *in ovo*, the eggs were moved to constant darkness (DD). On the second day of DD, the retinas were collected at 6 circadian time points for immunoblotting analyses. pAMPK exhibits a circadian rhythm with its peak at circadian time (CT) 4 (left panel), while the total amount of AMPK remains constant throughout a day (right panel). \*indicates that CT 4 is significantly different from CT 1, 8, and 16 (left panel). \* $p < 0.05$ .

(500  $\mu$ M) significantly damped the L-VGCC currents when cone photoreceptors were recorded in the middle of the night (ZT 16–19; Fig. 2b, c and e) but did not have any significant effect on these currents when cells were recorded during the middle of the day (ZT 4–7), late day (ZT 8–11), or late night (ZT 20–23; Fig. 2b–e).

When photoreceptors were treated with Compound C (1  $\mu$ M), an AMPK inhibitor, for 2 h prior to recordings, the L-VGCC currents were significantly enhanced when the cone photoreceptors were recorded during the middle of the day (ZT 4–7) compared to the control treated with 0.1% DMSO (Fig. 3a, b and d). Inhibition of AMPK did not affect L-VGCCs when photoreceptors were recorded during other time periods of the day (Fig. 3). These results indicate that AMPK has a circadian phase-specific effect on L-VGCCs in cone photoreceptors: when AMPK is activated by AICAR in the middle of the night (ZT 6–19), the L-VGCCs are significantly decreased, and when AMPK is inhibited by Compound C in the middle of the day (ZT 4–7), the L-VGCCs are significantly enhanced.

#### AMPK modulates the protein expression of L-VGCC $\alpha$ 1D

Since AMPK participated in the circadian phase-dependent modulation of L-VGCC currents in cone photoreceptors, we examined whether AMPK affected the protein expression of L-VGCCs. In both mammalian and avian retina, L-VGCC $\alpha$ 1D is extensively present in the inner segments, soma, and synaptic terminals of the photoreceptors (Ko *et al.* 2007; Kersten *et al.* 2010; Huang *et al.* 2013). The fluorescence intensity of L-VGCC $\alpha$ 1D in cones was significantly higher when cultured cells were fixed at CT 16 compared to cells fixed at CT 4 (Fig. 4; Ko *et al.*, 2007). Treatment with AICAR for 2 h prevented the increase in L-VGCC $\alpha$ 1D fluorescent intensity at night (CT 16) but not during the day (CT 4) in cone photoreceptors (Fig. 4a). On the other hand, treatment with Compound C for 2 h significantly increased the fluorescent intensity of L-VGCC $\alpha$ 1D in cone photoreceptors fixed at CT 4 but not at CT 16 (Fig. 4b). Hence, activation of AMPK by AICAR reduced the protein expression of L-VGCC $\alpha$ 1D, but inhibition of AMPK by Compound C enhanced the protein expression of L-VGCC $\alpha$ 1D in cone photoreceptors, which echoed the results from patch-clamp recordings of L-VGCC currents (Fig. 2).

#### Cyclic AMP-dependent signaling mediates AMPK activities

cAMP is a second messenger that plays important roles in many physiological processes including metabolism (Sutherland and Robison 1969), and its signaling is involved in the regulation of AMPK activities in a tissue-specific manner (Yin *et al.* 2003; Hurley *et al.* 2006; Omar *et al.* 2009; Djouder *et al.* 2010). We and others previously showed that not only is retinal cAMP under circadian control with its peak at night (Nikaido and Takahashi 1998; Ivanova and Iuvone 2003; Huang *et al.* 2012), cAMP-signaling is

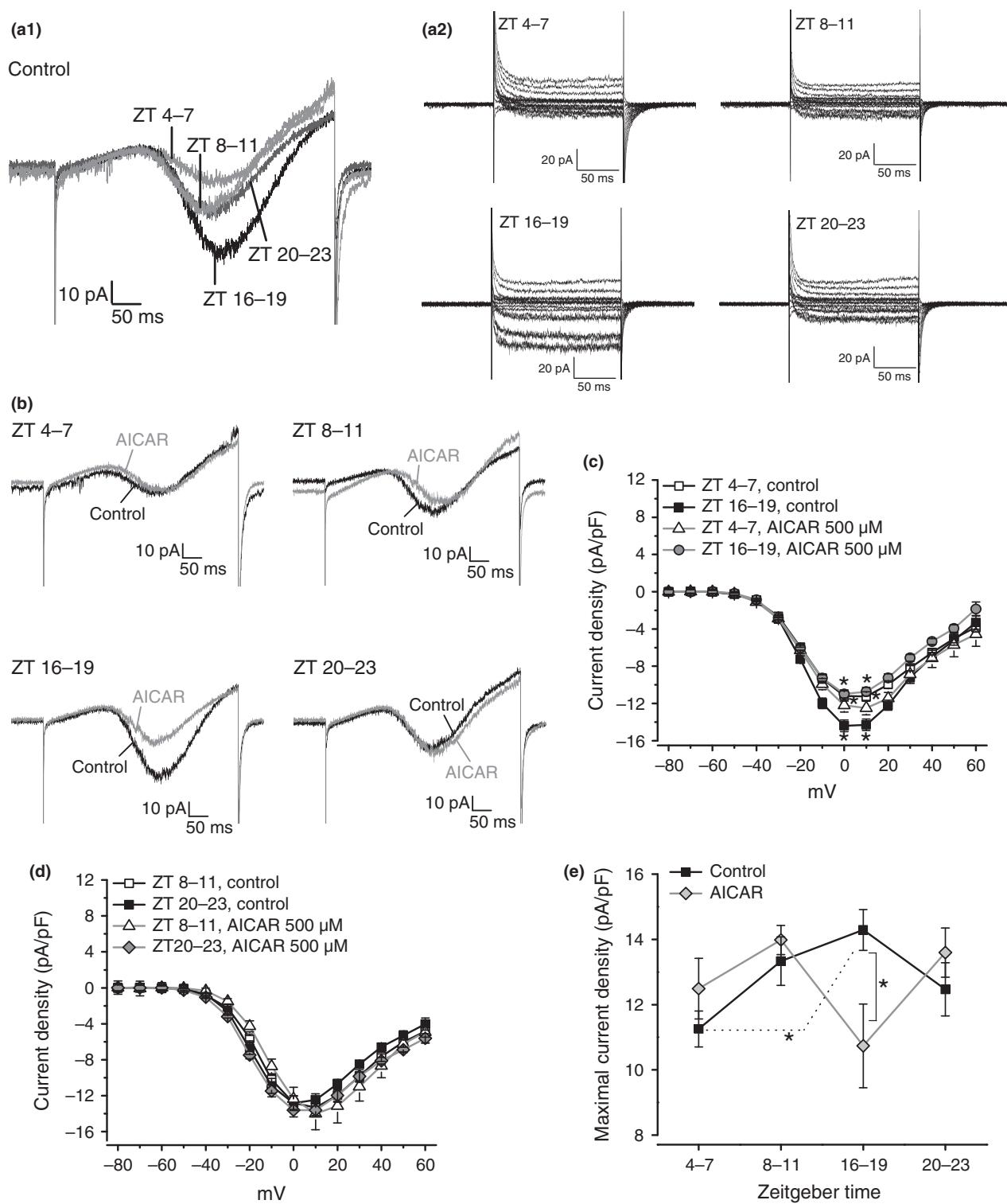
involved in the circadian regulation of Cyclic guanosine monophosphate (cGMP)-gated cation channels and L-VGCCs in avian photoreceptors (Ko *et al.* 2004; Huang *et al.* 2012). We therefore investigated the relationship between cAMP signaling and AMPK in the retina. Activation of adenylyl cyclase by forskolin (20  $\mu$ M) resulted in a diminished pAMPK during the subjective day (Fig. 5a) and significantly increased L-VGCC currents when cells were recorded during the day (ZT 4–8; Fig. 5b). Application of AICAR in the presence of forskolin was able to dampen the augmentation effect of forskolin on L-VGCC currents (Fig. 5b). In addition, treatment with the adenylyl cyclase inhibitor MDL-12330A (50  $\mu$ M) significantly increased the AMPK activity during the subjective night (Fig. 6a) and significantly decreased L-VGCC currents when cells were recorded at night (ZT 16–20; Fig. 6b), but inhibition of AMPK with Compound C was not able to reverse the effect of MDL-12330A (Fig. 6b) even though Compound C alone was able to increase L-VGCC currents when cells were recorded during the day (Fig. 3). These data suggest that cAMP signaling was upstream of AMPK in the circadian regulation of cone L-VGCCs and was a negative regulator of AMPK, with the circadian phase of retinal cAMP (Nikaido and Takahashi 1998; Ivanova and Iuvone 2003; Huang *et al.* 2012) anti-phase to that of pAMPK (Fig. 1).

#### AMPK regulates the mTORC1 signaling pathway

We previously showed that both mitogen-activated protein kinase (MAPK)-ERK and PI3K-AKT signaling pathways are downstream of cAMP and parallel to each other in the circadian regulation of L-VGCC trafficking (Ko *et al.* 2007, 2009; Huang *et al.* 2012, 2013). In addition, mammalian target of rapamycin complex 1 (mTORC1) signaling is a downstream target of PI3K-AKT in regulating the circadian rhythm of L-VGCCs (Huang *et al.* 2013). We further examined whether AMPK interacted with these signaling pathways in the circadian regulation of L-VGCCs. We found that activation of AMPK with AICAR decreased the phosphorylation of ERK (pERK; Fig. 7a and b) and S6 (pS6), a downstream target of mTORC1 signaling, at night (Fig. 6a and d) and dampened the circadian rhythm of phosphorylated AKT (pAKT) (Fig. 7a and c). Hence, AMPK was able to integrate with MAPK-ERK, PI3K-AKT, and mTORC1 as part of the complex signaling network in the circadian output regulation of L-VGCCs.

#### Discussion

In this study, we demonstrated that the overall retinal energy expenditure and production was under circadian control through the measurement of retinal ATP content and AMPK activation (measured as pAMPK), which were nearly anti-phase to each other. Total AMPK in the retina remained constant throughout the course of a day, which was different



from a previous report on the circadian oscillations of AMPK (both mRNA and protein expressions) in the liver. Since the liver is the major organ responsible for whole body metabolism, while the retina mainly utilizes glucose as its primary energy source with retinal glucose levels fluctuating

following systemic glycemia (Puchowicz *et al.* 2004; Ola *et al.* 2006), the difference of AMPK circadian rhythm in the liver versus retina might imply that the AMPK circadian rhythm could be tissue-specific to reflect the functional differences of AMPK in different tissues. Retinal AMPK

**Fig. 2** The AMP-activated protein kinase activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) dampens the circadian regulation of L-VGCC currents. The L-VGCC currents were recorded from cultured chick cone photoreceptors on the sixth day of light-dark entrainment during the day (Zeitgeber time, ZT 4–7 or ZT 8–11) or at night (ZT 16–19 or ZT 20–23). All cells were recorded using a ramp command from –80 to 60 mV in 500 ms, as well as a step command with holding potential at –65 mV and steps from –80 to 60 mV at 10 mV increments. (a) Representative day (4–7 or 8–11) and night (16–19 or 20–23) L-VGCC current traces recorded from the control with (a1) ramp command or (a2) step command. (b) Representative traces of L-VGCC currents from cells that were treated with AICAR (500 µM; gray) for 2 h prior to recordings compared to control cells (black) during different time periods: ZT 4–7, 8–11, 16–19, and 20–23. (c) The average current–voltage relationship is shown in current density (pA/pF) and step-voltage (mV). \*indicates that the current densities

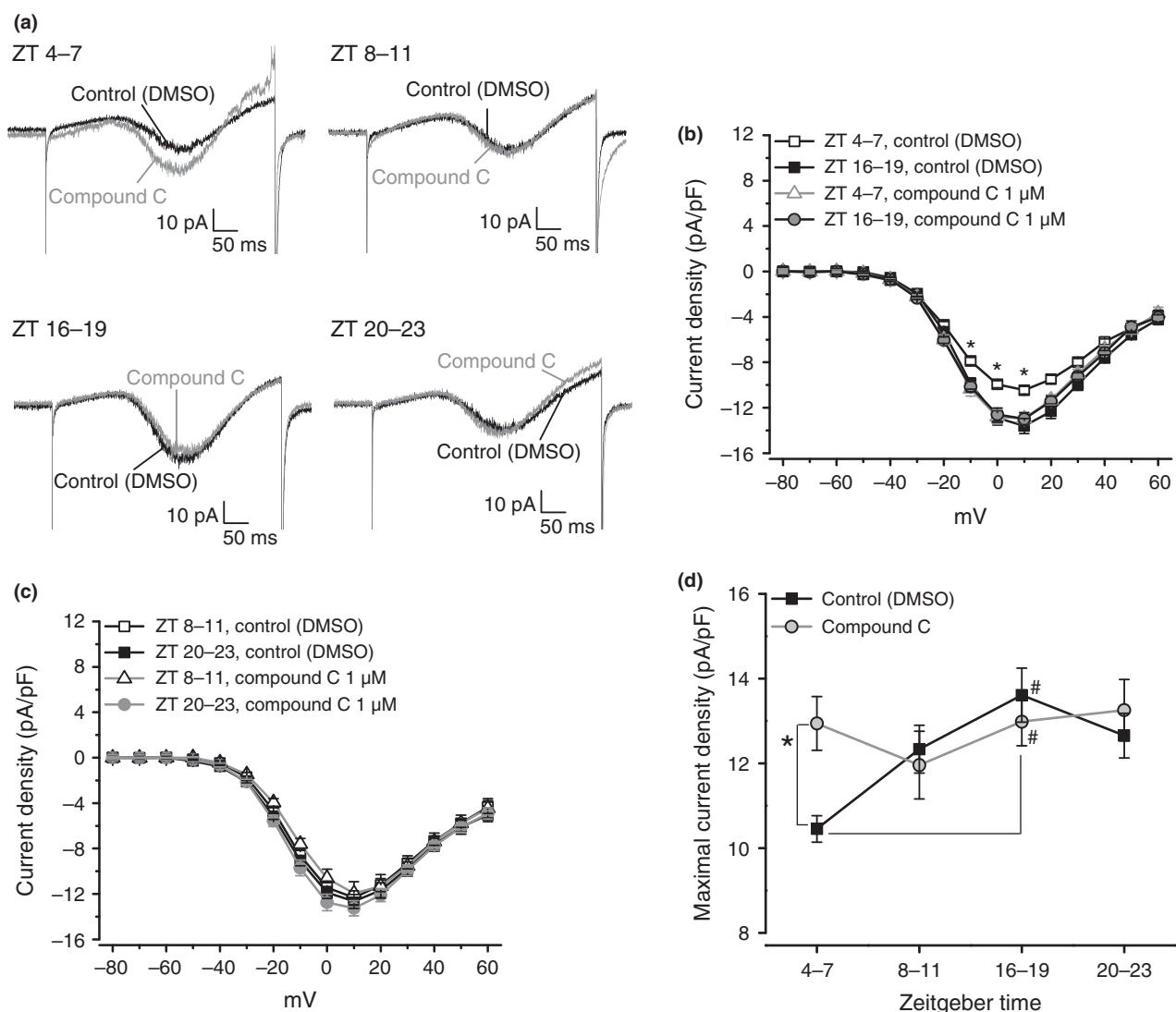
of L-VGCCs from the control recorded at night (ZT 16–19; black square) were significantly higher than the control (white square) and AICAR treated cells recorded during the day (ZT 4–7; white triangle). (d) The average current–voltage relationship is shown in current density (pA/pF) and step-voltage (mV) from cells recorded either at ZT 8–11 or ZT 20–23. White square: control cells recorded at ZT 8–11; black square: control cells recorded at ZT 20–23; white triangle: AICAR-treated cells recorded at ZT 8–11; gray diamond: AICAR-treated cells recorded at ZT 20–23. There was no statistical difference between these four groups. (e) The maximal current densities were elicited at 0 mV of the step command at different ZT time periods. \*indicates that the current densities recorded at night (control group, ZT 16–19) are significantly larger than during the day (control, ZT 4–7) and AICAR treated cells recorded at night (AICAR, ZT 16–19). Each group had at least 15 cells except the AICAR-treated group at ZT 8–11 (5 cells). \* $p < 0.05$ .

participated in the circadian regulation of L-VGCCs in the photoreceptors. Activation of AMPK by AICAR led to decreased protein expression and current densities of L-VGCCs at night, while inhibition of AMPK by Compound C resulted in the enhancement of L-VGCC currents during the day in cone photoreceptors. This result also echoes that normally during the day, retinal AMPK activity is higher (Fig. 1) with the current density of L-VGCCs being lower, and at night, retinal AMPK activity is lower with the L-VGCC current density reaching its peak. While AMPK was downstream of cAMP signaling, it integrated into the signaling pathways that regulate L-VGCC trafficking including MAPK-ERK (Ko *et al.* 2001, 2007), PI3K-AKT (Ko *et al.* 2009), and mTORC1-S6 (Huang *et al.* 2013). Even though the complexity of the signaling network that regulates L-VGCCs still requires more thorough investigation, our results showed that AMPK was capable of interacting with multiple signaling pathways, which indicates that AMPK might have multiple functions other than serving as an energy sensor.

In the retinal photoreceptors, energy consumption is highly compartmentalized (Linton *et al.* 2010; Wei *et al.* 2012). There is heavy energy expenditure in the outer segments of photoreceptors where phototransduction and protein transport for outer segment renewal are taking place in response to various light intensities (Korenbrot 1995; Koutalos and Yau 1996). In the dark, most of the energy consumption is in the inner segments and synaptic terminals to maintain the dark currents and neurotransmitter release (Wong-Riley 2010). Energy expenditure and production reaches a homeostatic state in any healthy cell. The retinal photoreceptors have a higher metabolic activity in the dark, which means that on one hand, ATP is hydrolyzed in an accelerated rate to support tonic neurotransmitter release. On the other hand, mitochondria will have to produce more ATP to sustain photoreceptor activities. As a result, the mitochondrial enzymes that are responsible for ATP production should be more activated in

darkness, which is supported by Huang *et al.* (2004) where the mitochondrial enzymes cytochrome C oxidase III and adenosine triphosphatase-6 are down-regulated by higher light intensity. Thus, the overall ATP production may be lower in the presence of bright light. We found that the overall energy state fluctuated in embryonic chick retina throughout the day (Fig. 1a): while the AMPK activity peaked during the mid-day, the ATP content (as the summation of energy expenditure and production) peaked at early night. By measuring the pH changes in the retina, Dmitriev and Mangel (2004) demonstrated that there is a circadian regulation of retinal energy metabolism. Therefore, the retinal energy expenditure and production can be both light intensity-dependent as a reflection of acute light/dark adaptation, as well as circadian clock-regulated as the retina ‘anticipates’ and adapts to the upcoming light changes at dawn and dusk.

Since we measured ATP from the whole retina, the ATP production might not be photoreceptor or retinal neuron specific. ATP receptors are present in all retinal neurons, and ATP is known to serve as a neurotransmitter in the retina and the brain (Ho *et al.* 2014). ATP and other purines (ADP, AMP, adenosine, adenine, and hypoxanthine) are tonically released in the retina particularly in the dark (Perez *et al.* 1986), and such release is increased by neuronal activity (Neal and Cunningham 1994). However, Müller glia cells are also able to generate, release, and accumulate extracellular ATP (Loiola and Ventura 2011). Interestingly, in cultured cortical glia cells, extracellular ATP accumulation displays circadian rhythms (Womac *et al.* 2009; Burkeen *et al.* 2011). Hence, extracellular ATP could serve as a neurotransmitter to communicate among multiple circadian oscillators existing in the different retinal cell types (Ruan *et al.* 2006; Liu *et al.* 2012). In addition, the extracellular ATP can further be converted into adenosine, which could lead to a circadian rhythm of retinal adenosine with a higher concentration at night than during the day (Ribelayga and Mangel 2005).

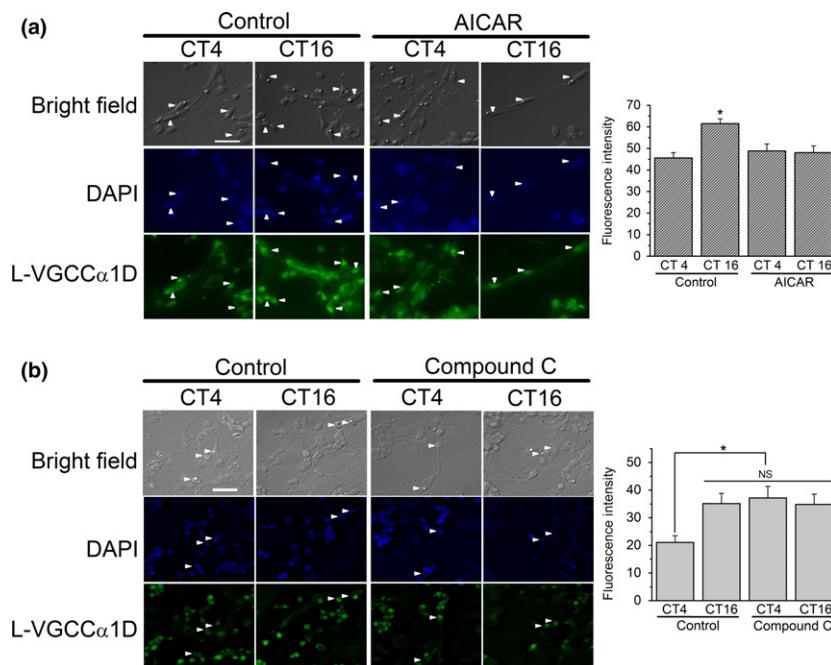


**Fig. 3** Inhibition of AMP-activated protein kinase increases the L-VGCC current densities during the day. (a) Representative L-VGCC current traces from 0.1% dimethylsulfoxide (DMSO)-treated cone photoreceptors (control; black) and 1  $\mu$ M compound C-treated cells (gray) that were recorded from Zeitgeber time (ZT) 4–7, 8–11, 16–19, and 20–23. (b) The average current–voltage relationship is shown in current density (pA/pF) and step-voltage (mV). \*indicates that the current densities of L-VGCCs from the control recorded at ZT 4–7 (white square) are significantly lower than the control recorded at night (ZT 16–19; black square) as well as compound C treated cells recorded during the day (ZT 4–7; white triangle) and at night (ZT 16–19; gray circle). (c) The average current–voltage relationship is shown in current density (pA/pF) and step-voltage (mV) from cells recorded

from ZT 8–11 or ZT 20–23. White square: control cells recorded at ZT 8–11; black square: control cells recorded at ZT 20–23; white triangle: compound C-treated cells recorded at ZT 8–11; gray circle: compound C-treated cells recorded at ZT 20–23. There was no statistical difference between these four groups. (d) The maximal current densities were elicited at 0 mV of the step command in different ZT phases. \*indicates that the current densities of the compound C group are significantly larger than the current densities of the control group recorded during the day (ZT 4–7). #indicates that the current densities of the control group as well as compound C-treated group recorded at night (ZT 16–19) are significantly greater than the control group recorded during the day (ZT 4–7). Each group had at least 15 cells. \* $p < 0.05$ .

Retinal adenosine is known to regulate circadian rhythms of photoreceptor coupling, as well as retinal light/dark adaptation (Ribelayga and Mangel 2007; Ribelayga *et al.* 2008; Li *et al.* 2013). Therefore, the circadian rhythm of retinal ATP might not only reflect the circadian control of

energy status, but it might implicate that ATP and its metabolite adenosine could serve as neuromodulators to coordinate the various circadian oscillators in the retina, so that the overall retinal circadian rhythm is integrated as a way for the retina to adapt to ambient light changes over 12 orders



**Fig. 4** There is a circadian phase-dependent effect of AMP-activated protein kinase activation on the protein expression of L-VGCCs in cone photoreceptors. Representative images from cultured retinal cells are shown. Retinal cells were dissociated and cultured on glass coverslips at E12 and entrained to 12 : 12 light-dark cycles for 5 days *in vitro* and kept in constant darkness (DD). On the second day of DD, cells were treated with 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (a) or Compound C (b) at circadian time (CT) 2 and CT 14 for 2 h followed by fixation. (a) The left panel shows the fluorescent images from the control

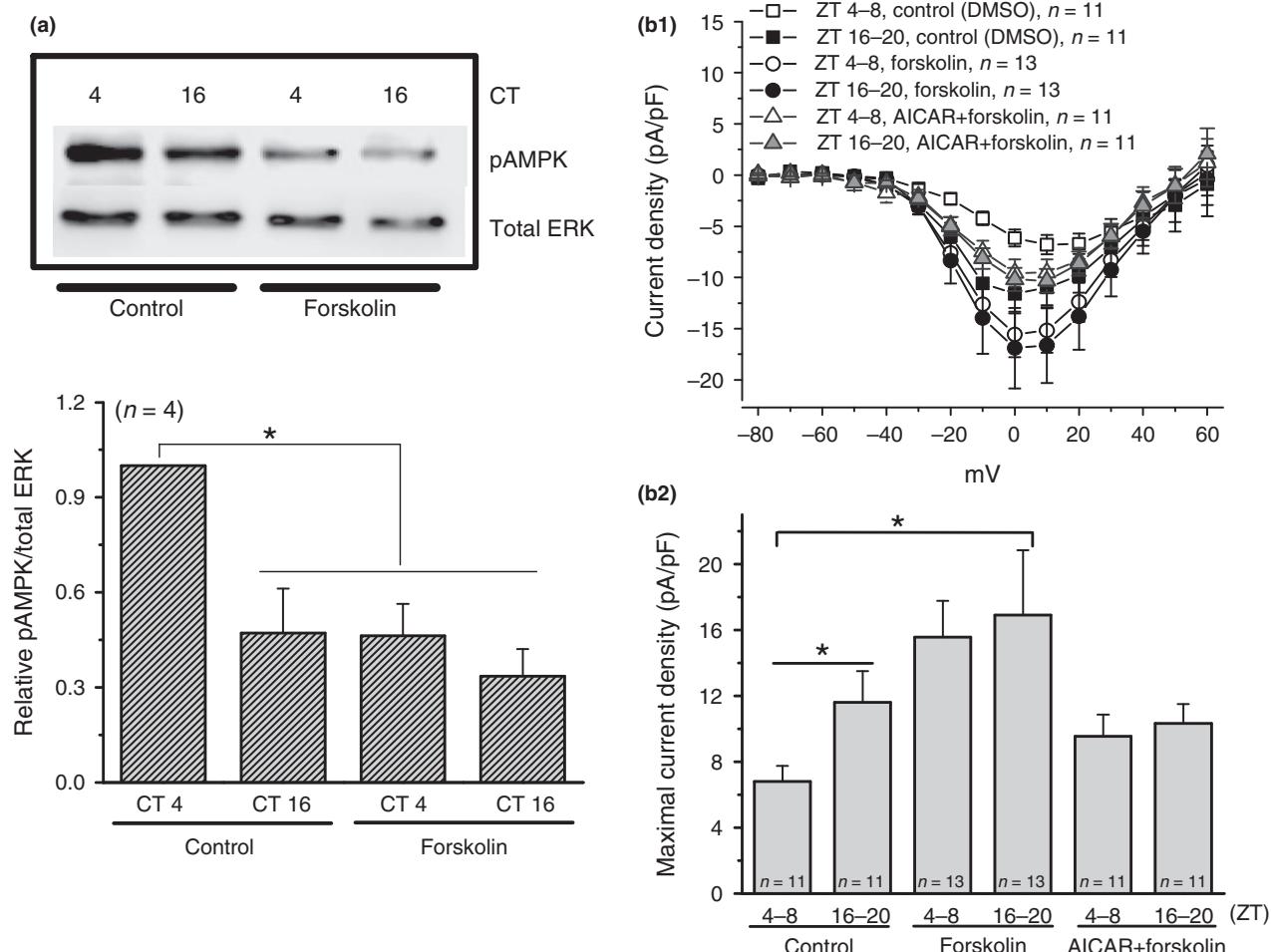
and AICAR treated cells. The right panel shows that the fluorescent intensity of L-VGCC $\alpha$ 1D in cone photoreceptors is significantly higher at CT 16 of the control compared to the other three groups, as denoted with \*. (b) The left panel shows the fluorescent images from the control and Compound C treated cells. The right panel shows that the fluorescent intensity of L-VGCC $\alpha$ 1D in cone photoreceptors is significantly lower at CT 4 of the control compared to all other groups. The arrowheads indicate the cone photoreceptors. The scale bar is 20  $\mu$ m. Each group has at least 20 cells from 4 different trials. \* $p$  < 0.05.

of magnitude over the course of a day (Green and Besharse 2004). It is our great interest to investigate the different sources of retinal ATPs (from retinal neurons vs. glia cells) and their contribution to the retinal circadian rhythm and light sensitivities in the future.

The activation of AMPK with AICAR led to attenuation of protein expression and current densities of L-VGCCs at night (Figs 2 and 4). AMPK and its upstream kinase LKB1 are involved in retina synaptic transmission (Samuel *et al.* 2014). Deletion of either LKB1 or AMPK in young mice reduced electroretinogram (ERG) a- and b-waves and caused ectopic synapses in the outer retina, where photoreceptors show abnormal axonal retractions, but bipolar and horizontal cells extend their dendrites into the outer nuclear layer (Samuel *et al.* 2014). These hallmarks caused by deletion of AMPK or LKB1 occur naturally in old mice as part of the aging process, and elevation of AMPK in old mice can rescue these aged-associated synaptic alterations (Samuel *et al.* 2014). Since L-VGCCs are essential for neurotransmitter release in the synaptic terminals of retinal neurons, the circadian phase-dependent regulation of L-VGCCs by AMPK might be critical for maintaining morphological and

functional synapses in the retina, as well as retinal light sensitivities, in addition to AMPK acting as an energy sensor.

As a cellular energy sensor, AMPK regulates many aspects of cellular physiological processes such as the metabolism of glucose, lipids, and proteins, and modulation of ion channels and transporters (Steinberg and Kemp 2009). How AMPK regulates ion channels is diverse and tissue/cell type-specific (Andersen and Rasmussen 2012; Dermaku-Sopjani *et al.* 2014). For example, constitutively active AMPK slows the inactivation of voltage-gated sodium channels ( $Na_v1.3$ ) and shifts the voltage-activation curve toward more hyperpolarized potentials in rat ventricular myocytes (Light *et al.* 2003). Co-expression of AMPK with  $Ca^{2+}$ -sensitive large conductance potassium channels (BK channel) in *Xenopus* oocytes enhances the current and protein expression of BK channels in the cell membrane, and BK channel expression in inner ear cells is reduced in AMPK $^{-/-}$  mice (Foller *et al.* 2012). Meanwhile, AMPK inhibits the current densities of BK channels in rat carotid body type I cells (Wyatt *et al.* 2007; Ross *et al.* 2011). Even though AMPK has been found to regulate sodium and potassium channels as well as various transporters, our study is the first to demonstrate the role of



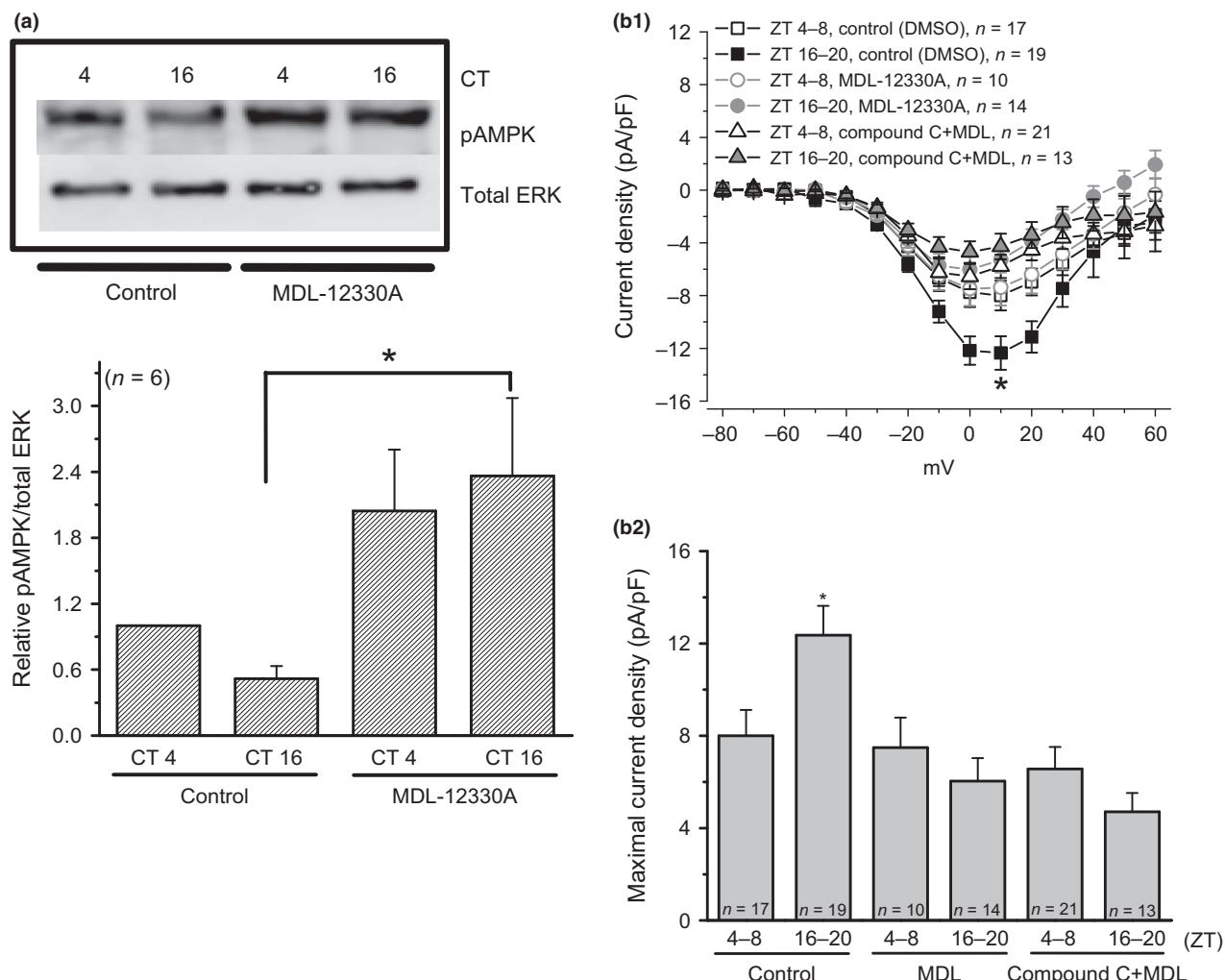
**Fig. 5** Activation of adenylyl cyclase with forskolin inhibits the activity of AMP-activated protein kinase (AMPK). The retinas were excised and cultured in constant darkness (DD) after chick embryos were entrained under light-dark cycles for 7 days. On the second day of DD, the cultured retinas were treated with dimethylsulfoxide (DMSO) (0.1%), forskolin, or forskolin+5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) for 2 h prior to harvest for immunoblotting at circadian time (CT) 4 and CT 16. (a) Treatment with forskolin

AMPK in regulating calcium channels, in which AMPK might be important for the regulation of calcium-dependent synaptic transmission in the nervous system. Since photoreceptors are non-spiking neurons (Barnes and Kelly 2002), calcium influx through L-VGCCs at the synaptic terminals allows for the continuous release of neurotransmitters from the ribbon synapses (Sterling and Matthews 2005). Here, we show that AMPK regulated the L-VGCCs in cone photoreceptors, which has not been reported previously. The L-VGCCs in cone photoreceptors exhibit circadian rhythms with the currents reaching maximum during the middle of the night (Ko *et al.* 2007). Activation of AMPK decreased the L-VGCC currents in cone photoreceptors in the middle of the night with a corresponding decrease in L-VGCC $\alpha$ 1D protein,

(20  $\mu$ M) at CT 4 significantly decreased the phosphorylation of AMPK at Thr172 (pAMPK) compared to the control (0.1% DMSO). \*indicates that pAMPK is significantly higher in the control at CT 4 than the other three groups. (b1 and b2) Treatment with forskolin alone significantly increases the L-VGCC current densities when cones were recorded during the day (Zeitgeber time, ZT 4–8), and AICAR dampens the augmentation effect of forskolin on L-VGCCs. \* $p < 0.05$ .

while inhibition of AMPK caused the opposite effect during the mid-day. These results give the first insight into the role of AMPK in circadian phase-dependent regulation of L-VGCCs, which indicates that AMPK might be important for photoreceptors to respond to various ambient lights throughout the course of a day.

The circadian regulation of L-VGCCs is in part through two parallel pathways: Ras-ERK and Ras-PI3K-AKT, and both are downstream of cAMP (Woods *et al.* 2005; Ko *et al.* 2007, 2009). cAMP had a negative action on AMPK, as we discovered that the activation of adenylyl cyclase by forskolin reduced AMPK activity, but inhibition of adenylyl cyclase increased AMPK phosphorylation (Fig. 5). While our result was similar to previous studies that showed activation of



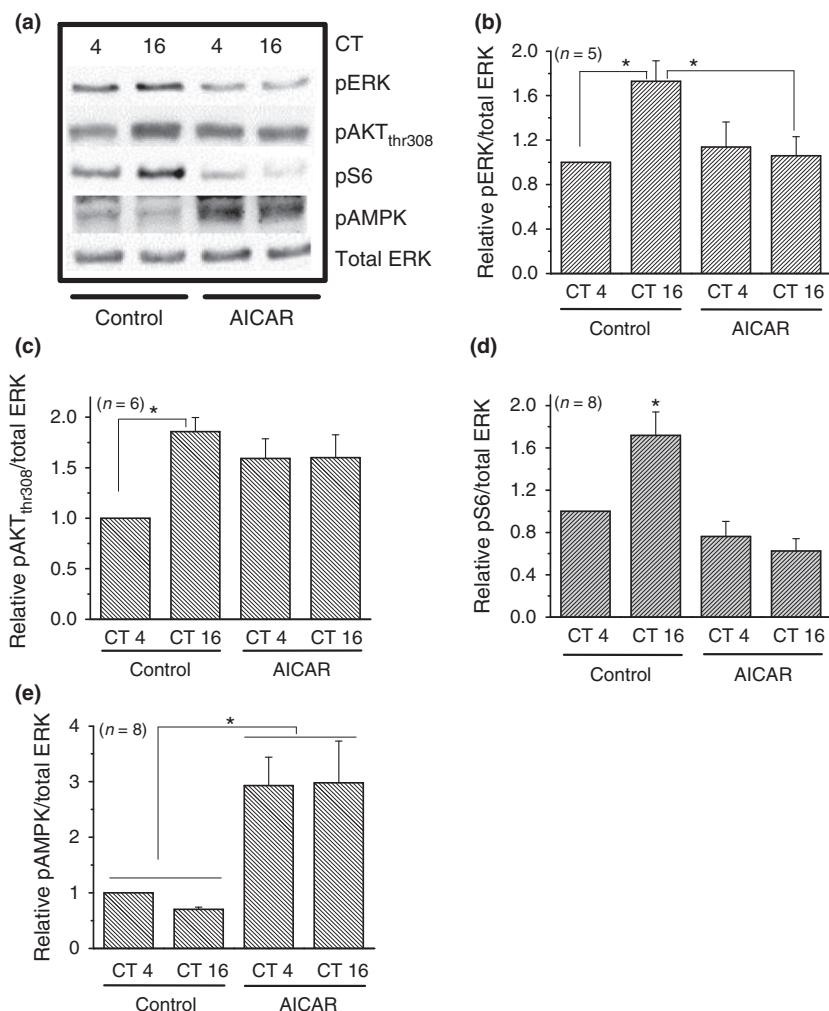
**Fig. 6** Adenylyl cyclase inhibitor MDL-12330A enhances the phosphorylation of AMP-activated protein kinase (AMPK). (a) Application of MDL-12330A (50  $\mu$ M) significantly increases AMPK phosphorylation at circadian time (CT) 16 compared to the control (at CT 16). (b) Treatment with MDL-12330A alone significantly decreases the L-

VGCC currents when cones were recorded at night (Zeitgeber time, ZT 16–20). Inhibition of AMPK with Compound C did not reverse the effect of MDL-12330A. \*indicates that the control at ZT 16–20 is significantly different from all other groups. \* $p < 0.05$ .

cAMP-protein kinase A (PKA) signaling decreases the phosphorylation of AMPK in several cultured cell-lines and adipocytes (Hurley *et al.* 2006; Djouder *et al.* 2010), cAMP signaling apparently increases AMPK phosphorylation on different amino acid residues in adipocytes (Yin *et al.* 2003; Omar *et al.* 2009). The seemingly conflicting reports on how cAMP signaling interacts with AMPK indicates that cAMP signaling triggered increase or decrease in AMPK phosphorylation could be ‘phosphorylation site’ and tissue specific. Phosphorylation of AMPK at different amino acids might contribute to different cellular functions, which will be very interesting for future investigation. Further, the activity of AMPK is influenced by other various factors including the intracellular  $\text{Ca}^{2+}$  concentration, the cellular AMP/ATP ratio, nitric oxide formation, and other metabolite signals (Viollet

*et al.* 2010; Mihaylova and Shaw 2011). We previously showed that the retinal nitric oxide content and the expression of neuronal nitric oxide synthase display circadian rhythms, and nitric oxide-dependent signaling further modulates L-VGCCs in cone photoreceptors (Ko *et al.* 2013). Hence, it is possible that  $\text{Ca}^{2+}$  influx through L-VGCCs, as well as nitric oxide-signaling might influence the activity of AMPK and subsequently promote AMPK to regulate L-VGCC trafficking in cone photoreceptors.

In our previous study, we found that mTORC1-S6K-S6 signaling is a downstream target of PI3K-AKT to modulate L-VGCC $\alpha$ 1D trafficking and translocation (Huang *et al.* 2013). The stimulation of AMPK significantly diminished the phosphorylation of S6 and moderately decreased the phosphorylation of ERK (Fig. 6). The integration of AMPK



**Fig. 7** AMP-activated protein kinase (AMPK) is a negative regulator of mTORC1 signaling. Chick embryos were light-dark entrained for 7 days. At E17, the retinas were dissected and cultured in constant darkness (DD). On the second day of DD, the cultured retinas were treated with the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) for 2 h. The retinal samples were collected at circadian time (CT) 4 and CT 16 for immunoblotting analyses. (a) The di-phospho-extracellular signal-regulated kinase (pERK), phospho-protein kinase B (AKT) at Thr308 (pAKT), phospho-S6 (pS6), and phospho-AMPK at Thr172 (pAMPK) were detected from samples treated with AICAR (500  $\mu$ M) or control. Since the total amounts of ERK, AKT, and S6 remained constant throughout the day, total ERK was used as loading control. (b) After treatment with AICAR, pERK is

decreased at CT 16 compared to the control group at CT 16. \*indicates that the control group harvested at CT 16 is significantly higher than CT 4 of control and the AICAR-treated at CT 16. (c) Treatment with AICAR seemed to increase pAKT at CT 4, but it did not reach statistical significance. \*indicates a significant difference between CT 4 and CT 16 of the control groups. (d) The level of pS6 is significantly dampened with AICAR treatments compared to the control group at CT 16. \*indicates the control group at CT 16 is significantly higher than the other three groups. (e) The phosphorylation of AMPK served as an internal control showing that treatment with AICAR for 2 h increases AMPK activities. \*indicates a significant difference between the AICAR groups and control groups at both CT 4 and CT 16, respectively. \* $p < 0.05$ .

into various signaling pathways to regulate L-VGCCs demonstrates the complexity of cell-signaling networks in the circadian outputs to regulate physiology and function at the cellular level. It also illustrates that AMPK may have multiple roles other than serving as an energy sensor in a cell. Regulation of ion channels, such as L-VGCCs, is energy consuming, since transporting synthesized channel proteins

to the plasma membrane and membrane retention of channel proteins require energy. The circadian fluctuation of photoreceptor AMPK activation might be a way for photoreceptors to be more energy efficient when light intensities change throughout the day.

Taken together, we showed that the activity of AMPK was under circadian control in the retina, with the retinal

ATP content displaying a diurnal rhythm. This report is the first to demonstrate the diurnal/circadian rhythm of the overall retina energy status. There was a circadian phase-dependent regulation of L-VGCCs by AMPK, and the action of AMPK was through crosstalk with ERK and mTORC1-S6, which was part of the complex signaling network in the circadian output regulation of photoreceptor physiology. Since L-VGCCs are essential in retinal neurotransmission, these results imply that AMPK plays an important role in the retina in regulating light sensitivities and adaptation to changes in ambient illumination across several orders of magnitude in the course of a day (Green and Besharse 2004).

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All experiments were conducted in compliance with the ARRIVE guidelines.

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