CIRCADIAN REGULATION OF L-TYPE VOLTAGE-GATED CALCIUM CHANNELS IN AVIAN RETINA

A Dissertation

by

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ABSTRACT

The circadian clock is an endogenous time-keeping mechanism that allows an organism to synchronize itself with external time cues and prepares the organism to anticipate upcoming environmental changes on a daily basis. The retina is a light-sensitive neuronal tissue located in the back of the eye. The circadian clocks in the retina enable the retina to anticipate daily ambient illumination over at least twelve orders of magnitude and initiate the adaptive processes with visual system throughout the course of a day. The retinal photoreceptors are responsible for phototransduction and transmitting the visual information into the brain. Unlike most neurons, photoreceptors do not fire action potentials, and they release neurotransmitter in a sustained manner, which is governed by the L-type voltage-gated calcium channels (L-VGCCs). The mRNA and protein expression of the α1 pore forming subunit of L-VGCCs are under circadian control, in which the protein expression of L-VGCCα1 with a corresponding increase in the L-VGCC current density is higher at night than during the day. Using the chicken embryo as a model system, an integrative strategy was used through combining biochemical, molecular, morphological, and electrophysiological analyses to investigate cellular mechanisms of the circadian regulation of L-VGCCs in the photoreceptors.

Three important cell signaling molecules and their pathways were investigated in this dissertation: calcineurin, mechanistic/mammalian target of rapamycin complex 1 (mTORC1), and AMP-activated protein kinase (AMPK). The activities of the protein

phosphatase calcineurin, as well as the protein kinase mTORC1 exhibited circadian oscillation with their activities higher at night than during the day, while the activities of AMPK are greater during the day compared to the activities at night. Inhibition of calcineurin and mTORC1 dampened the current densities and protein expression of L-VGCCs at night, while activation of AMPK decreased L-VGCC currents at night. These signaling molecules interacted with cAMP-Ras-MAPK and cAMP-Ras-PI3K-AKT signaling pathways to modulate the L-VGCC trafficking from the cytosol onto the plasma membrane in a circadian phase-dependent manner. The results demonstrated that the complex of cellular signaling pathways participated in the circadian regulation of L-VGCCs in the photoreceptors. Understanding the molecular mechanism underlying the circadian regulation of L-VGCCs in cone photoreceptors will provide important knowledge on how circadian clocks regulate retinal physiology and function in healthy states. Changes in L-VGCCs and these cell-signaling molecules might be indicators or biomarkers for age-related macular degeneration or other retinal degenerative diseases.

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NOMENCLATURE

AKT	Protein kinase B
AMPK	AMP-activated protein kinase
cAMP	Cyclic adenosine monophosphate
CT	Circadian time
ERG	Electroretinogram
ERK	Extracellular signal-regulated kinases
FKBP12	FK506 binding protein 12
GNL	Ganglion cell layer
L-VGCC	L-type voltage-gated calcium channel
INL	Inner nuclear layer
IPL	Inner plexiform layer
MAPK	Mitogen-activated protein kinases
mTORC1	Mechanistic/mammalian target of rapamycin
complex 1 ONL	Outer nuclear layer
OPL	Outer plexiform layer
PI3K	phosphatidylionositol 3 kinase
S6	S6 ribosomal kinase
SCN	Suprachaismatic nuclei
ZT	Zeitgeber time

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CHAPTER I

INTRODUCTION

1.1 Circadian biology

1.1.1: Circadian clock

The circadian clock, derived from the Latin roots “circa” meaning “about” and “diem” meaning “day”, is an endogenous time-keeping mechanism existing in living organisms, including bacteria, fungi, plants, and animals. This time-keeping mechanism self-oscillates continuously with a rough 24 hr period, even when the organisms are under constant conditions without any interruption from the external cues (Bell-Pedersen et al., 2005). The circadian clock is also able to provide a mechanism that allows an organism to synchronize its internal timing to the external time cues, as well as to anticipate upcoming environmental changes, such as daily fluctuations of temperature and ambient light. Therefore, the circadian clock is crucial for adaptation and survival of living organisms (Mohawk et al., 2012).

1.1.2: Circadian rhythm

There are a few keywords commonly used in chronobiology. Zeitgeber: zeitgeber is a German word meaning “time giver” (“Zeit” meaning “time” and “geber” meaning “giver”). A zeitgeber is an environmental signal that provides an external time cue to body’s endogenous circadian clocks. For example, the daily cycling light or temperature, and food can all serve as zeitgebers to provide external time reference to an

organism's circadian clock. Phase: the timing of a reference point in a rhythmic cycle (such as the peak of the cycle) relative to a fixed event (such as the beginning of the night phase) is the phase. Period: the time interval between phase reference points (such as the two peaks) is called the period. Simply, the duration of time to complete a rhythmic cycle is period. Amplitude: the difference between the peak and trough values is the amplitude (Fig1.1) (2006). Free running: the state of an organism that is under a constant condition without any external time cue is the "free-running" state.

The circadian rhythm, driven by the circadian clock in living organisms with a period of 24 h, is distinguished from other chrono-biological rhythms, such as ultradian rhythm with the period shorter than 24 h or infradian rhythm with the period longer than 24 h, by the following three criteria (Pittendrigh, 1960, Aschoff et al., 1971). First, an organism under the free-running state still maintains a self-sustained rhythmic period close to 24 h. Second, the phase and period of the daily rhythm in living organisms can be reset to 24 h by giving environmental cues. In most organisms, their own circadian rhythms do not run exactly at 24 h, but the zeitgeber can synchronize the organism's circadian rhythm to the environment, and such synchronization process is called circadian "entrainment". Lastly, the circadian clock has temperature compensation ability. It means that the period of an organism's circadian rhythm remains similar even under various ambient temperature conditions. Q10, a standard temperature dependency measurement of a biological process, is measured as the ratio of the rate of a biological reaction at the higher temperature divided by the rate at a temperature 10°C lower. For most of biochemical reactions, the value of Q10 is usually increased 2 or 3 fold when the

temperature increases every 10°C, while Q10 is approximately equal to 1 when the rate of biochemical reaction is independent from temperature fluctuations. In chronobiological research, Q10 is calculated as the ratio of the period rate at a higher temperature divided by the rate at lower temperature. Surprisingly, the Q10 values for free-running circadian rhythms in living organisms are from 0.8-1.4. Therefore, the period of circadian rhythms in the organisms is temperature compensated.

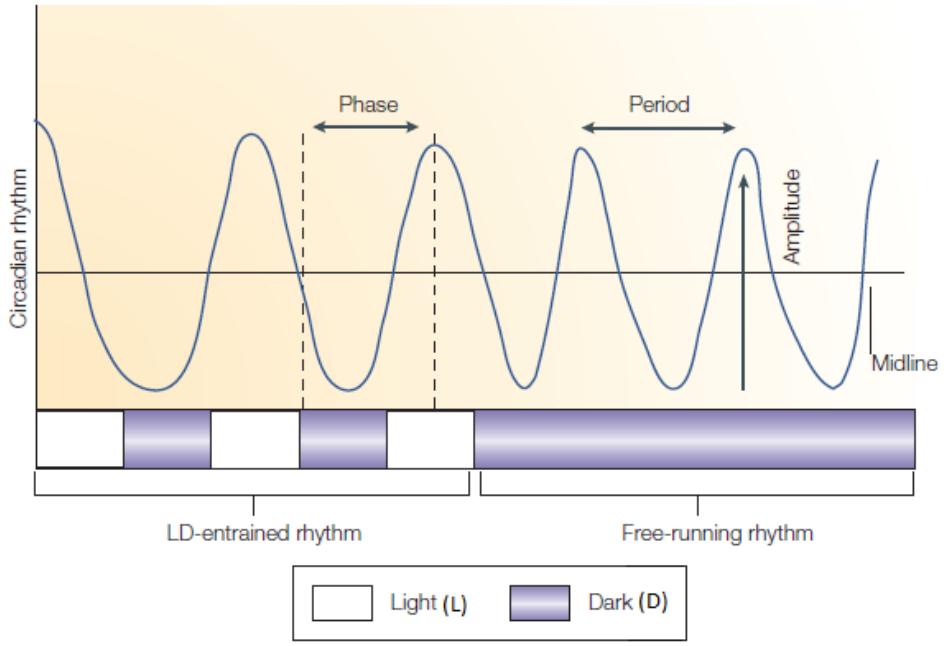


Figure 1.1 Terminology used for circadian rhythm. (Modified with permission from Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 6:544-556, Copyright 2005 by Nature Publishing Group.

1.1.3: The canonical circadian mechanism

There are three major components in the canonical circadian mechanism: the core oscillator, the input pathway, and the output pathway (Fig 1.2). The core oscillator, also known as the molecular clock, consists of a specific set of clock genes and their protein products that form interlocking transcription-translation feedback loops and generate oscillations with a period near 24 hr. The input pathway relays the external zeitgebers, such as light, temperature, odorants, and food, to the core oscillator, which allows the core oscillator to reset its timing according to the external cues. The core oscillator regulates expression of other genes in a time-of-a-day dependent manner, and hence, these "clock-controlled genes" serve as part of the circadian "output" pathway to modulate physiological responses, metabolism, and even behavior in the organisms (Wilsbacher and Takahashi, 1998, Reppert and Weaver, 2002, Lowrey and Takahashi, 2004, Bell-Pedersen et al., 2005).

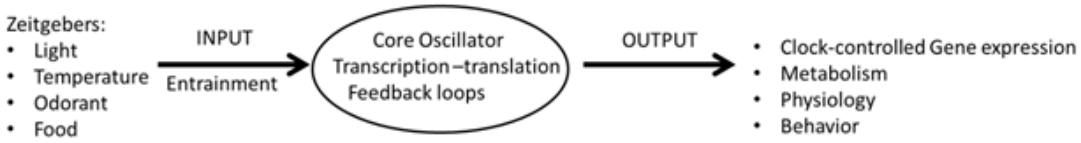


Figure 1.2 A schematic diagram of canonical circadian clock mechanism.

1.1.4: The molecular mechanism of the core oscillator

The mechanism of the cell-autonomous molecular clock in higher vertebrates is generated by a specific set of clock genes and their protein products forming two interlocking transcription-translation feedback loops thus to generate the circadian rhythm around 24 h (Fig 1.3). The two transcriptional activators that belong to the basic helix-loop-helix (bHLH) transcription factor family, CLOCK and BMAL1, heterodimerize and drive transcription through E-box enhancer sequences in the promoter region of their target genes , *Period (per*; with *per1*, *per2*, and *per3* in mammals*)* and *Cryptochrome (cry*; with *cyr1* and *cry2* in mammals*)*(King et al., 1997, Gekakis et al., 1998, Bunger et al., 2000). The protein products PER and CRY accumulate and form a complex in the cytoplasm, and then translocate back into the nucleus to inhibit their own transcription by repressing the transcriptional activity of CLOCK and BMAL1 (Kume et al., 1999, Shearman et al., 2000, Sato et al., 2006). The CLOCK-BMAL heterodimers activate another transcription-translation loop through activating transcription of retinoic acid-related orphan nuclear receptors, *Rev-erbα* and *Rorα.* Both Rev-ERBα and RORα subsequently translocate back into the nucleus and bind to retinoic acid-related orphan receptor response elements (ROREs) in the promoter region of *Bmal1* (Preitner et al., 2002, Sato et al., 2004, Akashi and Takumi, 2005). Rev-ERBα and RORα play opposite roles in the transcription of *Bmal1*. Rev-ERBα accumulates faster in the cytoplasm and quickly translocates and represses *Bmal1* transcription in the nucleus (Preitner et al., 2002, Guillaumond et al., 2005), while RORα accumulates more slowly, but it activates *Bmal1* transcription(Sato et al., 2004, Akashi

and Takumi, 2005, Guillaumond et al., 2005). Hence, the different actions of Rev-ERBα and RORα on *Bmal1* transcription lead to an antiphase oscillation of *Bmal1* expression.

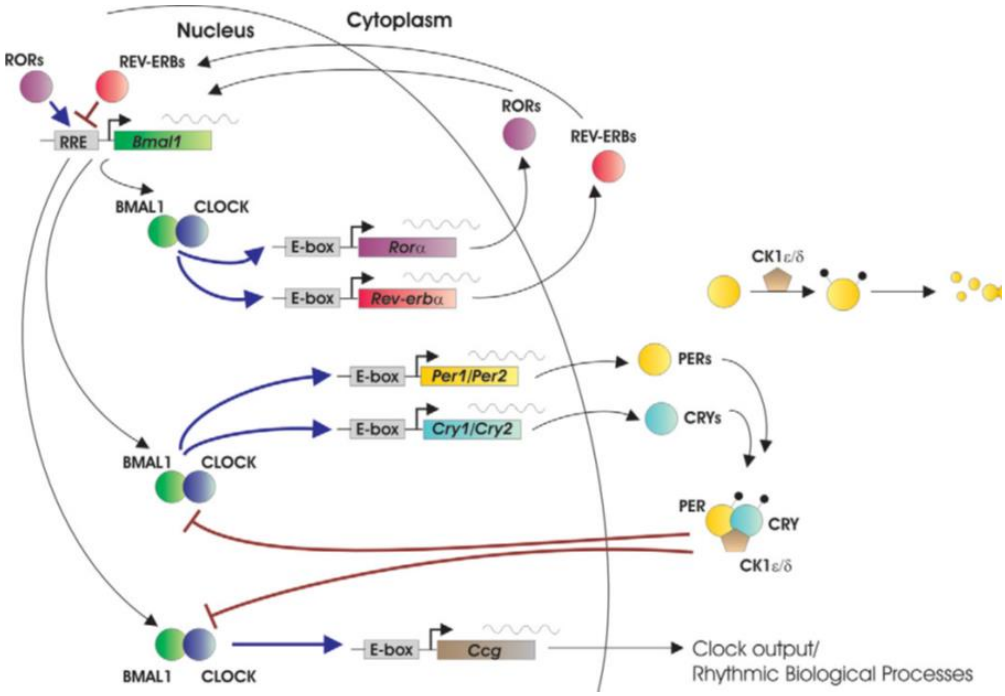


Figure 1.3 The molecular mechanism of the circadian clock in mammals.(Reprinted with permission from Caroline H. Ko and Joseph H. Takahashi (2006) Molecular components of the mammalian circadian clock. Human Molecular Genetic, 15(2): 271-277, copyright 2005 by Oxford University Press.)

1.1.5: The post-transcriptional regulation of the molecular clock

There are other post-transcriptional and post-translational regulation processes that modulate the molecular clock, so the stability and rhythmicity of the autoregulatory

feedback loops can remain near 24 h (Gallego and Virshup, 2007, Cibois et al., 2010). The first evidence of post-transcriptional process that the mRNA levels of the clock genes oscillate in a circadian fashion was found in *Drosophila* (So and Rosbash, 1997). Degradation of mRNA of clock genes results in the molecular clock instability, and that is at least partially mediated by the RNA binding proteins, (RNA-BPs) and/ or non-coding RNAs. The RNA-BPs polypyrimidine tract-binding protein 1 (PTBP1) and heterogenous nuclear riboprotein D (Hnrpd) are found to bind to the 3' untranslated region (3'UTR) of *Per2* and *Cry1* mRNAs, respectively, and promote the degradation of these mRNAs (Woo et al., 2009, Woo et al., 2010). Instead of causing mRNA degradation, the RNA-binding motif protein 4 (RBM4) binds to 3'UTR of *Per1* and stimulates its translation (Kojima et al., 2007). MicroRNAs, one of the non-coding RNA families, are also involved in maintaining the stability and translation of the clock gene mRNAs. Knockdown of microRNA 219 (miR-219) increases the length of the circadian period, while microRNA 132 (miR-132) modulates the light-induced phase-shifting and *Per1* transcription (Cheng et al., 2007).

1.1.6: The post-translational regulation of the molecular clock

The molecular clock is also governed by post-translational regulation, such as phosphorylation, sumoylation, acetylation, and ubiquitination. The first study on post-translational regulation of the molecular clock was observed from a tau mutant hamster, which exhibits a short circadian period with only 20 h (Ralph and Menaker, 1988). A later study revealed that the tau mutant hamster has a mutation in Casein Kinase I

epsilon (CK Iε), which binds to and phosphorylates PER (Lowrey et al., 2000). Phosphorylation of PER2 by CK Iε and another Casein Kinase Iδ (CK Iδ) causes its protein degradation through a proteasome-dependent pathway (Akashi et al., 2002, Eide et al., 2005), while CK Iε/CK Iδ regulates the nuclear entry of PER1. When PER1 alone is overexpressed in the human embryonic kidney 293 (HEK 293) cells, PER1 is abundantly expressed inside the nucleus (Vielhaber et al., 2000). Co-expression of PER1 with CK Iε or CK Iδ reduces nucleus accumulation and delays nuclear entry of PER1 by phosphorylating PER1 and retaining its protein product in the cytoplasm in HEK 293 cells (Vielhaber et al., 2000). The CK Iε/δ can also phosphorylate CRY when both proteins bind to PER and form a trimeric complex. CRY can overcome CK Iε-mediated cytoplasmic retention of PER1 and bring the complex into the nucleus and inhibit CLOCK-BMAL associated transcription in HEK 293 cells (Eide et al., 2002). Furthermore, the CRY1 is phosphorylated by AMP-activated protein kinase, which promotes F-box and leucine-rich repeat protein 3 (FBXL3 E3) ubiquitin ligase targeting for the proteasome-mediated degradation (Busino et al., 2007, Siepka et al., 2007), while the β-transducin repeat-containing protein 1 (β-TrCP1) ubiquitin ligase targets the phosphorylation of PER for degradation (Shirogane et al., 2005, Reischl et al., 2007). Taken together, the phosphorylation of clock proteins leads to protein degradation, which delays the repression of transcription and allows the molecular clock complete a cycle within 24 h.

There are other kinases and phosphatases involving in post-translational regulation of circadian clock. Glycogen synthase kinase-3 (GSK-3) is a serine-threonine

kinase with two isoforms, GSK-3α and GSK-3β, in mammals. GSK-3β can promote nuclear translocation of PER2 by phosphorylation of PER2 (Iitaka et al., 2005). In contrast, phosphorylation of CRY2, CLOCK and BMAL1 by GSK-3β leads to their degradation by the proteasome (Harada et al., 2005, Spengler et al., 2009, Sahar et al., 2010), while GSK-3β phosphorylates and stabilizes REV-ERBα (Yin et al., 2006). In addition, mitogen-activated protein kinase (MAPK) phosphorylates BMAL1 and inhibits the activation of BMAL1 –CLCOK complex-triggered transcription (Sanada et al., 2002). Although protein phosphorylation by kinases is important for transcription-translation feedback loops of the molecular clock, the level of phosphorylated proteins are also balanced in part by the dephosphorylation process from protein phosphatases. Protein phosphatase 5 (PP5) dephosphorylates CKIε autophosphorylation, which relieves the inhibitory effect of CKIε kinase activity (Partch et al., 2006). Protein phosphatase 1 can negatively regulate PER2 degradation through dephosphorylating PER2 (Gallego et al., 2006). In addition to modification of clock proteins by phosphorylation, the BMAL1 protein is also modified by other post-translational processes. The small ubiquitin-like modifier proteins (SUMO) bind to BMAL1 at the lysine residue for ubiquitin-dependent proteasomal degradation (Cardone et al., 2005, Lee et al., 2008). At the promoter regions of the *Per1* and *Per2*, the acetylation of histone H3 displays a circadian rhythm. This is in part due to the association of CLOCK with histone acetyltransferases (HATs) proteins to the *Per* promoter, which further regulates transcriptional activity in a time-dependent manner (Etchegaray et al., 2003). Interestingly, later research found that CLOCK itself exhibits an intrinsic HAT activity

that acetylates histones H3 and H4, and is able to rescue the circadian rhythm of PER1 in mouse embryonic fibroblast cells derived from homozygous CLOCK mutant mice when CLOCK transfects into cells (Doi et al., 2006). Acetylation of BMAL1 at lysine 537 by CLOCK accelerates recruitment of CRY for transcriptional repression (Hirayama et al., 2007), and the NAD+ dependent deacetylase sirtuin-1(SIRT1) interacts with CLOCK to regulate BMAL1 acetylation and circadian gene expression (Nakahata et al., 2008).

1.1.7: The master circadian clock

In higher vertebrates, there are multiple circadian oscillators existing in different organs. As for mammals, the master circadian clock is located in the suprachaismatic nuclei (SCN) of the hypothalamus in the brain (Moore and Eichler, 1972, Stephan and Zucker, 1972, Meijer and Rietveld, 1989). In the SCN neurons, the clock genes generate an autonomous 24 h circadian oscillation even when animals are kept in constant darkness (Klein et al., 1991, Reppert and Weaver, 2001). The SCN is also able to sustain the circadian oscillation in its neuronal activity, as well as gene expressions, even when it is isolated from the brain and cultured *in vitro* (Welsh et al., 1995). The SCN is capable of resetting the oscillation by receiving light input from retina through the retinohypothalamic tract. In the mammalian retina, there are intrinsic photosensitive ganglion cells (ipRGCs) that express melanopsin, a photosensitive opsin. The ipRGCs project their axons to the ventral core region of SCN and release glutamate and pituitary adenylate cyclase activating peptide, in order to transmit the light signal to the SCN (Provencio et al., 2000, Berson et al., 2002, Hannibal et al., 2002, Hattar et al., 2002, Do

and Yau, 2010). The SCN relays the "time information" to other brain regions, as well as peripheral tissues, to synchronize with external cues.

The SCN is a region with heterogeneous neurons forming a complex network. The neurons comprising the ventral core region of SCN express vasoactive intestinal polypeptide (VIP) and gastrin releasing peptide (GRP), while the neurons in the dorsomedial shell region of the SCN express neuropeptide arginine vasopressin (AVP). The dorsomedial shell region of SCN receives inputs from the ventral core region (Abrahamson and Moore, 2001). As a result, the change of clock gene expression in the dorsal SCN in responding to a light-induced shift is slower than the ventral SCN (Nagano et al., 2003). Even through the peak of the clock gene expression in the shell region differs from in the core region, and the individual SCN display circadian oscillations in different phases, the SCN neurons have the capacity to couple and synchronize with each other though synaptic transmission and gap junctions, and produce the coherent output signals, as well as keep the circadian rhythm with the period close to 24 h (Aton and Herzog, 2005, Welsh et al., 2010).

1.1.8: The peripheral clock

The circadian oscillators present outside of SCN are called peripheral clocks, which include the oscillators in the heart, lung, liver, skin, and other tissues. The peripheral clocks are under the influence of the SCN (Balsalobre et al., 2000b, Yamazaki et al., 2000, Stokkan et al., 2001, Storch et al., 2002, Nagoshi et al., 2004, Yamamoto et al., 2004, Yoo et al., 2004). In mice expressing the PER2::LUCIFERASE reporter,

peripheral tissues isolated from the body and maintained in cultures can self-sustain circadian oscillations. When the SCN is lesioned, peripheral tissues are able to maintain their own circadian rhythms, but the circadian phases and periods are varied among these tissues. The SCN mainly functions as a synchronizer between external zeitgeber and the peripheral clocks (Dibner et al., 2010). The axons of the SCN project to other brain regions, which subsequently coordinate the peripheral clocks through the autonomic system and hormonal signals (Ueyama et al., 1999, Balsalobre et al., 2000a, Yamazaki et al., 2000, Vujovic et al., 2008, Kalsbeek et al., 2010). The peripheral clocks can also be reset by low-amplitude temperature oscillations, such as the circadian rhythm of body temperature driven by SCN (Brown et al., 2002, Buhr et al., 2010).

1.2 The biology of the vertebrate retina

1.2.1: The organization of the retina

The retina is a highly organized tissue with different cell types (Dowling, 1970). The major neuronal layers are: the outer nuclear layer (ONL) composed of photoreceptors, the inner nuclear layer (INL) mainly composed of bipolar cells, horizontal cells, amacrine cells and Muller glia cells, and the ganglion cell layer (GNL) mainly composed of retinal ganglion cells and a few amacrine cells (Fig 1.4). While photoreceptors form synaptic connections with bipolar cells and horizontal cells in the outer plexiform layer (OPL), bipolar cells form synapses with ganglion cells and amacrine cells in the inner plexiform layer (IPL). Photoreceptors, bipolar cells and retinal ganglion cells are responsible for transmitting light information after

phototransduction takes place in the photoreceptors, with horizontal cells and amacrine cells serving as interneurons. While the majority of the retinal ganglion cells further relay the image-forming information into the thalamus as the primary visual pathway in the brain, a small number of ipRGCs project their axons into the SCN through the retinohypothalamic tract (Dowling, 1970, Kolb et al., 2001).

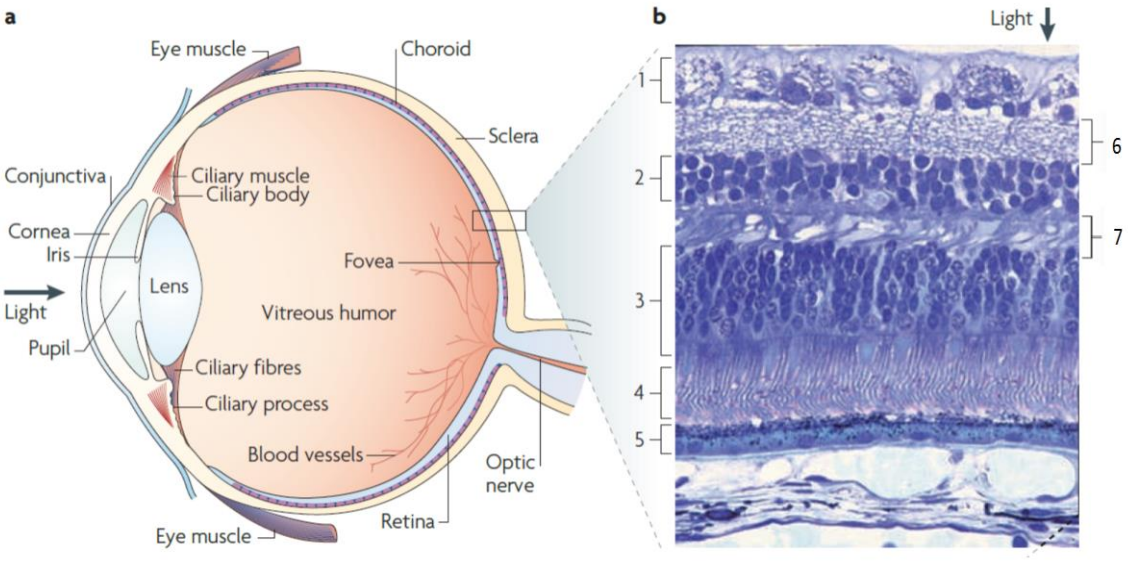


Figure 1.4 Structure of the human retina. (a) Schematic diagram of the human eye. (b) Cross-section of the human retina. (1) the ganglion cell layer (GNL), (2) the inner nuclear layer (INL), (3) the outer nuclear layer (ONL), (4) the outer segment of photoreceptors, (5) the retinal pigment epithelium, (6) the inner plexiform layer (IPL), and (7) the outer plexifrom layer (OPL). (Modified with permission from Wright A.,Chakarova, C., Abd El-Aziz M. and Bhattacharya S (2010) Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. Nature Reviews Genetics 11: 273-284, copyright 2010 by Nature Publishing Group.)

1.2.2: Photoreceptor cells

The structure of a photoreceptor consists of four parts: the outer segment, inner segment, nucleus and synaptic terminal (Fig1.5). The outer segment of a photoreceptor is responsible for phototransduction, a process that transmits the light signal into neuronal signals. The outer segments undergo a renewal process daily, with the outermost portion of the outer segment shed and removed by the retinal pigment epithelium (RPE) through phagocytosis. Cellular transcription, translation and metabolism happen in the inner segment as well as the nucleus of a photoreceptor. Materials needed for outer segment renewal is synthesized in the inner segment and transported through the connecting cilium. Lastly, the synaptic terminal is where the neurotransmitter is released from photoreceptors onto bipolar neurons or horizontal cells for signal communication.

There are two major types of photoreceptors in the vertebral retina: rods and cones. Rod photoreceptors are highly sensitive to photons, but they are easily saturated by bright light, so the rods are responsible for vision under dim light and at night. Since the outer segments of rods only contain one specific photosensitive pigment, rhodopsin, rods cannot detect colors. Cone photoreceptors are less sensitive to photons and not easily saturated by bright light, so cones are responsible for day-time vision. In primates, the outer segments of cones contain three different cone opsins, short-wavelength (S or blue -sensitive), medium-wavelength (M or green-sensitive) and long-wavelength (L or red-sensitive) opsins, so cones are able to detect different wavelengths

of light and responsible for color vision (Kolb, 1995, Kawamura and Tachibanaki, 2008). The outer segments of rods contain a set of discrete membrane discs with elongated shape, while the outer segments of cones are composed of continuous infolding membrane stacks with conical shape. In birds and reptiles, the cones but not rods have oil droplets at the base of the outer segments (Kolb and Jones, 1987). However, the oil droplets are not present in cone photoreceptors of mammalian retinas except for marsupials (Ahnelt et al., 1995).

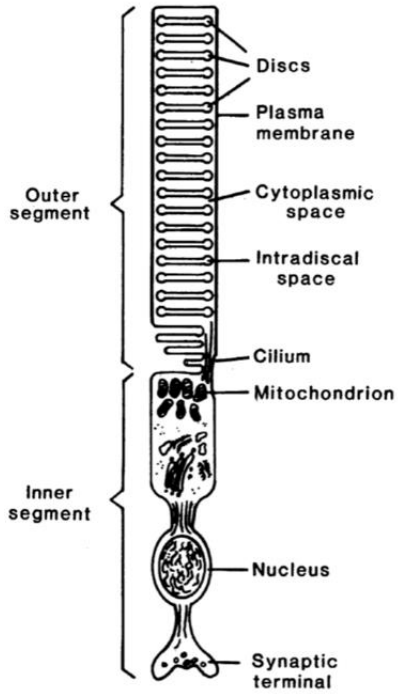


Figure 1.5 Schematic diagram of a rod photoreceptor. (Reprinted with permission from O'Brien, D.F. (1982) The chemistry of vision. Science, 218 (4576): 961-966, copyright 1982 by The American Association for the Advancement of Science)

1.2.3: Phototransduction

Phototransduction is the first step in vision, which occurs through a series of enzymatic steps in the outer segment of photoreceptors. When the light reaches the outer segment, the photon is absorbed by a vitamin-A-based retinaldehyde chromophore covalently bound to the opsin protein in the outer segment membrane. Absorption of a photon causes isomerization of retinal from 11-cis to all-trans configuration, and the all-trans retinal isomer no longer fits into the opsin binding site and results in the conformational change of opsin, so the opsin is enzymatically activated. The activated opsin stimulates the G protein, transducin, and leads to the α subunit of transducin releasing guanosine diphosphate (GDP) in exchange for guanosine triphosphate (GTP) in the cytoplasm. Gα-GTP dissociates from the β and γ subunits of transducing which further activates phosphodiesterase (PDE).

PDE is comprised of α, β, and γ subunits, and the catalytic α subunit of PDE is normally blocked by the γ subunit, so PDE is not able to hydrolyze cyclic guanosine monophosphate (cGMP). Gα-GTP of transducin binds to the PDE γ subunit and relieves the inhibitory effect of PDE. The activated catalytic site of PDE, therefore, is able to hydrolyze of cGMP to guanosine monophosphate (GMP). The decrease of the cytoplasmic concentration of cGMP results in closure of the cGMP-gated cation channels since there is less cGMP available. Hence, in the light, the cGMP-gated cation channels close, which causes a decline of cation influx. At the same time, potassium ions (K+) are continuously flowing out through the K+ selective leakage channels, so

together, the photoreceptor is hyperpolarized. The higher intensity of light activates more opsin molecules, which results in the closure of more cGMP-gated ion channels, and higher intensities of light will lead to more hyperpolarization of the photoreceptors. In the darkness, there are more cGMP molecules available to bind to the cGMP-gated cation channels and cause the opening of cGMP-gated cation channels, which leads to the influx of positively charged ions into the outer segment and depolarizes the photoreceptor. The inward current caused by the influx of cations through the cGMP-gated cation channels is therefore named the dark current (Lamb and Pugh, 2006, Fu and Yau, 2007).

1.2.4: Chemical synapse at the photoreceptor terminal

The chemical synapse at the photoreceptor terminal is a way of communication for the photoreceptors conveying light information to second order neurons, bipolar cells and horizontal cells. Synaptic transmission relies on the neurotransmitter glutamate released from the photoreceptor synaptic terminal at OPL onto dendrites of bipolar cells, or horizontals cells and this transmission is a calcium-dependent process. Photoreceptors are non-spiking neurons, so they do not fire action potentials, and hence, their neurotransmitter release is different from other spiking neurons. As the light evokes hyperpolarization of photoreceptors, L-type voltage-gated calcium channels (L-VGCCs) at the photoreceptor synaptic terminals close, thereby reducing calcium ions (Ca^{2+}) influx through L-VGCCs and decreasing neurotransmitter release. Conversely, in

darkness, the dark currents flowing into the outer segment keep the photoreceptor more depolarized, which allows L-VGCCs opening and sustained release of neurotransmitter.

There are two types of chemical synapses in the vertebrate retinas, the conventional synapse and the ribbon synapse. The conventional synapses in the retina are found in horizontal cells at OPL and amacrine cells at IPL, while the ribbon synapse are present in the photoreceptor cells as well as bipolar cells. In higher vertebrates, the conventional synapses are similar to synapses found in the nervous system, but ribbon synapses are only present in the retina and cochlea.

Ribbon synapses are characterized as a dense bar structure, the "ribbon", with synaptic vesicles arranged in an array tethering to the ribbon at the active zone. Since photoreceptors and bipolar cells are non-spiking neurons, instead of an action potential propagating to the axonal terminal and triggering the release of neurotransmitters, the ribbon synapse tonically releases neurotransmitter through a graded change of membrane potential and sustained Ca^{2+} influx at the presynaptic terminal. The SNARE complex is involved in synaptic vesicle fusion to the plasma membrane for docking and release at the ribbon synapse. However, the protein components of SNARE complex are slightly different from the SNARE at the conventional synapse. In the rodent retinal ribbon synapse, synataxin 3 replaces syntaxin 1 and forms a complex with SNAP-25 and synaptobrevin (Brandstatter et al., 1996, Morgans et al., 1996).

The proteins specifically associated with the ribbon synapse are, RIBEYE, bassoon, piccolo and KIF3A. RIBEYE is found exclusively located in the ribbon

structure but not in synaptic vesicles (Schmitz et al., 2000, Dieck et al., 2005). RIBEYE is a splice variant of transcriptional repressor C-terminal binding protein 2 (CtBP-2), with a proline-rich A domain and a B domain almost identical to CtBP2 (Schmitz et al., 2000). The A domain of RIBEYE constitutes a large scaffold and forms the backbone of the ribbon structure, whereas the B domain faces the cytoplasm and serves as a binding domains to other scaffold proteins including bassoon and piccolo. Although both bassoon and piccolo are found at the ribbon synapse (Brandstatter et al., 1999), they were first identified at the conventional synapses in brain (Garner et al., 2000). Bassoon functions as an anchoring protein for the ribbon and maintains the ribbon orientation with other synaptic structures (Dieck et al., 2005). In retina-specific bassoon knockout mice, the ribbon is unable to anchor to the presynaptic active zone, which results in the b-wave of electroretinogram (ERG) becoming diminished as the b-wave reflects the postsynaptic response of the bipolar cells. Therefore, bassoon is critical for normal photoreceptor synaptic transmission (Dick et al., 2003). A detailed study of molecular ribbon synapse shows that there are two compartments at ribbon complex, a ribbon-associated compartment including RIBEYE, piccolo, KIF3A, and a plasma membrane arciform density compartment. Bassoon is located at the border between the two compartments physically interacting with RIBEYE (Dieck et al., 2005). Piccolo is a cytomatrix scaffolding protein, which often co-localizes with bassoon and plays the role in assembly of the active zones (Takao-Rikitsu et al., 2004, Zhai and Bellen, 2004). Lastly, KIF3A, a member of the kinesin superfamily, functions as motor shutting that

transports the ribbon and synaptic vesicles from the soma to the active zone (Kondo et al., 1994, Muresan et al., 1998, Muresan et al., 1999, Garner et al., 2000).

1.2.5: Bipolar cells

Bipolar cells are non-spiking neurons in most mammalian retinas. The cell bodies of bipolar cells are located in the INL with their dendritic processes in the OPL to receive signals from photoreceptors and axons in the IPL and communicate with amacrine and ganglion cells (Kolb, 1970, Boycott and Kolb, 1973). In mammals, there is only one type of bipolar cell for rod photoreceptors, but there are different subtypes of bipolar cells for cones, which are species-dependent (cat:(Famiglietti, 1981, Kolb et al., 1981); rabbit:(Jeon and Masland, 1995); monkey:(Boycott and Wassle, 1991); rat:(Euler and Wassle, 1995). Despite the diverse subtypes of cone bipolar cells, cone bipolar cells can be broadly divided into two classes, "ON" cone bipolar cells and "OFF" cone bipolar cells, based on their different responses to light, but there are only ON rod bipolar cells.

The ON bipolar cells are depolarized in response to light when photoreceptors are hyperpolarized, while OFF bipolar cells are hyperpolarized in response to an increase of light intensity (Nelson and Kolb, 1983, Masu et al., 1995, Euler et al., 1996). In contrast, OFF bipolar cells are depolarized when the light is off and photoreceptors are depolarized. The opposite responses of ON and OFF bipolar cells to light are mainly due to distinct glutamate receptors that are expressed on bipolar cells. The ON bipolar cells possess metabotropic glutamate receptor 6 (mGluR6) (Yamashita and Wassle, 1991,

Nakajima et al., 1993, Nomura et al., 1994), while OFF bipolar cells possess α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite acid (KA) ionotropic glutamate receptors(Brandstatter et al., 1997, Hack et al., 1999, DeVries, 2000, Haverkamp et al., 2001b, a). The mGluR6 belongs to G_o/G protein-coupled receptor family, so after binding to glutamate, the ON bipolar cells become more hyperpolarized because of the intracellular signaling and coupled ion channels mediated by mGluR6 and Go. (Vardi, 1998, Morigiwa and Vardi, 1999, Nawy, 1999). The transient receptor potential M1 cation channels (TRPM1) has been identified downstream of mGluR6 (Shen et al., 2009, Koike et al., 2010a, Koike et al., 2010b)). TRPM1 is co-expressed with mGluR6 in dendrites of ON bipolar cells but absent in OFF bipolar cells, so in darkness, mGluR6 activation inhibits the cation influx through TRPM1 and causes bipolar cells to be hyperpolarized (Koike et al., 2010b). In darkness, when glutamate is released from photoreceptors, AMPA and KA receptors of OFF bipolar cells are activated, which causes AMPA and KA ion channels to open and allows the influx of cation. In summary, ON and OFF bipolar cells respond to light in opposite directions mainly because they express different types of glutamate receptors, and initiate independent visual pathways.

The bipolar cells release glutamate from their synaptic terminals at IPL to communicate with the third order neurons, the ganglion cells. The synaptic transmission relies on an increased intracellular Ca^{2+} concentration by Ca^{2+} influx through either T-type VGCCs (T-VGCCs) or L-type VGCCs (L-VGCCs) in a species-dependent manner. The T-VGCCs has been found in mouse bipolar cells (Kaneko et al., 1989), and the L-

VGCCs are present in glodfish bipolar cells (Kaneko et al., 1991). However, both T- and L-VGCCs are present in bipolar cells of zebrafish and salamanders.

1.2.6: Ganglion cells

Ganglion cells, the third-order spiking neurons that fire action potentials, are the final step of visual process in the retina. The ganglion cells can be divided into ON and OFF ganglion cells that receive inputs from bipolar and amacrine cells, and then send information into the different brain areas. In the vertebrate retina, the axon terminals of OFF cone bipolar cells lie in sublamina a of the IPL, in which they synapse with the dendrites of OFF ganglion cells, while the axon terminals of ON cone bipolar cells lie in sublamina b of the IPL, in which they contact with the dendrites of ON ganglion cells (Nelson et al., 1978, Bloomfield and Miller, 1986, Ammermuller et al., 1995, Euler et al., 1996, Ghosh et al., 2004, Pang et al., 2004). Once the ganglion cells receive signals from second-order neurons, the axons of ganglion cells form the optic nerve, which projects to the lateral geniculate nucleus for primary vision, to the superior colliculus for movement of head and eye in response to a stimulus, to the pretectal area for pupillary reflexes.

There are a small amount of light sensitive ipRGCs that also contribute image and non-image –forming vision (Hattar et al., 2002 , Gooley et al., 2003, Brown et al., 2010 , Ecker et al., 2010). Based on different morphology and physiological responses to light, there are at least five subtypes of ipRGCs identified (M1-M5) (Schmidt et al., 2008, Schmidt and Kofuji, 2009, Ecker et al., 2010). While the axons of M1 cells

project to SCN through the retinophypothalamic tract for circadian photoentrainment(Berson et al., 2002, Hattar et al., 2002) and to the pretectal area for pupillary reflexes, M2-M5 cells are involved in low-acuity vision by sending their axons to the lateral geniculate nucleus for image processing (Schmidt et al., 2011).

1.3 The circadian clock in the retina

1.3.1: Expression of clock genes in the retina

The retina, a light-sensitive neural tissue in the eye, possesses cell-autonomous peripheral oscillators that are not regulated by the SCN (Besharse and Iuvone, 1983, Tosini and Fukuhara, 2002). Thus far, the circadian oscillators in the retina have been found in many invertebrate and vertebrate species, including horseshoe crabs (Barlow, 1983), amphibians (Besharse and Iuvone, 1983), fish (Whitmore et al., 1998, Zaunreiter et al., 1998), avian (Pierce et al., 1993), and mammals (Pierce et al., 1993, Tosini and Menaker, 1996, 1998).

The clock genes are present in all retinal neurons, but the expression profiles of clock genes vary across species. In the rat retina, *Per1*, *Bmal1* as well as *Clock* mRNAs are abundant in the inner nuclear layer and the ganglion cell layer, but low expressed in the photoreceptor layer (Namihira et al., 1999, Namihira et al., 2001). Using laser capture microdissection, the *Per1*, *Per3*, *Cry1*, *Cry2*, *Clock*, *Bmal1*have been detected in the photoreceptor layer (Tosini et al., 2007, Schneider et al., 2010), and there is a robust circadian oscillation from the photoreceptor layer in the Per1: luciferase expressing rat (Tosini et al., 2007). In the mouse retina, the mRNAs of *Per1, Clock,* and *Bmal1* are

found in the photoreceptor layer, inner nuclear layer and ganglion cell layer (Gekakis et al., 1998). Although some studies supported that photoreceptors express all of the core clock genes (*Per1, Per2, Cry1, Cry2, Clock*, and *Bmal1*) (Yujnovsky et al., 2006, Dinet et al., 2007, Liu et al., 2012, Dkhissi-Benyahya et al., 2013), other studies reported that these genes are co-localized in the inner nuclear layer, as well as in the ganglion cell layer, but not all are expressed in the photoreceptor layer (Kuhlman et al., 2000, Witkovsky et al., 2003, Gustincich et al., 2004, Ruan et al., 2006, Dorenbos et al., 2007). The controversial results from mouse retina could due to the different techniques used in these studies. In non-mammalian vertebrate retinas, *Per2*, *Cry1* and *Cry2* mRNAs are expressed in the photoreceptors of *Xenopus* (Steenhard and Besharse, 2000, Zhu and Green, 2001b, a) and chicken (Bailey et al., 2002, Haque et al., 2002, Ko et al., 2003). *Bmal1* mRNA also exists in the chick retina (Chong et al., 2003). Even though the expression of the clock genes in which types of retinal neurons are not consistent among different species, the circadian clocks indeed are present in the retina, so the retina is able to anticipate the changes in ambient light intensities for visual adaptation. Although there are multiple circadian oscillators in the retina, these oscillators are coordinated through two neurotransmitters, dopamine and melatonin, to regulate the overall of daily adaptive physiology in the retina (Green and Besharse, 2004, Iuvone et al., 2005, McMahon et al., 2014).

1.3.2: Circadian clocks regulate retinal photoreceptor physiology

The circadian oscillator in the retinal photoreceptor regulates dynamic morphology and physiology (Cahill and Besharse, 1995, Tosini and Fukuhara, 2002). Outer segment disk shedding is balanced by the renewal process from the base of the outer segment in photoreceptors, and this process of shedding and renewal is under circadian control (LaVail, 1976, 1980). The rhythm of disk shedding in the photoreceptors is greater during the day than night in the chicken and mammals (Young, 1978, LaVail, 1980, Teirstein et al., 1980). In lower vertebrates, photoreceptors either elongate or shorten in response to changes in ambient light conditions and to circadian signals, and such movement is called retinomotor movement. In the light or during the day time, cones shorten and rods elongate, while in the dark or at night, movement are reversed (Welsh and Osborn, 1937, Levinson and Burnside, 1981).

The photoreceptor photopigment synthesis is also regulated by the circadian oscillator. The rod opsin mRNA rises during the light phase and decreases during the dark phase in fish and amphiabian species (Korenbrot and Fernald, 1989). The L cone photopigment iodopsin gene in the embryonic chick retinal cell cultures elevates its transcript at night (Pierce et al., 1993). mRNA levels of both rod rhodopsin and cone opsin are rhythmic throughout the course of a day and with a peak at the transition of light/dark cycle in the mouse retina (von Schantz et al., 1999). The circadian oscillators in the retina govern the synthesis and release of several neurotransmitters, including melatonin, dopamine (Wirz-Justice et al., 1984, Pozdeyev et al., 2000) and GABA

(Jaliffa et al., 2001). Retinal melatonin synthesis in photoreceptors peaks at night in lower vertebrates and most non-primate mammals (Pang et al., 1980, Cahill and Besharse, 1993, Tosini and Menaker, 1996, Lucas and Foster, 1997, Iuvone et al., 1999), and the enzymes required for its synthesis also display circadian rhythmicity (Besharse and Iuvone, 1983, Sakamoto and Ishida, 1998b, a, Ivanova and Iuvone, 2003b). Notably, there are certain mouse strains, such as C57BL/6 and BALB/c, that do not produce melatonin in the retina, while C3H/he mice do (Tosini and Menaker, 1998).

The photoreceptor ribbon synapses are under the circadian control, in which the arrangement of synaptic vesicles around the synaptic ribbon changes in a time-of-a-day fashion (Adly et al., 1999). Moreover, rod and cone photoreceptors are coupled by gap junctions, and this coupling is controlled by the circadian oscillator. The rod-cone coupling is strong at night, thereby allowing cones to receive dim light signals from rods and enhance the signal to noise ratio and detection of large objects. This rod-cone coupling is weak during the day, mainly because dopamine activates D2 receptors on both photoreceptors and decreases the conductance of rod-cone gap junctions in the daytime (Ribelayga et al., 2008). Lastly, the circadian oscillators govern ion channel activities in the retina. The affinity of cGMP-gated channels for cGMP existing in the outer segment is higher at night than during the day in chicken cone photoreceptors (Ko et al., 2001). Circadian regulation of L-type VGCCs has been found in the gold fish bipolar cells (Hull et al., 2006) and chicken cone photoreceptors (Ko et al., 2007), and in these retinal cells, the current amplitudes of L-type VGCCs are larger during the middle of night than during the middle of day.

1.4 The L-type voltage-gated calcium channels (L-VGCCs)

1.4.1: The physiological roles of VGCCs

Voltage-gated calcium channels (VGCCs) exist in many different cell types, such as muscles cells and neurons, and allow Ca2+ influx in response to membrane depolarization. In muscle cells, VGCCs initiate contraction, while in neurons, VGCCs mediate synaptic transmission. Calcium entering the cell through VGCCs serves as a second messenger in the cytosol to regulate various biochemical processes, including gene expression and enzyme activities, and activates Ca^{2+}- induced Ca^{2+} release through ryanodine receptors, a class of intracellular Ca^{2+} channels, to increase the intracellular Ca^{2+} concentration from nanomolar to micromolar level (Clapham, 2007). VGCCs can be distinguished into two major categories: high voltage-activated (HVA) channels that are activated when membrane is heavily depolarized, and low voltage-activated (LVA) channels that open channels by smaller changes of membrane voltage (Armstrong and Matteson, 1985, Bean, 1985). The HVA channels are heteromultimeric protein complexes that are composed of five subunits, a pore-forming α1 subunit and auxillary Cavα2,Cavβ, Cavδ, and Cav γ subunits, whereas the LVA channels are not found to associate with auxiliary subunits to date (Fig 1.6)(Perez-Reyes, 2003, Catterall et al., 2005, Perez-Reyes, 2006). The Cavα2 and Cavδ are linked by disulfide bonds into a signal subunit as Cavα2δ during post-translational modification (Takahashi et al., 1987).

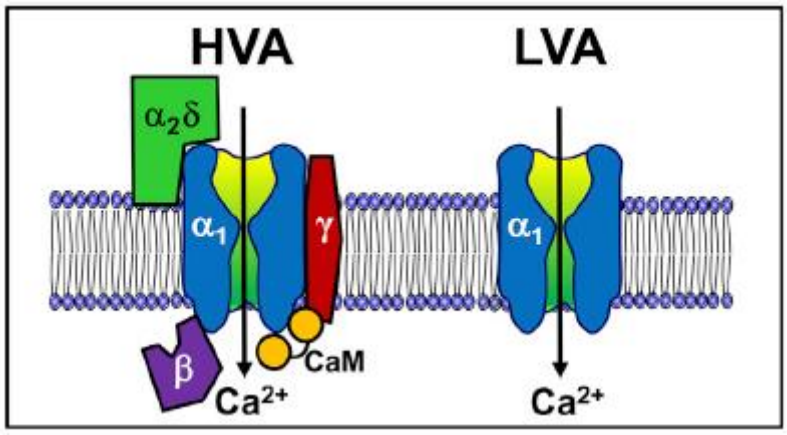


Figure 1.6 Subunit compositions of voltage- gated calcium channels. (Reprinted from Simms,B.A. and Zamponi, G.W. (2014) Neuronal Voltage-Gated Calcium Channels: Structure, Function, and Dysfunction. Neuron, 82(1): 24-45, copyright 2014 by Elsevier)

1.4.2: The classification of VGCCs

There are three major subfamilies of Cavα1: Cav1, Cav2 and Cav3, and each subfamily has several members (Table 1.1). The Cav1 channels, known as L-type channels (L presents as long-lasting), have four different members termed Cav1.1 (α1s), Cav1.2 (α1C), Cav1.3 (α1D) and Cav1.4 (α1F). The L-type VGCCs (L-VGCCs) display slow voltage-dependent inactivation, so they are long lasting when Ba2+ is used as a current carrier. L-VGCCs are sensitive to different dihydropyridine antagonists and agonists (Tsien et al., 1988, Randall and Tsien, 1995). L-VGCCs are expressed in the heart, lung, skeletal muscle, brain, retina (Bech-Hansen et al., 1998, Strom et al., 1998), lymphoid tissue (McRory et al., 2004), as well as pancreas (Yang and Berggren, 2006).

Cav2 channels have three members: Ca2.1 (α1A), Ca2.2 (α1B), and Ca2.3(α1E), and they are primarily localized in neurons. Ca2.1 channels, also named as P/Q-type channels, are blocked by ω-agatoxin IVA (Adams et al., 1993, Bourinet et al., 1999). P-type and Q-type currents were first recorded from Purkinje neurons and cerebellar granule neurons, respectively (Llinas and Yarom, 1981, Llinas et al., 1989, Randall and Tsien, 1995). Cav2.2 corresponds to N-type channels which are inhibited by ω-conotoxins GVIA and MVIIA (Dubel et al., 1992, Williams et al., 1992). N-type channels are activated at more negative voltage with relatively fast inactivation compared to L-type channels (Nowycky et al., 1985). The Cav2.3 channels encode R-type currents that are blocked by SNX-482 (Newcomb et al., 1998, Bourinet et al., 2001).

There are three members of Cav3 channels, Cav3.1 (α1G), Ca3.2 (α1H), Ca3.3 (α1I), which represent T-type channels expressed in neurons ,cardiac and smooth muscle cells (Cribbs et al., 1998, Perez-Reyes et al., 1998, Lee et al., 1999). Nickel is a potential blocker for Cav3.2, and some dihydropyridine antagonists at higher concentrations as well as kurtoxin have been used as Cav3 blockers, but these inhibitors may also interact with other VGCCs and voltage-gated sodium channels (Chuang et al., 1998, Heady et al., 2001, Perez-Reyes, 2003). Hence, there is no specific blocker for Cav3 channels thus far (Catterall, 2011). The Cav1 and Cav2 family belong to HVA family, and the Ca^{2+} currents are activated at more positive voltages, whereas, the Cav3 is designated as the LVA family activated at relative negative voltages.

Table 1.1 The classification of voltage-gated calcium channel subunits

Activation	Ca2+ current type	Pharmacological blocker	α1 subunits	Location
HVA	L	Dihydropyridine	Cav1.1(α1S)	Skeletal muscle
			Cav1.2(α1C)	Cardiac and smooth muscle, neurons, endocrine cells, sensory cells
			Cav1.3(α1D)	Sensory cells, cardiac muscle, endocrine cells, neurons.
			Cav1.4(α1F)	Sensory cells, lymphoid cells
HVA	P/Q	ω-agatoxin IVA	Cav2.1(α1A)	Neurons, neuroendocrine cells
HVA	N	ω-conotoxins GVIA and MVIIA	Cav2.2(α1B)	Neurons
HVA	R	SNX-482	Cav2.3(α1E)	Neurons, endocrine cells
LVA	T	None	Cav3.1(α1G)	Neurons, cardiac and smooth muscle
		None	Cav3.2(α1H)	Neuron, cardiac and smooth muscle
		None	Cav3.3(α1I)	Neurons

1.4.3: The structure of VGCCs

The Cavα1 subunit is a 190-250 kDa protein that is the principal subunit of VGCCs. The Cavα1 subunit contains four homologous transmembrane domains (I-IV) connected through cytoplasmic loops (Fig 1.7). Each domain has six transmembrane segments (S1-S6) and a pore-forming loop (P-loop) between S5 and S6. The four P-loops possess highly conserved negatively charged amino acids that form a pore for selecting and conducting cations, such as Ca^{2+} and Ba^{2+} (Yang et al., 1993, Sather and

McCleskey, 2003). The S4 segment is a voltage sensor that controls voltage-dependent activation of channel opening and closing.

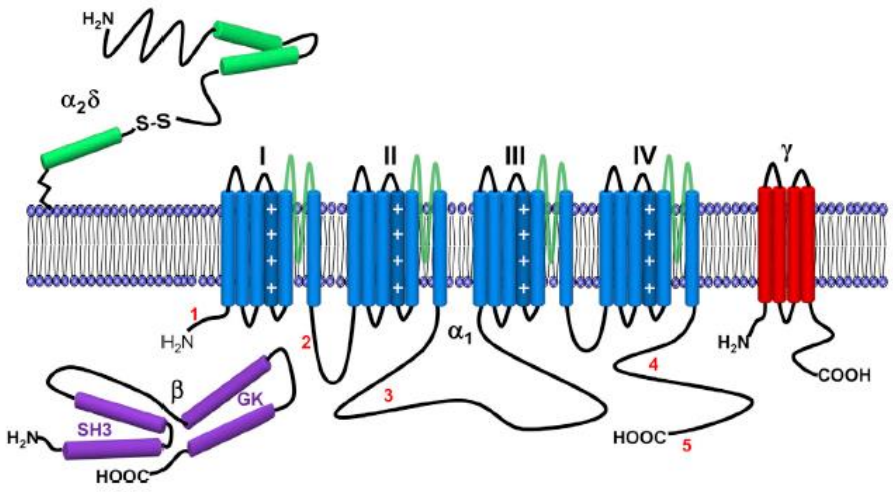


Figure 1.7 Subunit structures of voltage-gated calcium channels. (Reprinted from Simms,B.A. and Zamponi, G.W. (2014) Neuronal Voltage-Gated Calcium Channels: Structure, Function, and Dysfunction. Neuron, 82(1): 24-45, copyright 2014 by Elsevier).

Although the expression of Cavα1 subunit alone is sufficient to form a channel, co-expression of Cavα1 with auxiliary subunits enhances channel expression on the plasma membrane and alters channel gating properties to produce a fully functional channels (Lacerda et al., 1991, Singer et al., 1991). The Cavβ subunit plays an important role in the channel trafficking to the plasma membrane, in which Cavβ is essential for

the trafficking of Cavα1 to the plasma membrane. The Cavβ also modulates the channel gating of L-VGCCs. There are four Cavβ subunits (β1-β4) encoded by four distinct genes. All four Cavβ subunits can increase Ca2+ currents when they are co-expressed with Cav1 or Cav2α1 subunit through the following two mechanisms. First, the Cavβ can enhance expression of VGCC channels on the plasma membrane through preventing Cav1 and Cav2.2 channels undergo polyubiquitylation and proteasomal degradation (Altier et al., 2011, Waithe et al., 2011). Second, Cavβ can increase the channel open probability (Buraei and Yang, 2010, Dolphin, 2012). Most Cavβ subunits enhance the voltage-dependent activation and voltage-dependent inactivation to more hyperpolarized voltages (Lacerda et al., 1991, Dewaard and Campbell, 1995, Birnbaumer et al., 1998, Jones et al., 1998, Bogdanov et al., 2000). In addition to regulation of surface expression and gating of VGCCs, Cavβ subunits are involved in the G protein inhibition of Cav2 channels (Dolphin, 1998, Zamponi and Snutch, 1998, Dolphin, 2003) In the mammalian retina, only the Cavβ2 subunit is able to form a functional channel with Cavα1 for synaptic transmission from photoreceptors to bipolar cells (Ball et al., 2002).

There are four different α2δ subunits, and each α2δ subunit is encoded by a unique gene to a single protein that is cleaved post-translationally and linked by disulfide bonds into a 175kDa mature protein and attached to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor (Klugbauer et al., 1999, Davies et al., 2010, Dolphin, 2013). The Cavα2δ subunits have been found to increase Ca^{2+} currents by enhancing expression and retention of Cavα1 subunits on the plasma membrane (Gao et al., 2000, Klugbauer et al., 2003, Canti et al., 2005, Davies et al., 2006, Bernstein and

Jones, 2007, Hendrich et al., 2008, Davies et al., 2010). Additionally, the Cavα2δ subunits can function as the thrombospondin receptor to regulate synaptogenesis, which is independently from regulation of VGCCs (Eroglu et al., 2009, Kurshan et al., 2009). Recent study shows that overexpression of Cavα2δ in cultured hippocampal neurons modulates presynaptic function through increasing synaptic VGCCs and enhancing the probability of vesicle release (Hoppa et al., 2012).

There are eight different Cavγ subunits (γ1-γ8) that have various effects on VGCC activity. Ablation of Cavγ1 gene results in increased Ca2+ current of L-type VGCCs and shifts voltage-dependent inactivation to more depolarizing potentials (Freise et al., 2000). In contrast, co-expression of Cavγ2 and Cavγ4 subunits with Cav2.1α1subunit shifts voltage-dependent inactivation to more hyperpolarizing potentials (Klugbauer et al., 2000). When Cavγ2 subunit is expressed with Cavα1 and Cavα2δ subunits, the Ca2+ currents of P/Q type and N-type significantly decrease (Kang et al., 2001). Taken together, the Cavγ subunit is involved in the reduction of Ca2+ currents and inactivation properties of VGCCs.

1.4.4: The location of VGCCs in the retina

In the CNS synapse, P/Q type and N-type VGCCs localized at the active zones of presynaptic terminal mediate the neurotransmitter release (Wheeler et al., 1994). These calcium channels possess a synaptic protein binding site within the intracellular domain II-III of Cavα1 subunit that interacts with the SNARE complex, allowing channels close to synaptic vesicles (Sheng et al., 1994, Rettig et al., 1996, Sheng et al., 1996).

However, photoreceptors depend on L-type VGCCs (L-VGCCs) for synaptic transmission (Heidelberger and Matthews, 1992, Wilkinson and Barnes, 1996, Schmitz and Witkovsky, 1997, Thoreson et al., 1997, Hibino et al., 2002). Mutation of L-VGCCs results in blindness, since the absence of Ca2+ influx causes impairment of neurotransmitter release (Bech-Hansen et al., 1998). The L-VGCCα1C, L-VGCCα1D and L-VGCCα1F are present in the vertebrate retina, but the distribution of these channels is diverse in a species-and cell type-dependent manner (Table 1.2). The L-VGCCα1D are present in cell bodies and terminals of photoreceptors in non-mammalian and mammalian vertebrates (Morgans, 1999, Henderson et al., 2001, Hibino et al., 2002, Xu et al., 2002, Morgans et al., 2005, Ko et al., 2007). In adult chick retina sections, both L-VGCCα1C and L-VGCCα1D are distributed in the outer nuclear, inner nuclear and ganglion cell layers, while L-VGCCα1F is highly labelled in the outer plexiform layer (Firth et al., 2001). Interestingly, the L-VGCCα1F is exclusively expressed in rat rod and mouse rod bipolar cells in rodents (Morgans, 2001, Berntson et al., 2003).

Table 1.2 The distribution of L-VGCCs in the retina

α1 subunits	Species	Distribution in the retina
Cav1.2(α1C)	Chicken	Outer nuclear layer, ,inner nuclear layer, and ganglion cell layer
	Rat	Outer plexiform layer, inner plexiform layer, and inner nuclear layer
Cav1.3(α1D)	Rat	Outer nuclear layer and outer plexiform layer
	Mouse	Inner segment of photoreceptors, Outer plexiform layer (cone),inner plexiform layer
	Chicken	Outer nuclear layer, outer plexiform layer, inner nuclear layer and ganglion cell layer
	Tree shrew	Outer plexiform layer (cone)
Cav1.4(α1F)	Chicken	Outer plexiform layer, inner nuclear layer, inner plexiform layer,
	Mouse	Outer plexiform layer
	Rat	Outer plexiform layer, inner plexiform layer,

1.5 Objective and specific aims

The L-VGCCs in the cell bodies and terminals of photoreceptors mediate Ca2+ influx and therefore regulate cell metabolism, neurotransmission, cytoskeletal dynamics, gene expression and cell death (Krizaj and Copenhagen, 2002). Previous studies have demonstrated that the circadian oscillators existing in avian cone photoreceptors regulate the circadian rhythm of L-type VGCCs (Ko et al., 2007). Circadian regulation of L-type VGCCs is associated with the daily changes of ribbon synapses and glutamate release. Calcium entering through L-VGCCs serves as circadian input/ output signals to

modulate melatonin synthesis in avian photoreceptors. The mRNA levels and protein expression of the channel pore-forming α1 subunits are rhythmic. The current densities of L-VGCCs are larger when cells are recorded at night, compared to cells recorded during the day (Ko et al., 2007). However, the underlying mechanisms of circadian regulation of L-VGCCs are not well-understood. Once an ion channel protein is translated, it needs to be transported and inserted onto the plasma membrane to be fully functional. Hence, the goal of this dissertation is to investigate how the circadian oscillators control the trafficking and insertion of L-VGCCs in chicken cone photoreceptors. There are two parallel signaling pathways that have been characterized as part of the circadian output pathway regulating the circadian rhythms of L-VGCCs in cones: the Ras-ERK and the Ras-PI3K-AKT signaling pwthways (Ko et al., 2007, Ko et al., 2009). However, there are still major gaps between the circadian oscillators and L-VGCCs that need further investigations. The central hypothesis of this dissertation is that post-translational mechanisms, such as phosphorylation, dephosphorylation, and methylation, as well as various cellular signaling pathways are involved in the circadian regulation of L-VGCCs in avian cone photoreceptors. By using biochemical, molecular, electrophysiological and morphological analyses, three specific aims will be examined in this dissertation:

Specific aim 1: Examine the role of calcium/calmodulin-dependent serine/threonine phosphatase, calcineurin, in the circadian regulation of L-VGCCs in cone photoreceptors.

Specific aim 2: Determine the relationship between the mechanistic/mammalian target of rapamycin complex (mTORC) signaling pathway and the circadian rhythms of L-VGCCs in cone photoreceptors.

Specific aim 3: Evaluate AMP-activated protein kinase (AMPK), the cellular energy sensor, and its role in the circadian regulation of L-VGCCs in cone photoreceptors.

CHAPTER II

CALCINEURIN SERVES IN THE CIRCADIAN OUTPUT PATHWAY TO REGULATE THE DAILY RHYTHM OF L-TYPE VOLTAGE-GATED CALCIUM CHANNELS IN THE RETINA[1]

2.1 Overview

The L-type voltage-gated calcium channels (L-VGCCs) in avian retinal cone photoreceptors are under circadian control, in which the protein expression of the α1 subunits and the current density are greater at night than during the day. Both Ras-mitogen-activated protein kinase (MAPK) and Ras-phosphatidylionositol 3 kinase-protein kinase B (PI3K-AKT) signaling pathways are part of the circadian output that regulate the L-VGCC rhythm, while cAMP-dependent signaling is further upstream of Ras to regulate the circadian outputs in photoreceptors. However, there are missing links between cAMP-dependent signaling and Ras in the circadian output regulation of L-VGCCs. In this study, we report that calcineurin, a Ca2+/calmodulin-dependent serine (ser)/threonine (thr) phosphatase, participates in the circadian output pathway to regulate L-VGCCs through modulating both Ras-MAPK and Ras-PI3K-AKT signaling. The activity of calcineurin, but not its protein expression, was under circadian regulation.

[1]Reprinted with permission from "Calcineurin serves in the circadian output pathway to regulate the daily rhythm of L-type voltage-gated calcium channels in the retina" by Huang, C.C.Y., Ko, M.L., Vernikovskaya, D.I. and Ko, G.Y.P. (2012), Journal Cellular Biochemistry 113(3), 911–922 . Copyright 2011 Wiley Periodicals, Inc..

Application of a calcineurin inhibitor, FK-506 or cyclosporine A, reduced the L-VGCC current density at night with a corresponding decrease in L-VGCCα1D protein expression, but the circadian rhythm of L-VGCCα1D mRNA levels were not affected. Inhibition of calcineurin further reduced the phosphorylation of ERK and AKT (at thr 308) and inhibited the activation of Ras, but inhibitors of MAPK or PI3K signaling did not affect the circadian rhythm of calcineurin activity. However, inhibition of adenylate cyclase significantly dampened the circadian rhythm of calcineurin activity. These results suggest that calcineurin is upstream of MAPK and PI3K-AKT but downstream of cAMP in the circadian regulation of LVGCCs.

2.2 Introduction

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

phosphatidylionositol 3 kinase-protein kinase B (PI3K-AKT), and both are downstream of cAMP signaling (Ko et al., 2007, Ko et al., 2009).

Calcineurin, also known as protein phosphatase 2B (PP2B), is a Ca2+/calmodulin-dependent ser/thr phosphatase, which often dephosphorylates the targets of Ca2+/calmodulin-dependent kinase II (Wang and Kelly, 1996, Ghetti and Heinemann, 2000, Wen et al., 2004, Gerges et al., 2005). Calcineurin contains a 58–64 kDa calmodulin-binding catalytic subunit and a 19 kDa Ca2+-binding regulatory subunit(Klee et al., 1979, Klee et al., 1988). In the retina, calcineurin is expressed in various neurons including photoreceptors (Cooper et al., 1985, Nakazawa et al., 2001). Glutamate induces hyperpolarization of postsynaptic ON bipolar cells by binding to metabotropic glutamate receptors (mGluR6), and this depression of postsynaptic response is mediated through calcineurin (Snellman and Nawy, 2002). However, little is known about the role of calcineurin in the retina, or whether it participates in the visual process. Therefore, in this study, we set forth to investigate the role of calcineurin in the regulation of retinal photoreceptors.

Both FK-506 and cyclosporine A are widely used as immunosuppressants in post-organ transplantation and autoimmune diseases, partially through their actions as calcineurin inhibitors (Emmel et al., 1989, Tocci et al., 1989, Shapiro et al., 1991, Fruman et al., 1992). FK-506 and cyclosporine A inhibit calcineurin through interactions with FK-506 binding protein 12 (FKBP12) and cyclophilin A, respectively(Liu et al., 1991). Inhibition of calcineurin causes circadian phase shifts in mammals, which

indicates that calcineurin is involved in the circadian input pathway to reset or entrain the circadian clock in the suprachiasmatic nucleus (SCN; (Ding et al., 1998, Katz et al., 2008). In addition, other ser/thr phosphatase families, such as PP2A and PP1, are known to directly regulate the circadian clock mechanism in *Drosophila* and *Neurospora* (Sathyanarayanan et al., 2004, Yang et al., 2004, Schafmeier et al., 2005, Fang et al., 2007). Therefore, it is possible that calcineruin may have circadian phase-dependent actions in vertebrates. Here, we report that calcineurin served as part of the circadian output pathway, downstream from cAMP but upstream of Ras, to regulate photoreceptor L-VGCCs. These results suggest that in addition to phase-shifting as previously reported, calcineurin also serves in the circadian output pathway to regulate downstream targets.

2.3 Materials and methods

2.3.1: 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 days as described previously (Ko et al., 2007, Ko et al., 2009). Cultures were prepared in the presence of 20 ng/ml ciliary neurotrophic factor (CNTF; R&D Systems, Minneapolis, MN, USA), which yields cultures highly enriched with cone photoreceptors (Adler et al., 1984, Adler and Hatlee, 1989, Belecky-Adams et al., 1996) and 10% heat-inactivated horse serum. Cell culture incubators (maintained at 39°C and 5% CO2) were equipped with lights and

timers, which allowed for the entrainment of retinal circadian oscillators to 12h: 12 h light-dark (LD) cycles in vitro. Zeitgeber time zero (ZT 0) was designated as the time when the lights turned on and ZT 12 was the time when the lights went off. For in ovo entrainment, intact eggs at E10 were exposed to LD 12h: 12 h for 7 days. Retina cells were then dissociated, cultured, kept in constant darkness (DD), and used for biochemical and molecular biological assays on the second day of DD. In some experiments, after in ovo LD entrainment for 6 days, eggs were kept in DD for another day. On the second day of DD, retinas were collected at different circadian time (CT) points throughout a day for biochemical assays (Ko et al., 2007, Ko et al., 2009). The reason for using chick embryos from E12+6 for in vitro entrainment or E18 for in ovo entrainment is that more than 90% of the retina photoreceptors express functionally mature VGCC currents by E18 (Gleason et al., 1992).

2.3.2: Immunoblot analysis

Samples were collected and prepared as described previously (Ko et al., 2007, Ko et al., 2009). Briefly, intact retinas were homogenized in Tris lysis buffer including (in mM): 50 Tris,1 EGTA, 150 NaCl, 1% Triton X-100, 1% β-mercaptoethanol, 50 NaF, 1 Na3VO4; pH 7.5. Samples were separated on 10% sodium dodecyl sulfate–polyacrylamide gels by electrophoresis and transferred to nitrocellulose membranes. The primary antibodies used in this study were anti-pan calcineurin A (Cell Signaling Technology, Danvers, MA, USA), an antibody specific for di-phospho-ERK (pERK; Sigma, St. Louis, MO, USA), an antibody insensitive to the phosphorylation state of

ERK (total ERK, used for loading control; Santa Cruz Biochemicals, Santa Cruz, CA, USA), anti-VGCCα1D subunit (Alomone, Jerusalem, Israel), and anti-Ras (Millipore, Temecula, CA, USA). Blots were visualized using appropriate secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) and an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA). Relative protein expressions for all proteins involved in this study are reported as a ratio to total ERK, since total ERK remains constant throughout the day. Band intensities were quantified by densitometry using Scion Image (NIH, Bethesda, MD, USA). All measurements were repeated at least 3 times.

2.3.3: Calcineurin activity assay

Retina samples were lysed in a phosphatase lysis buffer including (in mM): 50 Tris, pH 7.5, 1 EGTA, 150 NaCl, 1%Triton X-100, and 1% β-mercaptoethanol. Calcineurin activities were assayed using a commercially available ser/thr phosphatase assay kit (Promega, Madison, WI, USA). This kit can distinguish between tyrosine (tyr) and ser/thr phosphatases by using a synthetic polypeptide, RRA(pT)VA, that is compatible with ser/thr phosphatases but is structurally incompatible for tyr phosphatases. To differentiate between PP2A, 2B, and 2C, the reaction buffer is made to favor one over the others since this class of enzyme has a diverse range of optimum conditions. For calcineurin (PP2B), the reaction buffer contained 250 mM imidazole (pH 7.2), 1 mM EGTA, 50 mM MgCl2, 5 mM NiCl2, 250 ug/ml calmodulin, and 0.1% β-mercaptoethanol, as described in the manufacturer's protocol. Free cytoplasmic

phosphate was first removed from the samples then dephosphorylation of the kit's calcineurin substrate proceeded for 30 min at room temperature (RT). This system determines the amount of free phosphate generated in a reaction by measuring the absorbance (600 nm) of a molybdate/ malachite green/ phosphate complex.

2.3.4: 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 BaCl2, 0.4 MgCl2, 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 MgCl2, 0.1 CaCl2, 1.1 EGTA, and 10 HEPES, pH 7.3 adjusted with CsOH. Recordings were made only from cells with elongated cell bodies with one or more prominent oil droplets (hallmark of avian cone photoreceptors). Currents were recorded at RT (23°C) using an Axopatch 200B (Axon Instruments/Molecular Devices, Union City, CA, USA) or 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). After Gigaohm seals were formed, the electrode capacitance was compensated. Cells were held at −65mV, and ramp voltage commands from −80 to +60 mV in 500 ms were used to evoke Ba2+ 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. FK-506 and cyclosporine A were obtained from A.G. Scientific (San Diego, CA, USA). The concentrations of FK-506 (Wilson et al., 2001, Okazawa et al., 2009, Mukherjee et al., 2010) and cyclosporine A (McDonald et al., 1996, Bambrick et al., 2006, Chen et al., 2009, Rana et al., 2009, Tan et al., 2011) used in this report were based on previous studies using these inhibitors in various neuronal tissue or cell preparations.

2.3.5: Quantitative real-time reverse transcription (RT) polymerase chain reaction (Q-PCR)

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

sequences were listed previously(Ko et al., 2004, Ko et al., 2007). All measurements were repeated 6 times.

2.3.6: cAMP assay

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

2.3.7: Ras activation assay

Ras activity was determined by a commercially available kit (Millipore), and the procedure was outlined previously(Ko et al., 2004). The procedure takes advantage of the fact that only activated Ras binds to the Ras binding domain of Raf-1 (Raf-1 RBD). The Raf-1 RBD is a GST (glutathione S-transferase) fusion-protein bound to glutathione agarose. Cultured retina cells (control and FK-506 treated) were lysed in a Mg2+ lysis buffer. A small portion (20 µl) of the supernatant was saved for total ERK (loading control) analysis by Western blotting. The remaining supernatant was incubated with Raf-1 RBD agarose for 45 min at 4°C. Subsequently, the agarose beads were pelleted,

washed, and boiled in 2× Lamelli buffer (20 µl). Samples were then subjected to Western immunoanalysis as described above. n=4.

2.3.8: Statistical analysis

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

2.4 Results

2.4.1: Calcineurin activity, but not its protein expression, is under circadian control

Calcineurin is known to be involved in circadian phase-shifting in mammals (Katz et al., 2008), but it is not clear whether calcineurin itself is expressed in circadian oscillations. We first examined whether the protein expression or the activity of calcineurin was under circadian control. Chick embryos were entrained to LD cycles for 7 days in ovo then kept in DD. On the second day of DD, retinas were collected at six different CT points for immunoblotting or calcineurin activity assay. Previously, we showed that the total amount of ERK protein is constant throughout the day, while the phosphorylation status of ERK (phosphorylated ERK) is under circadian control (Ko et al., 2001, Ko et al., 2007). Therefore, in this study, we used total ERK as the loading control. We found that calcineurin protein expression was constant throughout the day

(Fig. 2.1A), but its activity was under circadian control with peak activity during the middle of the subjective night (CT16) with a threefold difference between apex and trough values (Fig. 2.1B). Hence, there was a circadian regulation of calcineurin activity at the post-translational level in the chick retina.

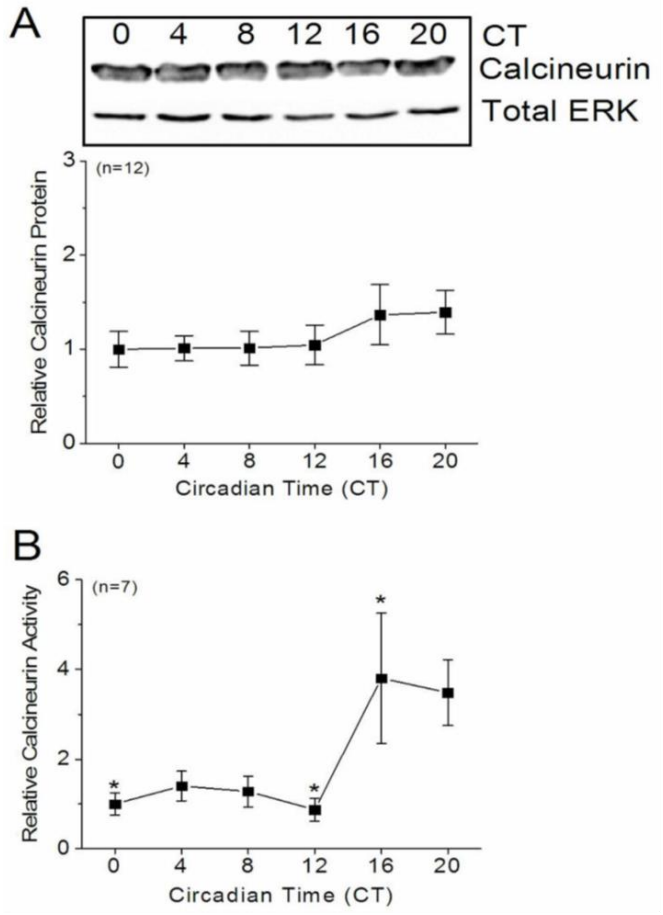


Figure 2.1 Calcineurin activity was under circadian control.
On the second day of DD after entrainment to 12 hr LD cycles for 7 days in ovo, intact retinas were collected at 6 different circadian time points (CT 0, 4, 8, 12, 16, and 20) for the following assays. (A) There was no significant change in the protein expression of calcineurin across 6 circadian times by Western immunoblotting. n=12 for each time point. (B) There was a circadian regulation of calcineurin activity. * indicates that the calcineurin activity is significantly higher at CT 16 compared to CT 0 and CT 12. n=7 for each time point. *$p<0.05$.

2.4.2: There is a circadian phase-dependent modulation of L-VGCCs by calcineurin

We previously found that L-VGCCs are under circadian control in cone photoreceptors(Ko et al., 2007, Ko et al., 2009), with maximal current density elicited at 0 mV significantly larger when cells are recorded during the subjective night than during the subjective day (Fig. 2.2;(Ko et al., 2007, Ko et al., 2009). The underlying mechanism of the L-VGCC circadian rhythm is in part attributed to the circadian regulation of both mRNA and proteinexpression of the L-VGCCα1 subunit (Ko et al., 2007), as well as α1 subunit trafficking andinsertion / retention into the plasma membrane (Ko et al., 2007, Ko et al., 2009, Shi et al., 2009a). Various signaling pathways are involved in the circadian regulation of L-VGCCs, including CaMKII, Ras-MAPK, and Ras-PI3K-AKT (Ko et al., 2007, Ko et al., 2009, Tan et al., 2011). Since CaMKII is involved in the circadian regulation of L-VGCCs, we hypothesized that calcineurin, a Ca2+-calmodulin dependent phosphatase that often dephosphorylates the same targets of CaMKII, might also participate in the circadian regulation of L-VGCCs. We examined whether inhibitors of calcineurin might have a circadian phase-dependent effect on L-VGCC currents. Whole-cell patch recordings were performed from cultured cone photoreceptors at either ZT (or CT) 4–8 or 16–20. We observed that calcineurin inhibitors evoked a circadian phase-dependent modulation of L-VGCCs. Application of the calcineurin inhibitor FK- 506 (10 μM) for 2 hr prior to recordings decreased L-VGCC current density when cells were recorded at night (ZT 16–20), but did not affect L-VGCC recordings during the day (ZT 4–8; Fig. 2.2B, 2.2C). Similarly, application of a structurally unrelated calcineurin inhibitor, cyclosporine A (2 μM), for 2 hr prior to

recordings also caused a significant decrease in photoreceptor L-VGCC current densities when recorded during the subjective night (CT16–20; Fig. 2.2D). Hence, treatment with a calcineurin inhibitor decreased L-VGCC currents at night under both LD (ZT 16–20) and DD (constant darkness, CT 16–20) conditions. Since calcineurin is known to cause circadian phase shifting in mammals (Katz et al., 2008), it is possible that the circadian phase-dependent regulation of L-VGCCs by calcineurin could be due to phase-shifting, in which L-VGCCα1 subunit mRNA would be affected after calcineurin inhibitor treatments. However, we found that while FK-506 dampened the circadian rhythm of L-VGCCα1D protein expression (Fig. 2.3A), it had no effect on the circadian rhythm of L-VGCCα1D mRNA levels (Fig. 2.3B). Hence, the circadian phase dependent action of calcineurin was not as a circadian input to shift the circadian phase of LVGCCs, since affecting the circadian input pathway or the molecular clock itself would alter the circadian rhythm/phase of L-VGCCα1D mRNA levels. Thus, we set forth to examine the role of calcineurin as part of the circadian output to regulate L-VGCCs.

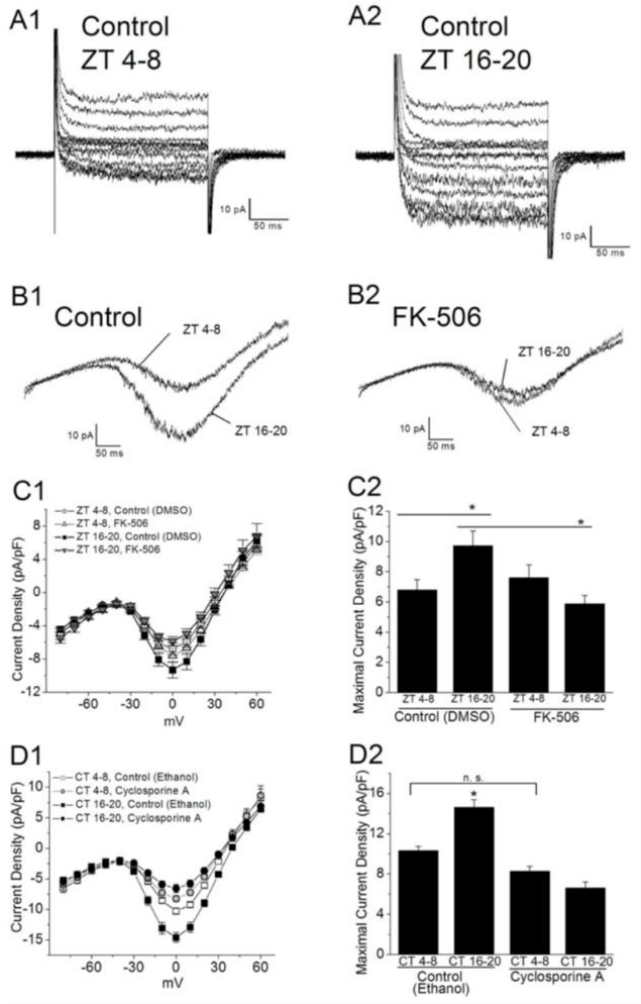


Figure 2.2 There was a circadian phase-dependent modulation of L-VGCCs by calcineurin.
L-VGCCs were recorded from cultured chick cone photoreceptors on the sixth day of LD entrainment during the day (ZT 4–8) or at night (ZT 16–20). Some cells were recorded on the second day of DD after LD entrainment during the subjective day (CT 4–8) or the subjective night (CT 16–20). Two representative L-VGCC current traces from cells treated with 0.1% DMSO (control) using step commands recorded during the (A1) day (ZT 4–8) and (A2) at night (ZT 16–20). (B1) Two representative L-VGCC traces were recorded under a ramp command during the day (ZT 4–8) or at night (ZT 16–20) under the control condition (0.1% DMSO). (B2) Two representative traces (ramp command) after treatment with FK-506 (10 μM), a calcineurin inhibitor, for 2 hr prior to recordings. (C1) The averagecurrent-voltage (I–V) relationships in current density (pA/pF) and step-voltage (mV). (C2) Maximal current densities were elicited at 0 mV of the step command. Treatment with FK-506 significantly dampened the rhythm of maximal L-VGCC current densities. * indicates that the L-VGCC current density recorded at night (ZT 16–20; n=12) is significantly higher than those recorded during the day (control, ZT 4–8; n=11) and FK-506 treated cells recorded at night (FK-506, ZT 16–20; n=8). There was no statistical difference in the values of maximal L-VGCC current densities recorded from cells treated with FK-506 during the day (ZT 4–8; n=9) or at night compared to the control recorded during the day (ZT 4–8). (D1 and D2) A similar effect was produced by treatment with another calcineurin inhibitor cyclosporine A (2 μM in 0.1% ethanol) for 2 hr prior to recordings. Cells treated with cyclosporine A for 2 hr were recorded at CT 4–8 and CT 16–20 on the second day of DD. There was no difference in the circadian rhythm of L-VGCC current densities in control groups treated with 0.1% ethanol for 2 hr. Control (ethanol), CT 4–8 and CT 16–20, n=9; cyclosporine A, CT 4–8, n=17; cyclosporine A, CT 16–20, n=14. *$p<0.05$.

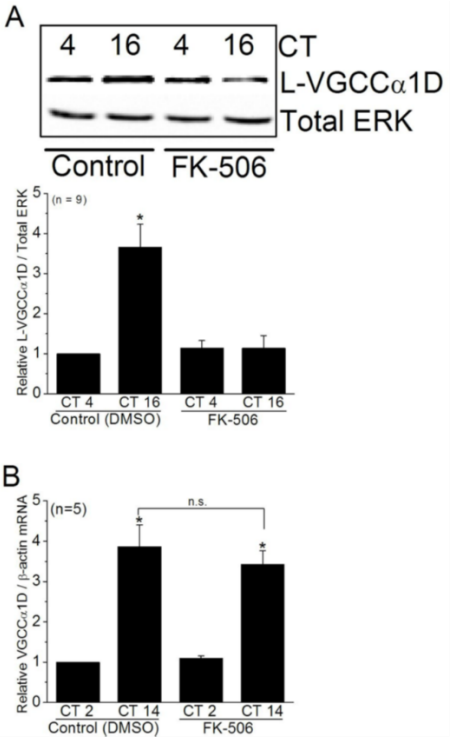


Figure 2.3 Inhibition of calcineurin dampens the circadian rhythm of L-VGCCα1D protein expression.
On the last day of LD entrainment in ovo, retinae were dissociated, cultured, and kept in DD for one day. On the second day of DD, cultures were treated with 0.1% DMSO (control) or 10 μM FK-506 for 2 hr prior to harvest at CT 4 or CT 16 for Western immnoblotting (A) or at CT 2 and CT 14 for Q-PCR (B) to measure the protein expression (A) or the mRNA level (B) of the L-VGCCα1D subunit. (A) FK-506 dampened the protein expression circadian rhythm of the L-VGCCα1D subunit. The protein level of L-VGCCα1D in control cells harvested at CT 16 was significantly higher than control cells harvested at CT 4, as well as cells treated with FK-506. n=9 for each group. (B) Inhibition of calcineurin with FK-506 had no effect on the mRNA level of L-VGCCα1D. Cells harvested from the control and FK-506 treated groups at CT 14 had significantly higher L-VGCCα1D mRNA compared to cells harvested at CT 2 from both the control and FK-506 treated groups. n=5 for each group. *p<0.05.(Note: Figure 2.3A was done by Dr. Michael L. Ko. Figure 2.3B was done by Mrs.Darya I. Vernikovskaya)

2.4.3: Calcineurin is upstream of MAPK and PI3K-AKT signaling in the circadian output regulation of L-VGCCs

Both MAPK and PI3K-AKT signaling pathways are known to regulate ion channel trafficking and insertion into the plasma membrane (Lhuillier and Dryer, 2000, 2002, Le Blanc et al., 2004, Keifer et al., 2007). We showed that both MAPK and PI3K-

AKT signaling serve as parallel circadian outputs to regulate L-VGCC trafficking and membrane insertion in photoreceptors (Ko et al., 2007, Ko et al., 2009). Since the circadian phase-dependent action of calcineurin on L-VGCCs was through post-translational modulation, we next examined whether calcineurin interacted with MAPK and/or PI3KAKT signaling pathways. Chick embryos were entrained in LD cycles, and on the last day of LD, retinal cells were cultured and kept in DD. On the second of DD, cells were treated with the calcineurin inhibitor FK-506 (10 uM) or cyclosporine A (2 uM) for 2 hr prior to harvest at CT 4 and CT 16 for Western blotting or PP2B activity assay. As shown previously (Ko et al., 2007, Ko et al., 2009) both phosphorylated ERK (pERK) and pAKT at thr308 (pAKTthr308) are under circadian control and significantly higher during the subjective night than during the subjective day (pERK rhythm, Fig. 2.4A and 2.4C; pAKTthr308 rhythm, Fig. 2.4B, 2.4D). Treatment with FK-506 or cyclosporine A dampened the circadian rhythm of pERK (Fig. 2.4A and 2.4C) and pAKTthr308 (Fig. 2.4B and 2.4D). However, inhibition of MAPK signaling using a MEK1 inhibitor PD98059 (50 μM) or the PI3K-AKT pathway with a PI3K inhibitor LY294002 (50 μM) did not alter the circadian rhythm of calcineurin activity (Fig. 2.5A and B). Hence, calcineurin was upstream of both MAPK and PI3K-AKT signaling as part of the circadian output pathway to regulate L-VGCCs.

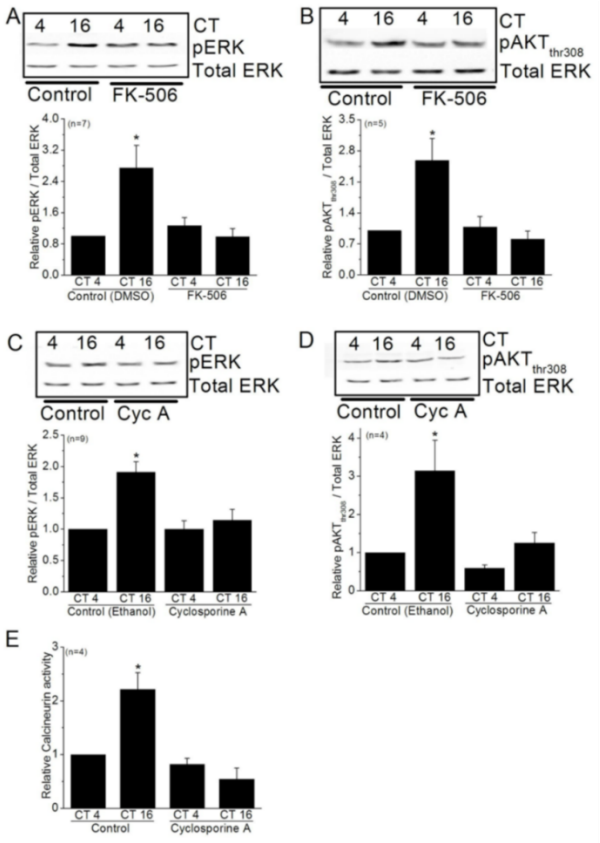


Figure 2.4 Inhibition of calcineurin dampens the circadian rhythm of pERK and pAKT.
(A) ERK phosphorylation (pERK) was higher during the subjective night (CT 16) than the subjective day (CT 4) in control retinal cells (0.1% DMSO). Treatment with FK-506 (10 μM) decreased pERK during the subjective night (CT 16). n=7 for each group. (B) Phosphorylation of AKT at thr308 (pAKTthr308) was higher during the subjective night (CT 16) than the subjective day (CT 4) in control cells (0.1% DMSO). Treatment with FK-506 decreased pAKTthr308 during the subjective night. n=5 for each group. (C and D) Treatment with cyclosporine A (2 μM) caused similar effects and decreased pERK during the subjective night (C) and diminished pAKTthr308 during both subjective day and night (D). (C) n=9 for each group. (D) n=4 for each group. (E) Serving as an internal control, treatment with cyclosporine A decreased calcineurin activities during both subjective day and night. n=4 for each group. * indicates that the levels of pERK, pAKTthr308, or calcineurin activity from control cells harvested at CT 16 are significantly higher than other groups. *$p<0.05$.

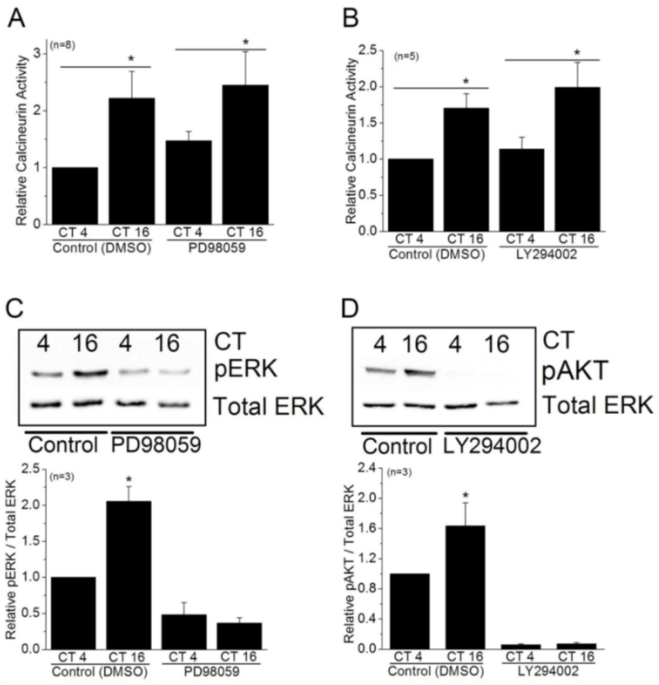


Figure 2.5 Inhibition of MAPK or PI3K-AKT signaling does not affect the circadian rhythm of calcineurin activity. (A) Calcineurin activity of control cells (0.1% DMSO) was higher during the subjective night (CT 16) than the subjective day (CT 4). Treatment with MEK1 inhibitor PD 98059 (50 μM) had no effect on the circadian rhythm of calcineurin activity. n=8 for each group. (B) Similarly, treatment with PI3K inhibitor LY294002 (50 μM) did not affect the circadian rhythm of calcineurin activity. n=5 for each group. (C and D) As internal controls, treatment with PD98059 inhibits pERK (C), while treatment with LY294002 inhibits pAKTthr308 (D). n=3 for each group. *$p<0.05$.

2.4.4: Calcineurin is downstream of cAMP but upstream of Ras in the circadian output regulation of L-VGCCs

In the retina, the activity of Ras is under circadian control with higher activity at night (Ko et al., 2004). Both MAPK and PI3K-AKT signaling pathways are downstream of Ras, since inhibition of Ras abolishes the circadian rhythm of pERK and pAKT (Ko et al., 2009), while inhibition of cAMP signaling dampens the circadian rhythm of Ras activity (Ko et al., 2004). Since calcineurin was also upstream of both MAPK and PI3K-

AKT signaling, we next investigated the potential interaction among calcineurin, Ras, and cAMP signaling. Using a commercially available cAMP assay kit, we found that cAMP content in the chick retina was rhythmic with its peak during the subjective night (Fig. 2.6A). This result was similar to previous reports (Nikaido and Takahashi, 1998, Ivanova and Iuvone, 2003a). Treatment with the calcineurin inhibitor FK-506 did not alter the circadian rhythm of cAMP (Fig. 2.6B), but the adenylate cyclase inhibitor MDL-12330A (50 μM) significantly dampened the circadian rhythm of calcienurin activity (Fig. 2.6C). Furthermore, inhibition of calcineurin with FK-506 abolished the circadian rhythm of Ras activity (Fig. 2.6D). Therefore, calcineurin was downstream of cAMP but upstream of Ras to serve as a circadian output to regulate LVGCCs.

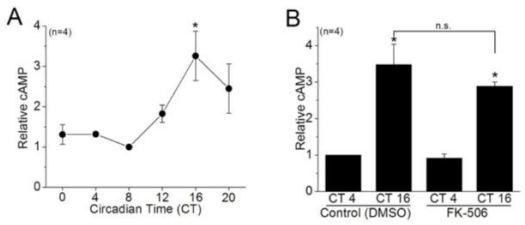


Figure 2.6 Calcineurin is downstream of cAMP signaling.
(A) The cAMP levels in the retina are under circadian control. The cAMP levels were measured in intact retinas taken on the second day of DD after several days of in ovo LD entrainment. The cAMP level peaked during the middle of the subjective night. n=4 for each circadian time point. * indicates that CT 16 was significantly different from CT 4 and 8. (B) Treatment with FK-506 did not affect the circadian rhythm of cAMP levels in cultured retina cells. * indicates that the cAMP levels of the control cells (0.1% DMSO) as well as FK-506 treated cells at CT 16 were significantly higher than the cells harvested at CT 4 from both treatments. There was no statistical difference (n.s.) between control cells and FK-506 treated cells at CT 16. n=4 for each group. (C) Treatment with an adenylate cyclase inhibitor MDL-12330A (50 μM) significantly dampened the circadian rhythm of calcineurin activity. * indicates that the calcineurin activity of the control cells (0.1% DMSO) was significantly higher at CT 16 than all other groups. n=5 for each group. *$p<0.05$. (D) Treatment with FK-506 significantly dampened the circadian rhythm of Ras activity. * indicates that Ras activity of the control cells (0.1% DMSO) was significantly higher at CT 16 than all other groups. n=4 for each group. *$p<0.05$.(Note: Figure2.6A and B were done by Mrs. Darya I. Vernikovskaya. Figure 2.6 D was done by Dr. Michael L. Ko)

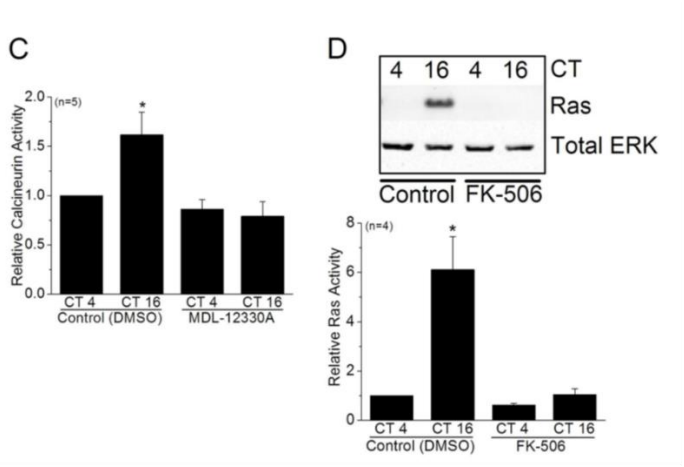


Figure 2.6 Continued.

2.5 Discussion

Calcineurin is involved in diverse biological processes, including regulating nuclear factor of activated T cells (NFAT) transcriptional activation (O'Keefe et al., 1992, Jain et al., 1993)and apoptosis (Yazdanbakhsh et al., 1995, Wang et al., 1999). In neurons, calcineruin participates in the modulation of synaptic plasticity (Mulkey et al., 1994, Wang and Kelly, 1997, Zhuo et al., 1999), neurotransmitter release (Halpain et al., 1990, Renstrom et al., 1996, Nishi et al., 1997), and gating of ion channels (Chen et al., 1995, Marcaida et al., 1996, Marrion, 1996, Oliveria et al., 2007). Since the visual system must anticipate large daily changes in ambient illumination, circadian oscillators in the retina provide a mechanism for the visual system to initiate more sustained adaptive changes throughout the course of a day (Cahill and Besharse, 1995, Green and

Besharse, 2004). In this study, we demonstrated that calcineurin was involved in the circadian phase-dependent modulation of L-VGCCs in the retina. We focus on the circadian regulation of the L-VGCCα1D subunit since in the avian retina, α1D is distributed mainly on the cell bodies of photoreceptors, bipolar cells, and ganglion cells, while L-VGCCα1C is abundant in Müller glia cells (Firth et al., 2001, Ko et al., 2007). In addition, L-VGCCα1D is present in the cell bodies and terminals of rodent photoreceptors, while VGCCα1C is not observed in rat photoreceptors (Xu et al., 2002, Morgans et al., 2005). Hence, α1D, but not α1C, is the dominant LVGCCα1 subunit in retinal photoreceptors across several vertebrate species.

We found that calcineurin activity was under circadian control (significantly higher during the subjective night than the subjective day), but its protein expression remained constant throughout the day (Fig. 2.1). Application of a calcineurin inhibitor, FK-506 or cyclosporine A, for 2 hr at night decreased L-VGCC current density in cone photoreceptors (Fig. 2.2) corresponding with a decrease in the protein expression of the L-VGCCα1D subunit in cultured retinal cells, but the circadian rhythm of L-VGCCα1D mRNA was not affected (Fig. 2.3). Therefore, the circadian phase-dependent action of calcineurin was not due to circadian phase-shifting of L-VGCCs. Instead, calcineurin served in the circadian output pathway to regulate L-VGCCs. We previously demonstrated that the circadian rhythm of LVGCCs is in part through both Ras-ERK and Ras-PI3K-AKT signaling, both of which are involved in the protein trafficking and membrane insertion of L-VGCCα1 subunits(Ko et al., 2007, Ko et al., 2009). Here, we showed that calcineurin regulated L-VGCCs in a circadian-phase dependent manner

through modulation of ERK and PI3K-AKT signaling, since calcineurin inhibitors dampened the circadian rhythms of phosphorylated ERK and AKTthr308 (Fig. 2.4), while inhibition of either signaling pathway did not alter calcineurin activity (Fig. 2.5).

In the chick retina, cAMP content is under circadian control as previously shown by others (Nikaido and Takahashi, 1998, Ivanova and Iuvone, 2003a, Chaurasia et al., 2006) and in this study (Fig. 2.6A), and the activity of Ras is also under circadian control and is downstream of cAMP signaling (Ko et al., 2004). We found that the adenylate cyclase inhibitor MDL-12230A dampened the circadian rhythm of calcineurin activity, but the calcineurin inhibitor FK-506, while having the ability to inhibit the Ras rhythm, had no effect on the circadian rhythm of retinal cAMP content (Fig. 2.6). Hence, calcineurin acted downstream of cAMP and upstream of Ras to regulate the circadian rhythm of L-VGCCs through regulating ion channel trafficking and membrane insertion (Fig. 2.7).

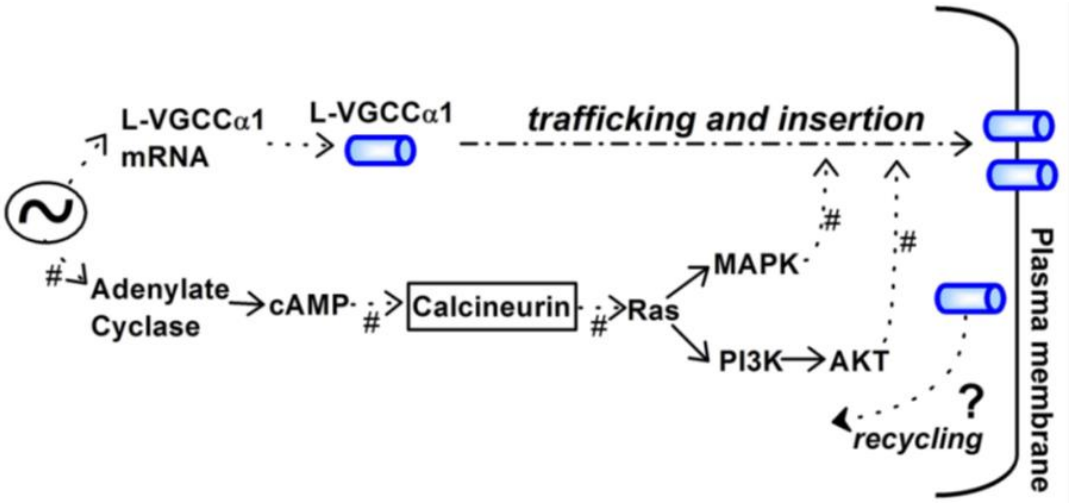


Figure 2.7 A schematic model of the circadian output regulation of L-VGCCs.
The calcineurin activity rhythm is driven by the circadian oscillator in photoreceptors. This calcineurin activity rhythm is downstream of the rhythmic oscillation of cAMP, while it is upstream of both MAPK and PI3K-AKT signaling that lead to the circadian regulation of LVGCCα1 subunit trafficking and membrane insertion. There are still numerous unknown steps (marked by #) that intervene between the various signaling components. Whether the sequestering and recycling (marked by ?) of the L-VGCC subunits are under circadian control will need further investigation.

Photoreceptors are non-spiking neurons, and its neurotransmitter release is continuous in the dark through voltage-dependent activation of L-VGCCs(Barnes and Kelly, 2002a). The circadian regulation of L-VGCCs has been shown in gold fish retinal bipolar cells (Hull et al., 2006)and avian cone photoreceptors (Ko et al., 2007). In each case, the L-VGCC current density is greater at night than during the day. The mechanism of this circadian rhythm is in part through the circadian regulation of mRNA and protein expression of the LVGCCα1 subunits (Ko et al., 2007), as well as channel subunit trafficking and insertion / retention into the plasma membrane (Ko et al., 2007, Ko et al., 2009, Shi et al., 2009a). There are two parallel signaling pathways that take part in the circadian regulation of L-VGCCs, Ras-MAPK and Ras-PI3K-AKT (Ko et al.,

2007, Ko et al., 2009), and both pathways are known to regulate ion channel trafficking (Lhuillier and Dryer, 2000, 2002, Le Blanc et al., 2004, Keifer et al., 2007). Since calcineurin was upstream of Ras, we concluded that the circadian phase-dependent regulation of L-VGCCs by calcineurin was also through the regulation of channel trafficking and insertion of VGCCα1.

Calcineurin is also involved in circadian phase-shifting in mammals, since in vivo administration of calcineurin inhibitors blocks circadian responses to light at night, produces circadian phase advances when applied during the subjective day, and disrupts circadian locomotor behavior rhythms when applied chronically in hamsters (Katz et al., 2008). These effects of calcineurin inhibitors on circadian phase-shifting are in part attributed to their interference with intracellular Ca2+ storage and release in SCN neurons (Ding et al., 1998). In addition, calcineurin is an important regulator of casein kinase-I (CKI) and glycogen synthase kinase 3β (GSK3β; (Cegielska et al., 1998, Lowrey et al., 2000, Liu et al., 2002, Kim et al., 2009). CKI and GSK3β are able to regulate the circadian core oscillator by phosphorylating circadian clock proteins (Vielhaber et al., 2000, Eide and Virshup, 2001, Iitaka et al., 2005, Yin et al., 2006). Therefore, calcineurin may well be involved in the circadian core oscillator mechanism through dephosphorylation of CKI and GSK3β in the mammalian SCN. Since we did not observe any changes in the circadian rhythm of LVGCCα1D mRNA levels after inhibition of calcineurin, we concluded that calcineurin is part of the circadian output pathway to regulate L-VGCCs post-translationally. However, we cannot rule out the

possibility that calcineurin might also serve in the circadian input pathway of retinal circadian oscillators, which will require further investigation.

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

Calcineurin is also an important regulator of L-VGCCs in various cell types. However, the effect of calcineurin on L-VGCCs varies, as both inhibition (Chad and Eckert, 1986, Armstrong, 1989, Schuhmann et al., 1997, Victor et al., 1997) and

enhancement (Norris et al., 2002, Tandan et al., 2009) have been observed. The L-VGCCα1C (Cav1.2) subunit can form macromolecular signaling complexes that comprise the β-adrenergic receptor, G(s) protein, adenylate cyclase, protein kinase A (PKA), as well as PP2A and calcineurin in the heart and brain (Xu et al., 2010). Calcineurin is capable of binding the C-terminus of the cardiac L-VGCCα1C (Xu et al., 2010). In the present study, we found that inhibition of calcineurin decreased L-VGCC currents in cone photoreceptors only at night (Fig 2.2), and this inhibition was due to decreased L-VGCCα1D protein expression (Fig 2.3A). Therefore, we rule out direct dephosphorylation as the circadian phase-dependent action of calcineurin on L-VGCCs in cone photoreceptors. Even though we demonstrated that calcineurin was downstream of cAMP signaling and upstream of Ras, it is still not known which molecule is the direct target of calcineurin. Missing links remain between cAMP signaling and calcineurin, as well as between calcineurin and Ras, and the complexity of the signaling network in the circadian regulation of L-VGCCs are not completely understood (Fig. 2.7). Thus far, we have shown that at the post-translational level, the trafficking and membrane insertion / retention of L-VGCCs are under circadian control (Ko et al., 2007, Ko et al., 2009), in which calcineurin was part of the output pathway as shown in this study. However, we do not know whether the internalization and recycling of the channel subunits are also under circadian control. The circadian rhythm of L-VGCCs could be the focal point between channel protein expression, insertion / retention into the plasma membrane, and sequestering / recycling of the channel subunits. In conclusion,

our present study provides new insight on the mechanism underlying the circadian regulation of L-VGCCs in chick cone photoreceptors.

CHAPTER III

A NEW FUNCTIONAL ROLE FOR MECHANISTIC/MAMMALIAN TARGET OF RAPAPMYCIN COMPLEX 1 (MTORC1) IN THE CIRCADIAN REGULATION OF L-TYPE VOLTAGE-GATED CALCIUM CHANNELS IN AVIAN CONE PHOTORECEPTORS[1]

3.1 Overview

In the retina, the L-type voltage-gated calcium channels (L-VGCCs) are responsible for neurotransmitter release from photoreceptors and are under circadian regulation. Both the current densities and protein expression of L-VGCCs are significantly higher at night than during the day. However, the underlying mechanisms of circadian regulation of LVGCCs in the retina are not completely understood. In this study, we demonstrated that the mechanistic/mammalian target of rapamycin complex (mTORC) signaling pathway participated in the circadian phase-dependent modulation of L-VGCCs. The activities of the mTOR cascade, from mTORC1 to its downstream targets, displayed circadian oscillations throughout the course of a day.

[1] Reprinted from Huang CC-Y, Ko ML, Ko GY-P (2013) "A New Functional Role for Mechanistic/Mammalian Target of Rapamycin Complex 1 (mTORC1) in the Circadian Regulation of L-Type Voltage-Gated Calcium Channels in Avian Cone Photoreceptors." PLoS ONE 8(8): e73315. doi:10.1371/journal.pone.0073315. Creative Commons Attribution License.

Disruption of mTORC1 signaling dampened the L-VGCC current densities, as well as the protein expression of L-VGCCs at night. The decrease of L-VGCCs at night by mTORC1 inhibition was in part due to a reduction of L-VGCCα1 subunit translocation from the cytosol to the plasma membrane. Finally, we showed that mTORC1 was downstream of the phosphatidylionositol 3 kinase-protein kinase B (PI3K-AKT) signaling pathway. Taken together, mTORC1 signaling played a role in the circadian regulation of LVGCCs, in part through regulation of ion channel trafficking and translocation, which brings to light a new functional role for mTORC1: the modulation of ion channel activities.

3.2 Introduction

The mechanistic/mammalian target of rapamycin (mTOR) signaling pathway governs diverse cellular physiological functions including cell growth, cell survival, energy balance, and metabolism in response to environmental signals such as nutrients and stress(Gao et al., 2002, Kim et al., 2002, Inoki et al., 2003, Brugarolas et al., 2004, Gangloff et al., 2004, Laplante and Sabatini, 2012). mTOR, a conserved serine (ser) / threonine (thr) protein kinase, is composed of two distinct complexes, namely mTOR complex 1 (mTORC1) and mTORC2. mTORC1 regulates cell growth by increasing protein synthesis through phosphorylation of downstream targets, p70 ribosomal S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1(4EBP1) (Gingras et al., 1999, Nojima et al., 2003, Schalm et al., 2003, Chiang and Abraham, 2005, Holz and Blenis, 2005), while mTORC2 regulates cell survival and

cytoskeletal organization (Jacinto et al., 2004, Sarbassov et al., 2004, Sarbassov et al., 2005, Sarbassov et al., 2006). In the retina, mTOR signaling is important for cell survival and axon regeneration. Stimulation of mTOR signaling by insulin prolongs the survival of retinal neurons(Wu et al., 2004, Punzo et al., 2009), and depletion of the negative regulators of mTOR promotes axon regeneration in retinal ganglion cells after optic nerve injury(Park et al., 2008, Leibinger et al., 2012). Under hyperglycemic conditions, the suppression of mTOR activity in diabetic retinas causes apoptosis (Fox et al., 2012). Therefore, the mTOR signaling pathway is essential for maintaining retinal metabolic homeostasis and health.

While mTOR is essential in metabolism and cell survival, it is also involved in the circadian regulation of both vertebrates and invertebrates (Cao et al., 2008, Cao et al., 2010, Zheng and Sehgal, 2010). The circadian clocks regulate metabolism, physiological processes, and behaviors across the course of a day, and these internal time-keeping mechanisms allow organisms to anticipate and adapt to daily external environmental changes such as cycling ambient illumination and temperature fluctuations(Ko and Takahashi, 2006, Nozue et al., 2007). The canonical core mechanism underlying the circadian oscillations is composed of a specific set of "clock genes" and their protein products, which form self-regulated transcriptional-translational feedback loops with a period close to 24 hours(Ko and Takahashi, 2006, Nozue et al., 2007). However, other post-translational mechanisms such as phosphorylation, methylation, and ubiquitination, as well as various cellular signaling pathways are also involved in the circadian mechanism or the circadian regulation of downstream targets

(Gallego and Virshup, 2007). mTOR signaling is involved with the core circadian oscillator components and affects the rhythmicity. Disruption of mTOR signaling alters the light-induced expression of the Period gene, a core oscillator component (Cao et al., 2010), as well as light induced phase shifting in animal activity rhythm (Cao et al., 2010), while activation of mTOR signaling impacts the nuclear accumulation of the clock protein TIMELESS and lengthens the circadian period in Drosophila (Zheng and Sehgal, 2010). Hence, mTOR signaling may participate in the core circadian mechanism.

In the vertebrate retina, many physiological aspects are under circadian control, since the visual system has to adapt to large changes in ambient illumination throughout the day (Cahill and Besharse, 1995, Green and Besharse, 2004). In particular, the circadian oscillators in retinal photoreceptors regulate daily changes in retinomotor movement (Pierce and Besharse, 1985, Burnside, 2001), outer segment shedding and renewal (LaVail, 1980), gene and protein expression(Korenbrot and Fernald, 1989, Pierce et al., 1993, Haque et al., 2002, Liu et al., 2012); morphological changes at synaptic ribbons (Adly et al., 1999), as well as ion channel activities (Ko et al., 2001, Ko et al., 2007). We previously showed a circadian regulation of L-type voltage-gated calcium channels (L-VGCCs) in cone photoreceptors (Ko et al., 2007). The L-VGCCs are essential for neurotransmitter release from photoreceptors and other retinal neurons (Barnes and Kelly, 2002a). We further demonstrated that both Ras-mitogen activated protein kinase (MAPK) and Ras-phosphatidylionositol 3 kinase-protein kinase B (PI3K-AKT) signaling pathways are part of the circadian output pathway mediating L-VGCC trafficking and insertion in a circadian phase-dependent manner (Ko et al., 2007, Ko et

al., 2009). Since mTOR is involved in the circadian mechanism, we investigated whether it also participates as part of the circadian output pathway to regulate L-VGCCs in cone photoreceptors. We combined biochemical, morphological, and electrophysiological analyses to examine the potential circadian phase-dependent modulation of L-VGCCs by mTOR and its potential interaction with other signaling pathways.

3.3 Materials and methods

3.3.1: 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 day12 (E12) and cultured for 6 days as described previously(Ko et al., 2007, Ko et al., 2009). Cultures were prepared in the presence of 20 ng/mlciliary neurotrophic factor (CNTF; R&D Systems, Minneapolis, MN, USA), which yields cultures highly enriched with cone photoreceptors (Adler et al., 1984, Adler and Hatlee, 1989, Belecky-Adams et al., 1996)and 10% heat-inactivated horse serum. Cell culture incubators (maintained at 39°C and 5% CO2) were equipped with lights and timers, which allowed for the entrainment of retinal circadian oscillators to 12h: 12h light-dark (LD) cycles *in vitro*. Zeitgeber time zero (ZT 0) was designated as the time when the lights turned on and ZT 12 was the time when the lights went off. For *in ovo* entrainment, intact eggs were exposed to LD 12h: 12h at E10-E11 for 7 days. Retina cells were then dissociated, cultured, kept in constant darkness (DD), and used for biochemical and molecular biological assays on the second day of DD. In some

experiments, after in ovo LD entrainment for 6 days, eggs were kept in DD. On the second day of DD, retinas were collected at different circadian time (CT) points throughout a day for biochemical assays (Ko et al., 2007, Ko et al., 2009). The reason for using chick embryos from E12+6 for in vitro entrainment or E18 for in ovo entrainment is that more than 90% of the retina photoreceptors express functionally mature VGCC currents by E18 (Gleason et al., 1992).

3.3.2: Immunoblot analysis

Samples were collected and prepared as described previously (Huang et al., 2012). 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 3VO4; 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-ERK (pERK; Sigma, St. Louis, MO, USA), anti- ERK (total ERK, used for loading control; Santa Cruz Biochemicals, Santa Cruz, CA, USA), anti-VGCCα1D (Alomone, Jerusalem, Israel), anti-phosphomTORC1 (ser2448; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-mTORC1 (ser2481; Cell Signaling Technology), anti-mTORC (total mTORC, Cell Signaling Technology), anti-phospho-S6 (ser240/244; Cell Signaling Technology), anti-S6 (Cell Signaling Technology), antiphospho- p70S6K (thr389; Cell Signaling Technology), and antip70S6K (Cell Signaling Technology). Blots were visualized using appropriate secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology)

and an enhanced chemiluminescence (ECL) detection system (Pierce,Rockford, IL, USA). Relative protein expressions for all proteins involved in this study are reported as a ratio to total ERK since total ERK remains constant throughout the day. Band intensities were quantified by densitometry using Scion Image(NIH, Bethesda, MD, USA). All measurements were repeated at least 3 times.

3.3.3: 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 BaCl2, 0.4 MgCl2, 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 MgCl2, 0.1 CaCl2, 1.1 EGTA, and 10 HEPES, pH 7.3 adjusted with CsOH. Recordings were made only from cells with elongated cell bodies with one or more prominent oil droplets (hallmark of avian cone photoreceptors) (Gleason et al., 1992, Pierce et al., 1993, Ko et al., 2001). Currents were recorded at room temperature (RT, 23°C) using an Axopatch 200B (Axon Instruments/Molecular Devices, Union City, CA, USA) or 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). Electrode capacitance was compensated after gigaohm (GΩ) seals were formed. Cells were held at -65mV, and ramp voltage commands from -80 to +60 mV in 500 ms were used to evoke Ba2+ 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. Rapamycin and PP242 were obtained from A.G. Scientific (San Diego, CA, USA) and Chemdea (Ridgewood, NJ, USA), respectively. Both rapamycin and PP242 were dissolved in DMSO (the final concentration of DMSO vehicle was 0.1%).

3.3.4: Calcineurin activity assay

Retina samples were lysed in a phosphatase lysis buffer including (in mM): 50 Tris, pH 7.5, 1 EGTA, 150 NaCl, 1% Triton X-100, and 1% β-mercaptoethanol, and calcineurin activities were assayed using a commercially available ser/thr phosphatase assay kit (Promega, Madison, WI, USA). This kit can distinguish between tyrosine (tyr) and ser/thr phosphatases by using a synthetic polypeptide, RRA(pT) VA, that is compatible with ser/thr phosphatases but is structurally incompatible for tyr phosphatases. To differentiate between PP2A, 2B, and 2C, the reaction buffer is made to favor one over the others since this class of enzyme has a diverse range of optimum conditions. For calcineurin (PP2B), the reaction buffer contained 250 mM imidazole (pH

7.2), 1 mM EGTA, 50 mM MgCl2, 5 mM NiCl2, 250 μg/ml calmodulin, and 0.1% β-mercaptoethanol as described in the manufacturer's protocol. Free cytoplasmic phosphate was first removed from the samples then dephosphorylation of the kit's calcineurin substrate proceeded for 30 min at RT. This system determines the amount of free phosphate generated in a reaction by measuring the absorbance (600 nm) of a molybdate/ malachite green/ phosphate complex.

3.3.5: Immunocytochemistry

Dissociated retinas were cultured on coverslips and entrained under LD cycle for four days. Cell were then fixed at CT4 or CT16 with Zamboni fixative for 30 min at RT, washed in phosphate buffer (0.1M PB, pH7.4), and permeabilized in 1% Triton-X PB for 10min. Samples were blocked in 10% goat serum in 0.1% Triton-X/PB for 2 hr at RT, then incubated with VGCCα1D primary antibody (1:100) overnight. The cells were washed with 0.1% Triton-X/PB and incubated with fluorescent conjugated secondary antibody (Alexa 488nm goat anti-rabbit; Molecular Probes, Carlsbad, CA, USA) for 2 hr in the dark. Coverslips were then re-washed and mounted with ProLong® Gold antifade reagents with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Eugene, OR, USA) on a glass slide and stored at 4°C for later observation on a Zeiss microscope (Thornwood, NY, USA) with epi-fluorescence to determine the localization of VGCCα1D and the nucleus (with DAPI). 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. The fluorescence intensity analyses were carried out blindly. The experiment was repeated at least four times.

3.3.6: Statistical analysis

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

3.4 Results

3.4.1: mTORC1 signaling is under circadian control

mTORC1 signaling participates in light-induced phase-shifts in mammals, as well as in the changes of circadian period in Drosophila (Cao et al., 2008, Cao et al., 2010, Zheng and Sehgal, 2010). We first investigated whether mTOR signaling was under circadian control in the avian retina, since the phosphorylation states of mTORC1 signaling display circadian rhythms in the suprachiasmatic nucleus (SCN) (Cao et al., 2011). Chicken retinal samples were collected at six different circadian time (CT) points on the second day of DD after LD entrainment and used for Western blotting analysis. Since the total amount of ERK (total ERK) is constant throughout the day (Ko et al., 2001), we used total ERK as the loading control. We found that the phosphorylation status of mTORC1 was under circadian regulation, and it was a site-specific regulation.

The phosphorylation at ser 2448 of mTORC1 (pTORC1ser2448), the site that monitors mTORC1 activity (Scott et al., 1998, Reynolds et al., 2002), peaked at CT 12 (Figure 3.1A), but phosphorylation at ser2481 (pTORC1ser2481) on the regulatory domain, as well as total mTORC1, remained constant (Figure3.1B). Downstream of mTORC1, the phosphorylation states of p70S6 kinase (pp70S6K) and S6 ribosomal kinase (pS6) were also rhythmic with peaks at CT 12 (Figure 3.1C, 3.1D), while total p70S6K and S6 protein remained constant. Hence, the activation state of mTORC1 signaling was under circadian regulation in the retina.

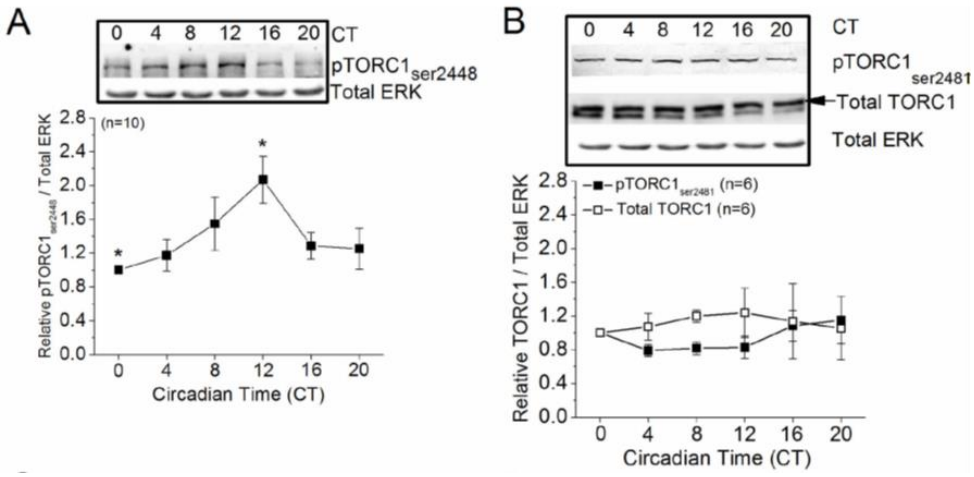


Figure 3.1 mTORC1 signaling is under circadian control.
On the second day of DD after circadian entrainment to 12: 12 hr LD cycles for 7 days in ovo, intact retinas were collected at 6 different circadian time points (CT 0, 4, 8, 12, 16, and 20) for immunoblotting analysis. (A) Phosphorylation of mTORC1 at ser2448 (pTORC1ser2448) showed a circadian rhythm with its peak at CT 12. (B) The total amount of mTORC1 (total TORC1) and mTORC1 phosphorylation at ser2481 (pTORC1ser2481) did not display circadian rhythmicity. (C) Phosphorylation of the downstream target of mTORC1, p70-S6 kinase 1(pp70S6K), displayed a circadian rhythm with a peak at CT 12 (upper panel), while total p70S6K (lower panel) remained constant. (D) Phosphorylation of S6 ribosomal protein (pS6), the downstream target of p70S6 kinase, exhibited a circadian rhythm with a peak at CT 12 (upper panel), with total S6 protein (lower panel) constant throughout the day. * indicates a statistical significance at CT 12 compared to CT 0, CT4, or CT8. *$p<0.05$.

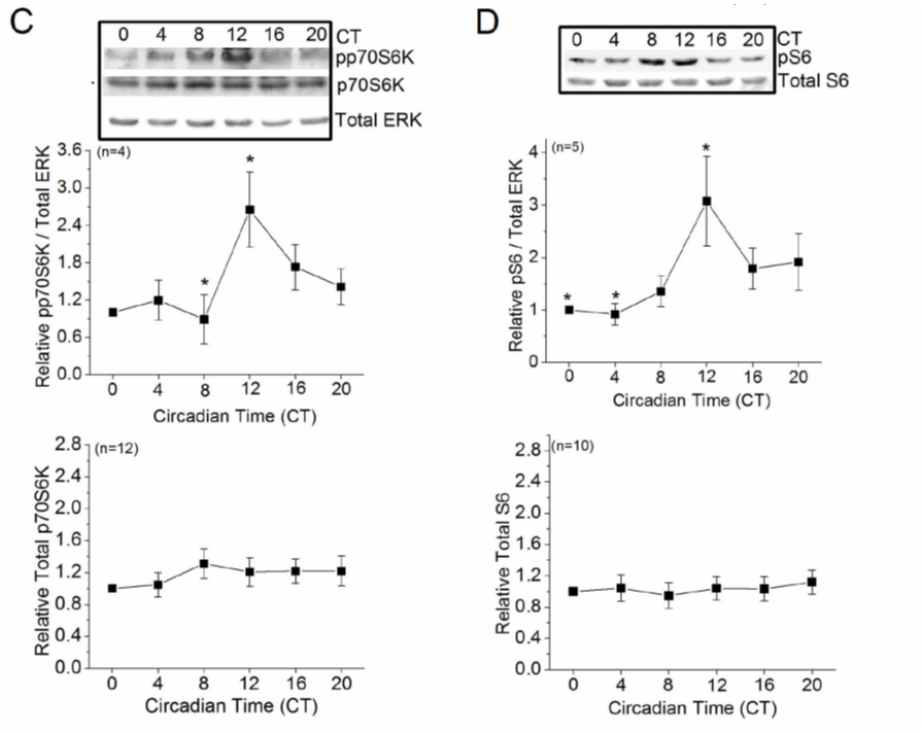


Figure 3.1 Continued.

3.4.2: mTORC1 is involved in the circadian regulation of LVGCC currents

In the retina, the L-VGCCs are essential in neurotransmitter release from photoreceptors and other retinal neurons (Barnes and Kelly, 2002b), and these channels are under circadian control in cone photoreceptors (Ko et al., 2007) and bipolar cells (Hull et al., 2006). The maximal currents of L-VGCCs elicited at 0 mV are significantly larger at night than during the day (Figure 3.2A, 3.2B (Ko et al., 2007)). To investigate whether mTORC1 was involved in the circadian regulation of LVGCCs, we applied rapamycin to inhibit mTORC1 in the following experiments. Rapamycin forms a complex with tacrolimus (FK506) binding protein 12 (FKBP12), an intracellular receptor protein, to inhibit mTORC1 activity (mTORC1 is more sensitive to rapamycin

inhibition thanmTORC2 (Ballou and Lin, 2008)). After 2 hr treatment of rapamycin (1 μM), the LVGCC current densities in cone photoreceptors were significantly dampened at night (ZT 16-20; Figure 3.2C, 3.2D), while there was no effect on currents recorded during the daytime (ZT 4-8; Figure 3.2C, 3.2D). Similar results were observed at a higher rapamycin concentration (10 μM; Figure 3.2E, 3.2F). To verify our observations were truly caused by the inhibition of mTOR, we applied PP242, another mTOR inhibitor not related to rapamycin structurally or mechanistically. PP242 inhibits both mTORC1 and mTORC2 by competing for ATP binding sites (Feldman et al., 2009). Treatment with PP242 (400 nM; 2 hr) significantly decreased the circadian rhythm of L-VGCC current densities at night (Figure 3.3A, 3.3D, 3.3E). Similar results were obtained at lower concentrations (100 nM) where PP242 inhibits mTORC1 more specifically than mTORC2 (Feldman et al., 2009) (Figure 3.3A, 3.3B, 3.3C).Therefore, mTOR signaling caused a circadian phase-dependent modulation of L-VGCCs in chick cone photoreceptors, in which inhibition of mTOR significantly reduced L-VGCC currents at night.

We previously also demonstrated that calcineurin, a ser/thr phosphatase, regulates the L-VGCCs in a circadian phase-dependent manner, where inhibition of calcineurin dampens LVGCC currents at night (Huang et al., 2012). Rapamycin and FK506 are structurally related immunosuppressants that inhibit the lymphocyte-activation pathway through binding to FKBP12 (Harding et al., 1989, Siekierka et al., 1989, Bierer et al., 1990, Dumont et al., 1990). While the rapamycin-FKBP12 complex inhibits mTORC1 signaling, the FK506-FKBP12 complex targets calcineurin (Liu et al.,

1991). Because of the structural similarities between rapamycin and FK-506 (Findlay and Radics, 1980, Tanaka et al., 1987, Clardy, 1995), we further verified that the action of rapamycin was not though interference of calcineurin activity at the concentrations used in this study. We examined calcineurin activity after cultured retinal cells were treated with rapamycin (10 μM or 1 μM), PP242 (400 nM), or DMSO (0.1%, control) for 2 hrs. At these concentrations, rapamycin and PP242 did not inhibit calcineurin activity (Figure 3F), consistent with other reports (Fruman et al., 1992, Yu et al., 2006), while FK-506 clearly inhibits calcineurin activity as we and others have previously shown (Fruman et al., 1992, Huang et al., 2012). Hence, we used 1 μM rapamycin and 400 nM PP242 to inhibit mTORC signaling for the following experiments.

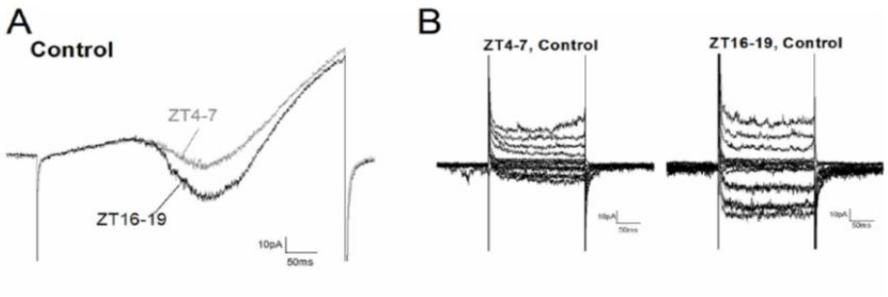
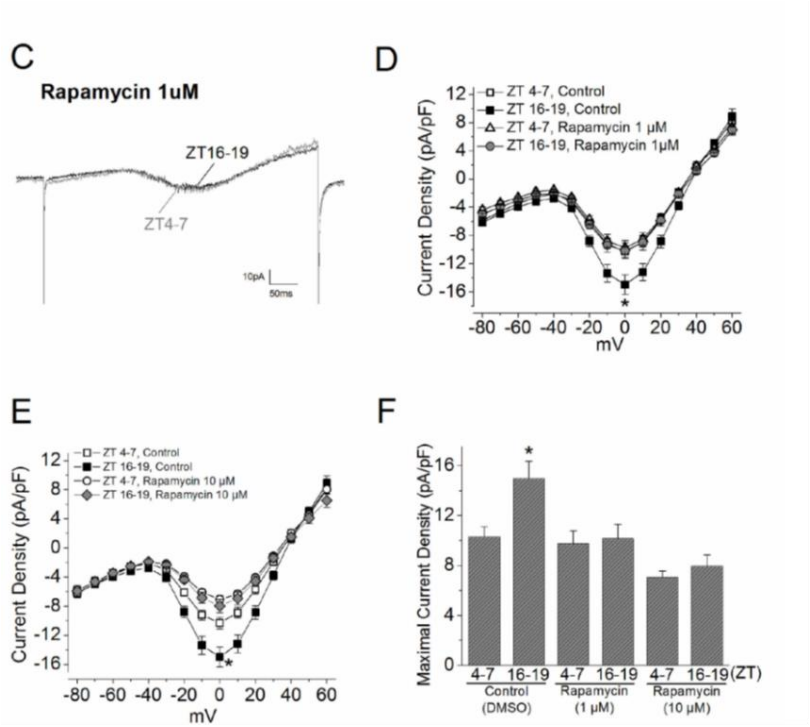


Figure 3.2 mTORC1 inhibitor dampens the circadian rhythm of L-VGCC currents.
L-VGCCs were recorded from cultured chick cone photoreceptors on the sixth day of LD entrainment during the day (ZT 4-7) or at night (ZT 16-19). (A) Representative day (ZT 4-7) and night (ZT 16-19) control L-VGCC current traces from cells treated with 0.1% DMSO; (A) ramp command, (B) step commands. (C) Two representative traces from cells that were treated with rapamycin (1 μM) for 2 hr prior to recordings. (D) and (E) The average current-voltage (I–V) relationships are shown in current density (pA/pF) and step-voltage (mV). * indicates that the control group at ZT 16-19 is significantly different from the other groups. (F) The maximal current densities were elicited at 0 mV. * indicates that the L-VGCC current density recorded at night (control, ZT 16-19; n=23) is significantly higher than those recorded during the day (control, ZT 4-7; n=21), rapamycin treated cells recorded during the day (ZT 4-7; 1 μM n=16; 10 μM n=13) and night (ZT 16-19; 1 μM n=18; 10 μM n=13). * indicates that the L-VGCC current density recorded from the control cells at night (control, ZT16-19) is significantly higher than all other groups. *$p<0.05$.



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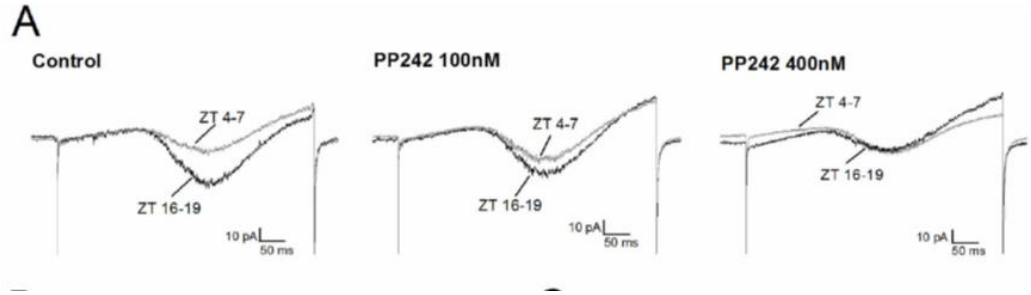


Figure 3.3 mTOR signaling regulates the circadian phase-dependent modulation of L-VGCCs.
(A) Representative L-VGCC current traces from cells treated with 0.1% DMSO (control) or PP242 (100 nM or 400 nM) are shown. (B) The average currentvoltage (I–V) relationships from cells treated with 0.1% DMSO (control) or PP242 (100 nM) are shown in current density (pA/pF) and step-voltage (mV). (C) The maximal current densities were elicited at 0 mV of the step command. * indicates that the L-VGCC current density of the controls recorded at night (ZT 16-19) is significantly higher than control and PP242 (100 nM) treated cells recorded during the day (ZT 4-7). PP242-treated cells that were recorded at night (ZT 16-19) have no statistical difference (n.s.) in L-VGCC current densities when compared to PP242-treated cells recorded during the day (ZT 4-7) or the controls recorded at night (ZT 16-19). Each group had at least 15 cells. (D) The average current-voltage (I–V) relationships from cells treated with 0.1% DMSO (control) or PP242 (400 nM) are shown in current density (pA/pF) and step-voltage (mV). * indicates that the L-VGCC current density of the controls recorded at night (ZT 16-19) is significantly higher than the other three groups. (E) Treatment with PP242 at 400 nM significantly dampened the circadian rhythm of maximal L-VGCC current densities. Each group had at least 15 cells. * indicates that the L-VGCC current density of the control group at night (ZT 16-19) is significantly higher than the other three groups.(F) Treatment with either rapamycin (10μM, 1μM) or PP242 (400nM) does not inhibit calcineurin activity compared to the control(0.1% DMSO) cultures. n=6 for each group. *$p<0.05$.

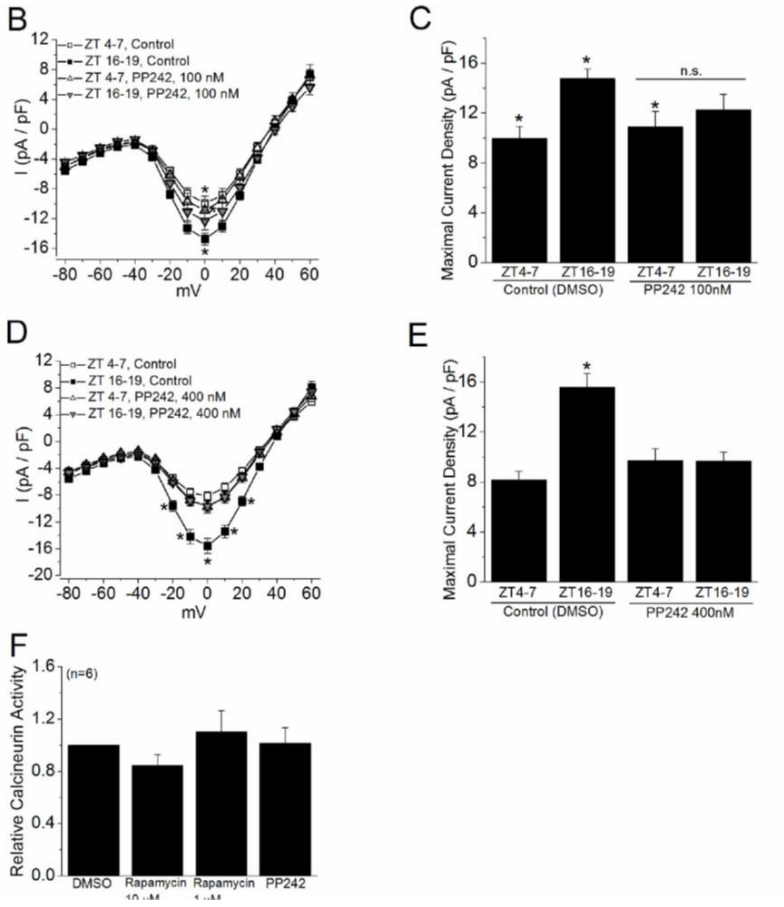


Figure 3.3 Continued.

3.4.3: mTORC1 is involved in the circadian regulation of LVGCCα1 protein expression and channel trafficking

Since mTORC1 was involved in the circadian phase-dependent modulation of L-VGCC currents in cone photoreceptors, we next examined whether mTORC1 affected the protein expression of L-VGCCα1D and its trafficking from the cytosol to the plasma membrane. In the mammalian retina, the distribution of L-VGCCα1D in photoreceptors

is wide ranging from the inner segment layer, outer nucleus layer, and outer plexiform layer. In avian cone photoreceptors, LVGCCα1D is concentrated in the inner segment (including soma, Fig. 3.4 (Ko et al., 2007)). There was a significantly higher LVGCCα1D fluorescence intensity in cone photoreceptors when cultures were fixed at CT 16 compared to CT 4 (Fig. 3.4A, top panel, Fig. 3.4B). Treatment with rapamycin for 2 hr significantly decreased the fluorescence intensity of L-VGCCα1D in photoreceptors fixed at CT 16 (Fig. 3.4A, lower panel, Fig. 3.4B). Using Western blot analysis, we found that retinal cultures treated with rapamycin or PP242 for 2 hr decreased the protein expression of L-VGCCα1D when cultures were harvested at night (CT 16; Figure 3.5A). We further used biotinylation assays to differentiate plasma membrane-bound versus cytosolic LVGCCα1Dand found that inhibition of mTORC1 significantly decreased the plasma membrane-bound L-VGCCα1D when cells were harvested at night (CT 16; Figure 3.5B1, 3.5B2). These results indicated that mTORC1 participated in the circadian regulation of protein expression and translocation of LVGCCα1D. Since physiologically functional ion channels have to be transported and inserted into the plasma membrane first, these results echo the earlier data that mTORC1 is involved in the circadian regulation of L-VGCC currents (Figures 3.2, 3.3).

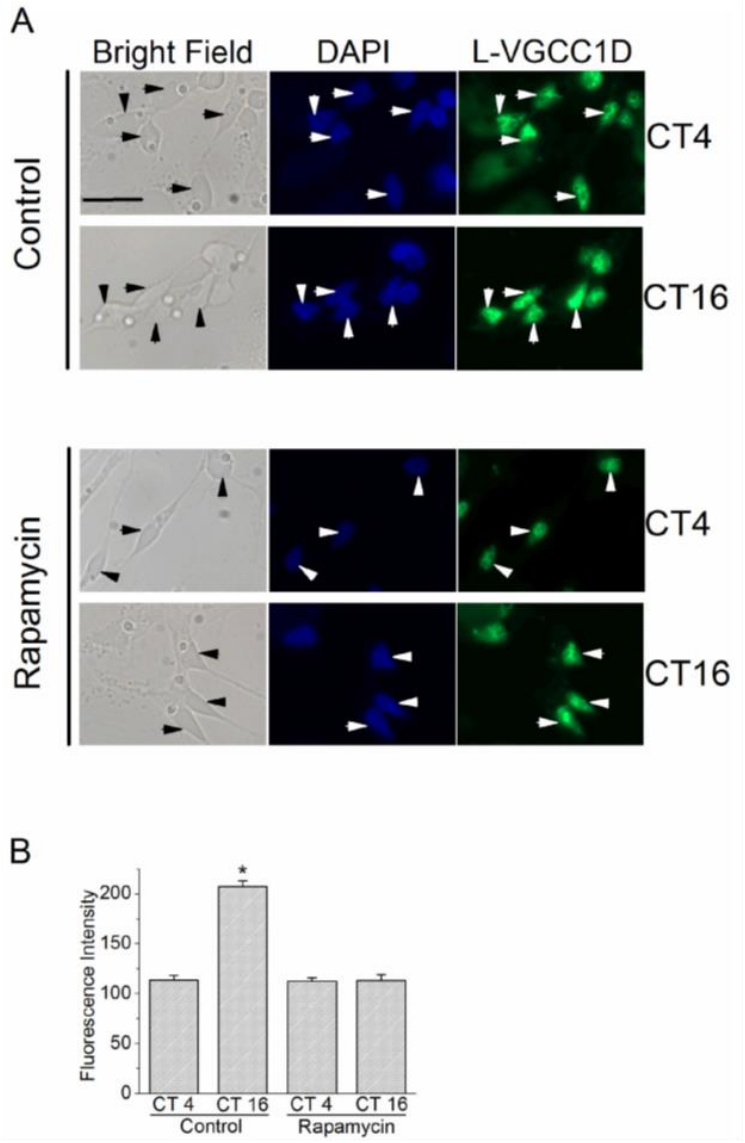


Figure 3.4 mTORC1 inhibition dampens the circadian rhythm of L-VGCCα1D.
Retinal cells were cultured on glass coverslips and entrained to 12 hr LD cycles for four days in vitro and kept in DD. On the second day of DD, cells were treated with rapamycin at CT 2 and CT 14 for 2 hr followed by fixation at CT 4 and CT 16. After washing and blocking, cells were processed for LVGCCα1Dimmunofluorescent staining. (A) Epi-fluorescent photos from the control (top panel) and rapamycin treated (lower panel) groups. The photographs shown here were slightly over-exposed to provide clearer images but were not used for statistical analysis in (B). The arrowheads indicate the cone photoreceptors. (B) The fluorescence intensity of L-VGCCα1D was significantly higher at CT 16 (control) compared to all other groups. Each group has at least 15 cells from 4 different trials. *$p<0.05$.

3.4.4: mTORC1 is a downstream target of the PI3K-AKT pathway

We previously showed that the circadian regulation of LVGCCs is in part through the regulation of channel trafficking (Ko et al., 2009). The MAPK-ERK and PI3K-AKT signaling pathways are involved in ion channel translocation in neurons or cardiomyocytes (Lhuillier and Dryer, 2002, Viard et al., 2004, Ko et al., 2009), and both are downstream of Ras and parallel to each other in the circadian regulation of LVGCC trafficking (Ko et al., 2009). The phosphorylation/activation states of ERK and AKT are also under circadian control in the retina (Ko et al., 2001, Ko et al., 2007, Ko et al., 2009). More specifically, the di-phosphorylation of ERK (pERK) and phosphorylation of AKT at thr308 (pAKTthr308) are significantly higher at night than during the day (Ko et al., 2004, Ko et al., 2007, Ko et al., 2009, Huang et al., 2012). Since mTORC1 was involved in the circadian phase-dependent modulation of L-VGCCα1D trafficking and translocation (Figures 3.4 and 3.5), we next investigated whether there was any cross-talk between mTORC1 and MAPK-ERK or PI3K-AKT signaling. As parallel pathways, inhibitors that block MAPK-ERK signaling do not affect the circadian rhythm of pAKT and vice versa. We found that treatment with rapamycin did not affect the circadian rhythm of pAKTthr308 (Figure 3.6A) or pERK (Figure 3.6F). Treatment with PP242 inhibited pAKTthr308 both at night (CT 16) and during the day (CT 4; Figure 3.6A) but did not affect pERK (Figure 3.6F). The effect of PP242 on pAKT might be due to its non-specific inhibition of both mTORC1 and mTORC2, since AKT is a known downstream target of mTORC2 (Hresko and Mueckler, 2005, Sarbassov et al., 2005). As a positive control, both rapamycin and PP242 completely abolished the phosphorylation

of S6 (pS6), a direct downstream target of mTORC1 (Figure 3.6B). When cultured retinas were treated with PD98059 (50 μM), a MEK1inhibitor, the circadian rhythm of pAKTthr308 (Figure 3.6C (Ko et al., 2009)) and pS6 (Figure 3.6D) were not affected, but the phosphorylation of ERK (pERK) was completely inhibited since MEK1 is directly upstream of ERK (Figure 3.6E). When cultured retinal cells were treated with the PI3K inhibitor LY294002 (50 μM), the phosphorylation of AKT thr308 (Figure 3.6C) and S6 (Figure 3.6D) were completely abolished but did not affect the circadian rhythm of pERK (Figure 3.6E (Ko et al., 2009)). Through these pharmacological studies, we concluded that mTORC1 was downstream of PI3K-AKT, but independent from MAPK-ERK signaling, to regulate the circadian rhythm of L-VGCCs.

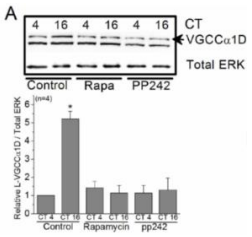
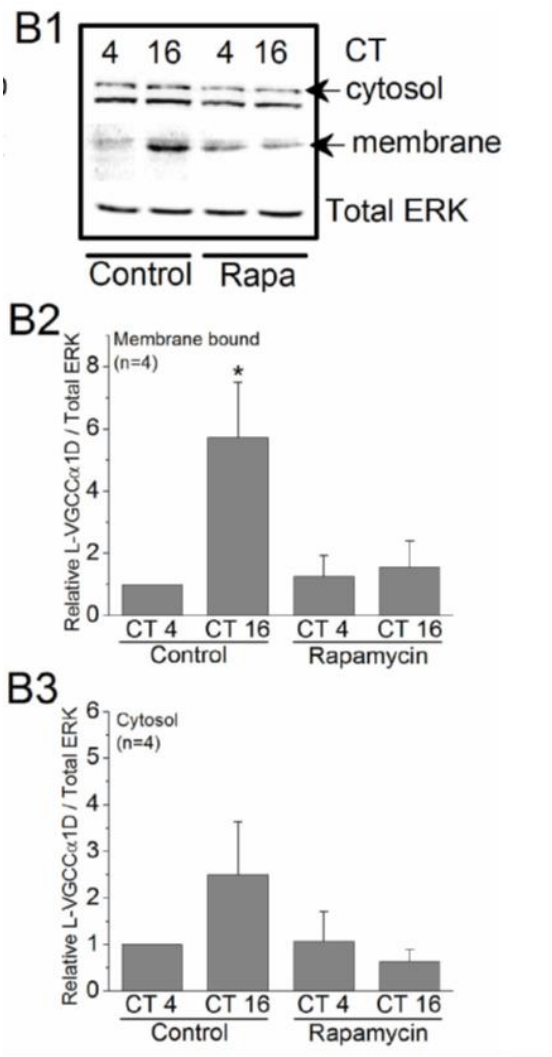


Figure 3.5 Inhibition of mTORC1 causes a circadian phase-dependent decrease in the protein expression and plasma membrane distribution of L-VGCCα1D.
Chick embryos (E11) were entrained in LD cycles for 7 days in ovo, and retinas were dissected, cultured, and kept in DD. On the second day of DD, cultured retinal cells were treated with 0.1% DMSO (control), rapamycin, or PP242 for 2 hr prior to harvest at CT 4 and CT 16. (A) In the whole cell lysate, the total protein expression of LVGCCα1D was significantly higher at night (CT 16) of the control compared to all other groups. Treatment with either rapamycin orPP242 dampened the circadian rhythm of L-VGCCα1D protein levels (B1-B3). After treatment with DMSO (control) or rapamycin for 2 hr, the distribution of L-VGCCα1D on the plasma membrane or cytosol was analyzed using biotinylation assays (B1). Representative blots of L-VGCCα1D from the cytosolic compartment (cytosol) and membrane-bound fraction (membrane). Total ERK served as loading control (B2). In the membrane-bound fraction, the L-VGCCα1D was significantly higher in control cells harvested at CT 16 compared to other groups (B3). In the cytosolic compartment (cytosol), there was no difference in the quantity of L-VGCCα1D among all groups. *$p<0.05$. (Note: This figure was done by Dr. Michael Ko)



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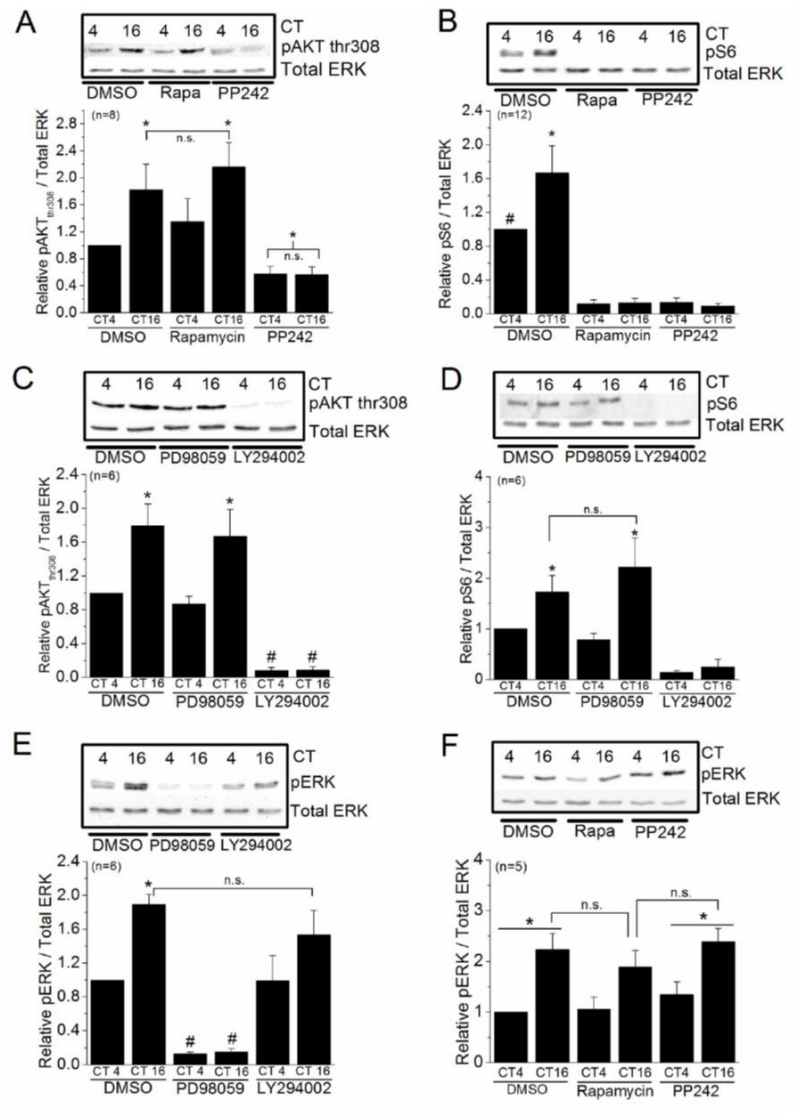

Figure 3.6 The mTORC1 pathway is downstream of PI3K-AKT.
Chick embryos (E11) were entrained in LD cycles for 7 days in ovo, and retinas were dissected, cultured, and kept in DD. On the second day of DD, cultured retinas were treated with 0.1% DMSO (control) or various inhibitors for 2 hr prior to harvest for immunoblotting at CT 4 and CT 16. (A) The phosphorylation of AKT at thr308 (pAKTthr308) was significantly higher in the control and rapamycin treated cells at CT 16 compared to other groups. "n.s." indicates that there is no statical difference between the two CT 16 groups. * indicates statistical differences. (B) The phosphorylation of S6 (pS6), a downstream target of mTORC1, is significantly higher in the control at CT 16 (*) compared to all other groups. The level of pS6 in the control at CT 4 (#) is significantly higher than groups treated with rapamycin or PP242. (C) The levels of pAKTthr308 are significantly higher in the control at CT 16 (*) and PD98059 treated group at CT 16 (*) compared to all other groups. The levels of pAKTthr308 are significantly lower in both groups (CT 4 and CT 16) treated with LY294002 (#). (D) The levels of pS6 are significantly higher in the control at CT 16 (*) and PD98059 treated group at CT 16 (*) compared to all other groups. (E) The level of di-phosphorylated ERK (pERK) is significantly higher in the control at CT 16 (*), and there is no statistical difference between the control at CT 16 and LY294002 treated group at CT 16. The pERK levels in both PD98059 treated groups (CT 4 and CT 16) are significantly lower (#) compared to all other groups. (F) Treatment with rapamycin or PP242 does not affect the circadian rhythm of pERK, in which the pERK level is significantly higher at night (CT 16, *) compared to the day time level (CT 4). *, # $p<0.05$.

3.5 Discussion

Through four independent lines of investigation (electrophysiology, immunofluorescent staining, Western blotting, and surface biotinylation assays), we uncovered a new functional role for mTORC1, the circadian regulation of ion channels in cone photoreceptors. Inhibition of mTORC1 caused a circadian phase-dependent decrease of L-VGCC currents, as well as the distribution of L-VGCCα1D in the plasma membrane. Our results suggest that mTORC1 in part was involved in the channel trafficking and translocation from the cytosol to the plasma membrane, membrane insertion, and/or membrane retention of L-VGCCα1D. This conclusion was based on our previous observation, as well as others, that the PI3K-AKT pathway is involved in ion channel trafficking (Lhuillier and Dryer, 2002, Viard et al., 2004, Ko et al., 2009). Since mTORC1 is downstream of PI3K-AKT (Figures 3.6 and 3.7), it is reasonable to conclude that in part, mTORC1 is involved in the circadian regulation of L-VGCCin the plasma membrane at night compared to the day (Ko et al., 2009)(Figure 3.5B), mechanisms involved in the circadian regulation of L-VGCC plasma membrane insertion, membrane retention (Shi et al., 2009a), or recycling are all possible actions of mTORC1, which will require further investigation.

The phosphorylation state of mTORC1-dependent signaling, but not its total protein expression, was also under circadian control. mTORC1 can be activated by phosphorylation at ser2448 (Scott et al., 1998, Reynolds et al., 2002), while ser2481 is an autophosphorylation site in the regulatory domain (Peterson et al., 2000). We found

that the phosphorylation of mTORC1 at ser2448 (pTORC1ser2448), but not ser2481 (pTORC1ser2481), displayed circadian rhythm with a peak at CT 12, which indicated that the activity of mTORC1 was under circadian control, and the circadian regulation of mTORC1 was phosphorylation-site specific. The activation of p70S6 kinase (p70S6K) is pTORC1ser2448 dependent (Chiang and Abraham, 2005), which further phosphorylates S6 to initiate other cellular processes(Ferrari and Thomas, 1994). We showed that the phosphorylation of p70S6K (pp70S6K) and S6 (pS6) in the retina also displayed circadian oscillations in synch with pTORC1ser2448.

Since we previously characterized the circadian regulation of L-VGCCs is in part through both MAPK-ERK and PI3K-AKT signaling (Ko et al., 2007, Ko et al., 2009, Huang et al., 2012), we further deciphered whether there was any cross-talk among mTORC1, MAPK-ERK, and PI3K-AKT signaling. PI3K-AKT signaling activates mTORC1 phosphorylation, while mTORC2 is upstream of PI3K-AKT (Zoncu et al., 2011). Even though MAPK-ERK signaling may also stimulate the mTORC1 dependent pathway (Carriere et al., 2011), we did not observe any cross-talk between them in the retina. Through a series of pharmacological studies, we found that mTORC1-dependent signaling was downstream of PI3K-AKT and independent from MAPK-ERK (Figure 3.7). Hence, mTORC1-dependent signaling served as part of the circadian output pathway to regulate LVGCCs in the retina.

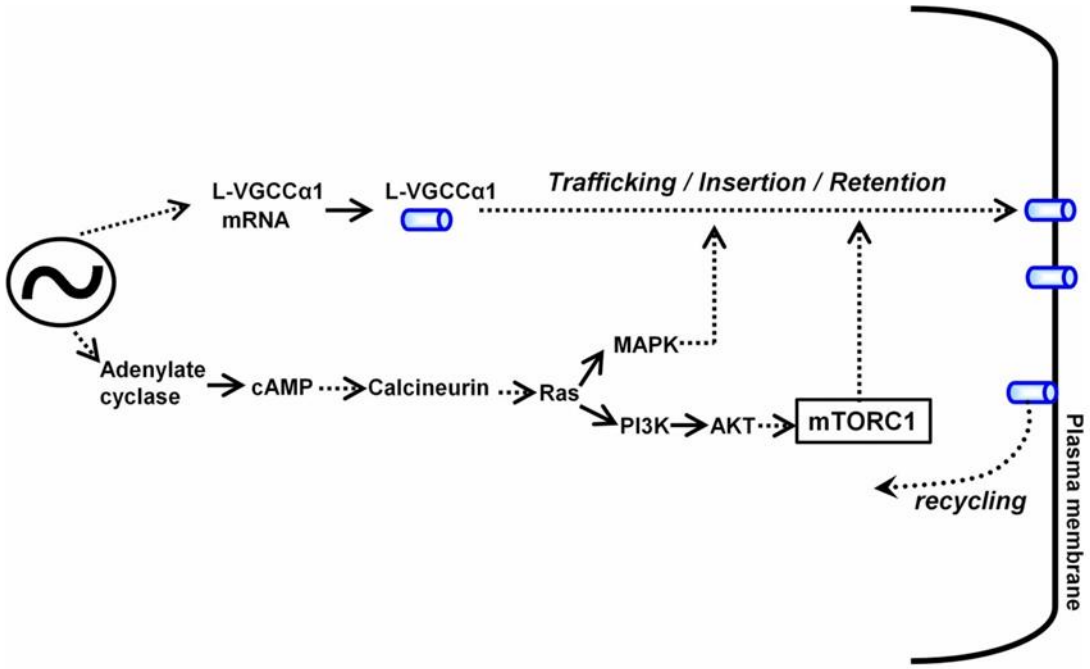

Figure 3.7 A schematic model summarizes the complex cell signaling in the circadian regulation of L-VGCCs. The circadian clock in the photoreceptor regulates the mRNA levels and protein expression of the channel forming L-VGCCα1 subunits. The circadian clock also regulates the activities / phosphorylation states of cell signaling molecules. In part, the complex cell signaling involves the trafficking, translocation, plasma membrane insertion, and/or membrane retention of L-VGCCα1. This model is based on our previous observations (Ko et al., 2001, 2004, Ko et al., 2007, Ko et al., 2009, Huang et al., 2012), as well as the current results.

mTORC signaling participates in many cellular processes including protein and lipid synthesis, metabolism, cell survival, growth (Zoncu et al., 2011), and is an important regulator of ribosome biogenesis (Proud, 2009). When cellular energy levels are high, mTORC signaling promotes energy expenditure in processes such as protein translation and prevents autophagy (He and Klionsky, 2009). When cells are under stress or nutrient-deprived conditions, mTORC signaling has the opposite action (Reiling and Sabatini, 2006). In the retina, activation of mTORC delays retinal cell death and promotes axon regeneration (Wu et al., 2004, Park et al., 2008, Punzo et al., 2009, Fox et

al., 2012, Leibinger et al., 2012), but its inhibition results in the loss of cone photoreceptor opsins and retinal degeneration (Punzo et al., 2009). Therefore, mTORC promotes survival and neural protection. In addition, mTORC1 signaling is known to be involved in the circadian rhythms of both vertebrates and invertebrates (Cao et al., 2008, Cao et al., 2010, Zheng and Sehgal, 2010). The mammalian master circadian clocks are located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore, 1983). The phosphorylation state of mTORC1-dependent signaling displays circadian rhythms in the mouse SCN (Cao et al., 2011). mTORC1 activity is light-inducible and involved in light-dependent circadian phase-shifting (Cao et al., 2008, Cao et al., 2010). Disruption of mTORC1 signaling alters the light-induced expression of Period gene, a core component of the molecular clock, in the SCN (Cao et al., 2010). In Drosophila, activation of mTORC1 impacts the nuclear accumulation of the circadian oscillator genes and lengthens the period of circadian oscillations (Zheng and Sehgal, 2010). Hence, mTORC1 plays an important role in regulating circadian rhythms.

In the present study, we discovered that mTORC1 signaling was involved in the circadian regulation of L-VGCCs in part through promoting L-VGCCα1D subunit translocation into the plasma membrane at night, and the activation of mTORC1 signaling was also under circadian control. Retinal photoreceptors are non-spiking neurons (Barnes and Kelly, 2002b), and many of their intracellular processes including calcium homeostasis are highly compartmentalized (Wei et al., 2012). In the dark, calcium influx through L-VGCCs at the synaptic terminals allows for the continuous release of neurotransmitters from the ribbon synapses (Sterling and Matthews, 2005). In

response to various light intensities, the phototransduction cascade and changes in local intracellular calcium take place in the outer segment (Korenbrot, 1995, Koutalos and Yau, 1996, Firth et al., 2001). Hence, calcium plays different roles in different localized compartments of photoreceptors.

In mammalian and avian photoreceptors, L-VGCCα1D is mainly distributed in the inner segment, soma, and synaptic terminals (Morgans, 1999, Firth et al., 2001, Yu et al., 2006, Ko et al., 2007), where calcium is involved in the regulation of metabolism and neurotransmitter release (Krizaj and Copenhagen, 2002). While the circadian oscillators in photoreceptors regulate daily changes in various cellular processes, from gene and protein expressions (Korenbrot and Fernald, 1989, Pierce et al., 1993, Haque et al., 2002, Ko et al., 2007)to light sensitivities (Ribelayga et al., 2008), all of these processes are energy dependent. In addition, there are circadian regulations of both cGMP-gated cation channels (Ko et al., 2001, 2003, 2004) and L-VGCCs (Ko et al., 2007, Ko et al., 2009, Huang et al., 2012), which might ultimately regulate calcium homeostasis in photoreceptors. In vertebrates, glucose metabolism is under circadian control (la Fleur et al., 2001), and hence, the circulating plasma glucose that reaches the retina for neuronal fuel might be oscillating daily. We postulate that the circadian regulation of L-VGCCs through mTORC1 signaling might be essential to photoreceptor metabolism and energy expenditure, since metabolism and gene expression occur in the inner segment and the soma (Krizaj and Copenhagen, 2002, Johnson et al., 2007), where LVGCCα1Dis also heavily distributed. The circadian oscillation in mTORC1 activation and the circadian phase-dependent increase of L-VGCCs in the plasma membrane of

inner segments and the soma would allow for local increases of calcium influx, which would further enhance calcium-dependent gene / protein expressions, potentially for subsequent needs in intersegmental transport (Besharse et al., 2003), outer segment renewal (LaVail, 1980), and energy requiring retinomotor movement (Pierce and Besharse, 1985, Burnside, 2001, Menger et al., 2005). Disruption of mTORC1 activation and L-VGCC circadian rhythm could further alter intracellular calcium homeostasis, which might lead to photoreceptor pathophysiological conditions and degeneration. In summary, we showed that the activation of mTORC1-dependent signaling was under circadian control, and the circadian rhythm of L-VGCCs in cone photoreceptors was in part through the PI3K-AKT-mTORC1 pathway. More specifically, mTORC1 participated in the circadian phase-dependent modulation of L-VGCCα1D trafficking and translocation. Hence, mTORC1 signaling is indispensable in maintaining healthy physiological function in the retina.

CHAPTER IV

THE CELLULAR ENERGY SENSOR AMP-ACTIVATED PROTEIN KINASE (AMPK) AND ITS ROLE IN THE CIRCADIAN REGULATION OF L-TYPE VOLTAGE-GATED CALCIUM CHANNELS IN THE PHOTORECEPTORS

4.1 Overview

L-type voltage-gated calcium channels (L-VGCCs) are responsible for neurotransmitter release from photoreceptors in the retina and under circadian regulation, in which the current densities and protein expression of L-VGCCs are significantly higher at night than during the day. The underlying mechanisms of circadian regulation of L-VGCCs are not fully clear. The processes of ion channel trafficking from the cytosol onto the plasma membrane require energy consumption. In a cell, AMPK is the energy sensor that maintains energy balance through regulating metabolism. In the present study, we demonstrated that AMPK plays a role in the circadian regulation of L-VGCCs. Activation of AMPK by AICAR dampened the L-VGCC currents at night with a corresponding decrease in protein expression of L-VGCCα1D subunits, while inhibition of AMPK increased L-VGCC current during the day. Activity of AMPK displayed a circadian rhythm in the retina, which in part participated in the circadian phase-dependent modulation of L-VGCCs. We previously showed that both MAPK-ERK and PI3K-AKT –mTORC1 signaling pathways, serve in parallel to modulate the circadian rhythm of L-VGCC trafficking and translocation.

Although stimulation of AMPK inhibited mTORC1 signaling and pERK, there was no interaction between AMPK and AKT signaling. Therefore, we concluded that AMPK is involved in the circadian regulation of L-VGCCs through modulating both mTORC1 and pERK signaling.

4.2 Introduction

The sensory systems of an organism constantly detect and process external environmental changes at both active and resting states, which result in high energy expenditure of the body. The retina is considered one of the highest energy-consuming sensory tissues since the retina has to adjust to daily ambient illumination over at least twelve orders of magnitude (Niven and Laughlin, 2008). The processes of phototransduction, protein transport for outer segment renewal, neurotransmitter release, and various physiological states ranging from hyperpolarization to depolarization of photoreceptors and other retinal neurons are highly energy dependent. The AMP-activated protein kinase (AMPK) acts as the cellular energy sensor to maintain cellular energy balance through regulating lipid, protein and carbohydrate metabolism (Steinberg and Kemp, 2009). Disregulation of AMPK is associated with retinal diseases and degeneration (Qin and De Vries, 2008, Spasic et al., 2008, Kubota et al., 2011, Kamoshita et al., 2014). However, little is known about how the role of AMPK in normal retinal physiology.

The AMPK is a heterotrimeric protein kinase containing a catalytic subunit (α) and two regulatory subunits (β and γ). The activity of AMPK depends on the cellular

ratio between AMP and ATP: AMPK is activated when intracellular AMP to ATP ratio rises. Activated AMPK promotes catabolic pathways to generate more ATP and inhibits anabolic pathways to balance the cellular metabolism (Hardie, 2007). When the intracellular ATP level is low, AMP or ADP binds to the AMPK γ subunit, which leads to activation of AMPK through phosphorylation on its threonine-172 (Oakhill et al., 2011, Xiao et al., 2011). Upstream kinases, such as live kinase B1 (LKB1) and Ca^{2+} /calmodulin-dependent protein kinase kinase β (CaMKKβ) are able to directly phosphorylate AMPK at threonine residue 172 (Thr172) (Hawley et al., 2003, Woods et al., 2005, Mihaylova and Shaw, 2011). Protein phosphatases also play important roles in regulating AMPK activities. Both protein phosphatase 2A (PP2A) and PP2C dephosphorylate AMPK at Thr-172 *in vitro* (Davies et al., 1995, Kudo et al., 1996, Wu et al., 2007). Hence, the intracellular AMP level, and other upstream kinases as well as phosphatases are involved in regulation of AMPK.

The circadian clocks in living organisms regulate the physiology and behavior and provide mechanisms for adaption, so organisms can anticipate the upcoming daily environment changes, such as temperatural fluctuations and the cycling ambient illumination. The canonical molecular 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 hours (Ko and Takahashi, 2006). Post-translational modifications such as phosphorylation, ubiquitination, and methylation, as well as various signaling pathways, contribute to the regulation of circadian oscillation. Circadian clocks participate in the metabolic processes across the

course of a day, so metabolites, enzymes, transporters as well as receptors that regulate metabolism, display daily fluctuations (Panda et al., 2002). Disruption of clock genes perturbs the energetic homeostasis, and eventually leads to the development of various metabolic syndromes, such as diabetes (Bass and Takahashi, 2010, Froy, 2010). Mutation of *Per2* gene exhibits a diabetic phenotype in the retina (Bhatwadekar et al., 2013). On the other hand, metabolic signals can feedback to modulate the circadian oscillation (Jordan and Lamia, 2013). AMPK phosphorylates one of the clock genes, *Cry1*, and the phosphorylated CRY1 protein binds to ubiquitin-mediated FBXL3 protein, which leads to degradation of *Cry* (Lamia et al., 2009). AMPK also phosphorylates casein kinase Iε, which degrades the clock gene *Per2* and shortens the length of the circadian period (Um et al., 2007). Furthermore, deficiency of AMPK alters circadian wheel running behavior and expression of circadian genes (Um et al., 2011). While AMPK modulates Clock genes through post-translational modification, AMPK is also under circadian control with its activity displaying rhythmicities in the liver and the hypothalamus (Lamia et al., 2009, Um et al., 2011). Therefore, AMPK serves as both input and output signals in the circadian mechanism.

In the vertebrate retina, circadian clocks exist in different retinal cell types and involved in many aspects of retinal function, physiology and morphology. 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, Ko et al., 2007). The L-VGCCs located in the inner segment and synaptic terminals of

photoreceptors are essential for cellular metabolism and neurotransmission. The circadian clock in cone photoreceptors regulates L-VGCC activity in a circadian phase-dependent manner, in which the maximal current densities of L-VGCCs are larger at night than during the day (Ko et al., 2007). The mRNA level and protein expression of L-VGCCα1D show circadian rhythmicity in cone photoreceptors (Ko et al., 2007). However, the underlying mechanisms of the circadian regulation of L-VGCCs still remain unclear. Since the process of ion channel transport from the cytosol onto the plasma membrane requires highly energy consumption, the goal of this study was to investigate whether the cellular energy regulator, AMPK, participates in circadian regulation of L-VGCCs.

4.3 Materials and methods

4.3.1: 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 day12 (E12) and cultured for 6 days as described previously (Ko et al., 2007, Ko et al., 2009). Cultures were prepared in the presence of 20 ng/mlciliary neurotrophic factor (CNTF; R&D Systems, Minneapolis, MN, USA), which yields cultures highly enriched with cone photoreceptors (Adler et al., 1984, Adler and Hatlee, 1989, Belecky-Adams et al., 1996)and 10% heat-inactivated horse serum. Cell culture incubators (maintained at 39°C and 5% CO2) were equipped with lights and timers, which allowed for entrainment of retinal circadian oscillators to 12h: 12h light-

dark (LD) cycles *in vitro*. Zeitgeber time zero (ZT 0) was designated as the time when the lights turned on and ZT 12 was the time when the lights went off. For *in ovo* entrainment, intact eggs were exposed to LD 12h: 12h at E10 for 7 days. Retina cells were then dissociated, cultured, kept in constant darkness (DD), and used for biochemical and molecular biological assays on the second day of DD. In some experiments, after in ovo LD entrainment for 6 days, eggs were kept in DD. On the second day of DD, retinas were collected at different circadian time (CT) points throughout the day for biochemical assays (Ko et al., 2007, Ko et al., 2009). The reason for using chick embryos from E12+6 for in vitro entrainment or E18 for in ovo entrainment is that more than 90% of the retina photoreceptors express functionally mature VGCC currents by E18 (Gleason et al., 1992).

4.3.2: Immunoblot analysis

Samples were collected and prepared as described previously (Huang et al., 2012, Huang et al., 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 3VO4; 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-ERK (pERK; Sigma, St. Louis, MO, USA), 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-AKT (Thr308, Cell Signaling Technology). Blots were visualized using appropriate secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) and an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA). Relative protein expressions for all proteins involved in this study are reported as a ratio to total ERK since total ERK remains constant throughout the day. Band intensities were quantified by densitometry using Scion Image (NIH, Bethesda, MD, USA). All measurements were repeated at least 3 times.

4.3.3: 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 BaCl2, 0.4 MgCl2, 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 MgCl2, 0.1 CaCl2, 1.1 EGTA, and 10 HEPES, pH 7.3 adjusted with CsOH. Recordings were made only from cells with elongated cell bodies with one or more prominent oil droplets (hallmark of avian cone photoreceptors) (Gleason et al., 1992, Pierce et al., 1993, Ko et al., 2001). Currents were recorded at room temperature (RT, 23°C) using an Axopatch 200B (Axon Instruments/Molecular Devices, Union City, CA, USA) or 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). Electrode capacitance was compensated after gigaohm (GΩ) seals were formed. Cells were held at -65mV, and ramp voltage commands from -80 to +60 mV in 500 ms were used to evoke Ba2+ 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. Current densities (pA/pF) were obtained by dividing current amplitudes by membrane capacitances. AICAR and compound C were obtained from EMD Millipore (Billerica, Massachusetts, USA),and Sigma (St. Louis, MO), respectively. AICAR was dissolved in water, while compound C was dissolved in 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.

4.3.4: 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. Cell were fixed at CT4 or CT16 with Zamboni fixative and then permeabilized in 1% Triton-X phosphate buffer (PB). Samples were blocked in 10% goat serum in 0.1% Triton-X/PB and incubated with VGCCα1D primary antibody (Alomone, Jerusalem, Israel) at 4°C overnight. The fluorescent conjugated secondary antibody (Alexa Fluor® 488 goat anti-rabbit; Molecular Probes, Carlsbad, CA, USA) was applied on the coverslips at room temperature for 2 h in the dark. Coverslips were then re-washed and mounted with ProLong® Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; 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 epi-fluorescence to determine the localization of VGCCα1D and the nucleus (with DAPI). 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 carried out blindly. The experiment was repeated at least 4 times.

4.3.5: Immunohistochemistry

The whole chicken eyes were excised and fixed with Zamboni's fixative for 24 hr at 4°C. The frozen sections (12μm) were incubated with a blocking solution containing 10% goat serum in PBS for 2 hr at room temperature after permeabilization procedure. The sections were then incubated with primary antibody in 2% goat serum-PBS at 4°C overnight. After washing with PBS 3 times, the sections were incubated with secondary

antibody in 2% goat serum-PBS at room temperature for 2 hr in darkness. After washed with PBS 3 times, the sections were mounted with the ProLong® Gold antifade reagent and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA). The images were taken with a Zeiss Stallion microscope (Carl Zeiss AG, Oberkochen, Germany). The primary antibodies used were anti-L-VGCCα1D subunit antibody (Alomone, Jerusalem, Israel) at 1:100. The secondary antibodies used were Alexa Fluor® 568 goat anti-rabbit IgG (Molecular Probes, Carlsbad, CA, USA).

4.3.6: Statistical analysis

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

4.4 Results

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

In the retina, the neurotransmitter release from photoreceptors to other retinal cells relies on Ca^{2+} influx through L-VGCCs (Barnes and Kelly, 2002a). The L-VGCCs are under circadian control in both cone photoreceptors and bipolar cells (Hull et al., 2006, Ko et al., 2007). Maximal currents of L-VGCCs elicited at 0 mV are peaked in the middle of night (ZT 16-19) and troughed during the middle of day (ZT4-7; (Ko et al.,

2007) (Fig 4.1A). To investigate whether AMPK modulated the circadian dependent activities of L-VGCCs, the AMPK activator, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), an analog of AMP, was used. Retinal cells were cultured and entrained to LD 12:12 cycles for five days, and prior to the patch-clamp recordings, cells were treated with AICAR (500μM) for 2hr. L-VGCC current densities recorded in the middle of night from AICAR treated cone photoreceptors were significantly dampened (ZT 16-19; Fig 4.1B (iii), 4.1C, 4.1E), while treatments with AICAR did not have any significant effect on L-VGCC currents when cells were recorded during the middle of day (ZT 4-7; Fig 4.1B (i), 4.1C, 4.1E). Treatments with AICAR did not have any significant effect on L-VGCCs when cones were recorded during the late day (ZT 8-11) or late night (ZT 20-23).

We further treated cultured retinal cells with an AMPK inhibitor, compound C (1μM), for 2 hr prior to recordings to examine whether inhibition of AMPK might affect the circadian rhythms of L-VGCCs. In compound C treated cells, the L-VGCC currents were significantly enhanced when cone photoreceptors were recorded during the middle of day (ZT 4-7) compared to control cells treated with DMSO (0.1%) (Fig 4.2A, 4.2C, 4.2E). Inhibition of AMPK did not affect L-VGCCs when photoreceptors were recorded during other time periods of the day (Fig 4.2B, 4. 2D, 4.2E). These results indicate that AMPK has a circadian phase-specific effect on L-VGCCs in cone photoreceptors: when AMPK was activated in the middle of night (ZT 6-19), the L-VGCCs were significantly decreased, and when AMPK was inhibited in the middle of day (ZT 4-7), the L-VGCCs were significantly enhanced.

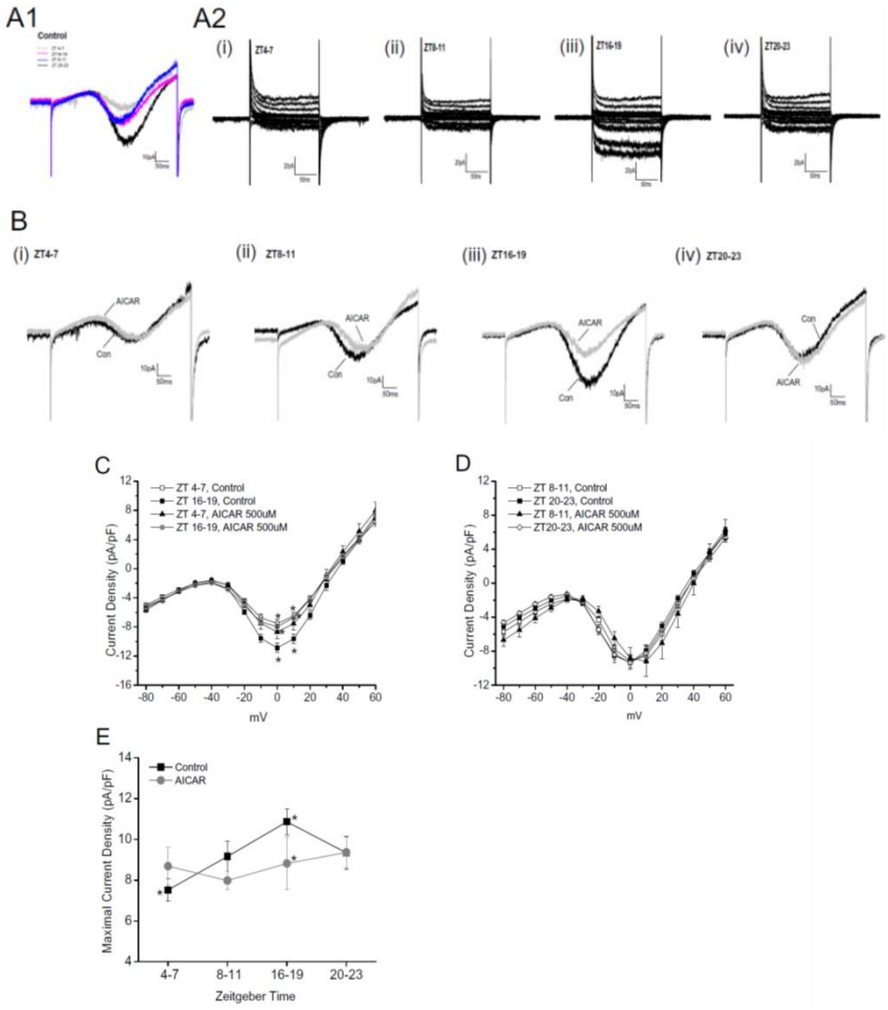

Figure 4.1 The AMPK activator dampens the circadian regulation of L-VGCC currents.
L-VGCC currents were recorded from cultured chick cone photoreceptors on the sixth day of LD entrainment during the day (ZT 4-7 or ZT 8-11) or at night (ZT 16-19 or ZT 20-23). All cells were recorded using a ramp command 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 (4-7or 8-11) and night (16-19 or 20-23) L-VGCC current traces recorded from control with (A1) ramp command, or (A2) step command. (B) Representative traces of L-VGCC currents from cells that were treated with AICAR (500μM; gray) for 2 hr prior to recordings compared to control cells (black) during different time periods [ZT4-7:B(i); ZT8-11:B(ii); ZT16-19: B(iii); ZT20-23: B(iv)]. (C) Average current-voltage relationships are shown in current density (pA/pF) and step-voltage (mV). * indicates that 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 (black triangle) recorded during the day (ZT 4-7). (D) The average current-voltage relationships are shown in current density (pA/pF) and step-voltage (mv) from the cells were recorded either from ZT 8-11 or ZT 20-23. Open square: control cells recorded at ZT8-11; gray square: control cells recorded at ZT20-23; black triangle: AICAR-treated cells recorded at ZT8-11; white diamond: AICAR-treated cells recorded at ZT 20-23. There was no statistical difference in these four groups. (E) The maximal current densities were elicited at 0 mV of the step command at different ZT time periods. * indicates that 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 that recorded during the nighttime (AICAR, ZT 16-19). Each group had at least 15 cells except the AICAR-treated group at ZT 8-11with 5 cells.

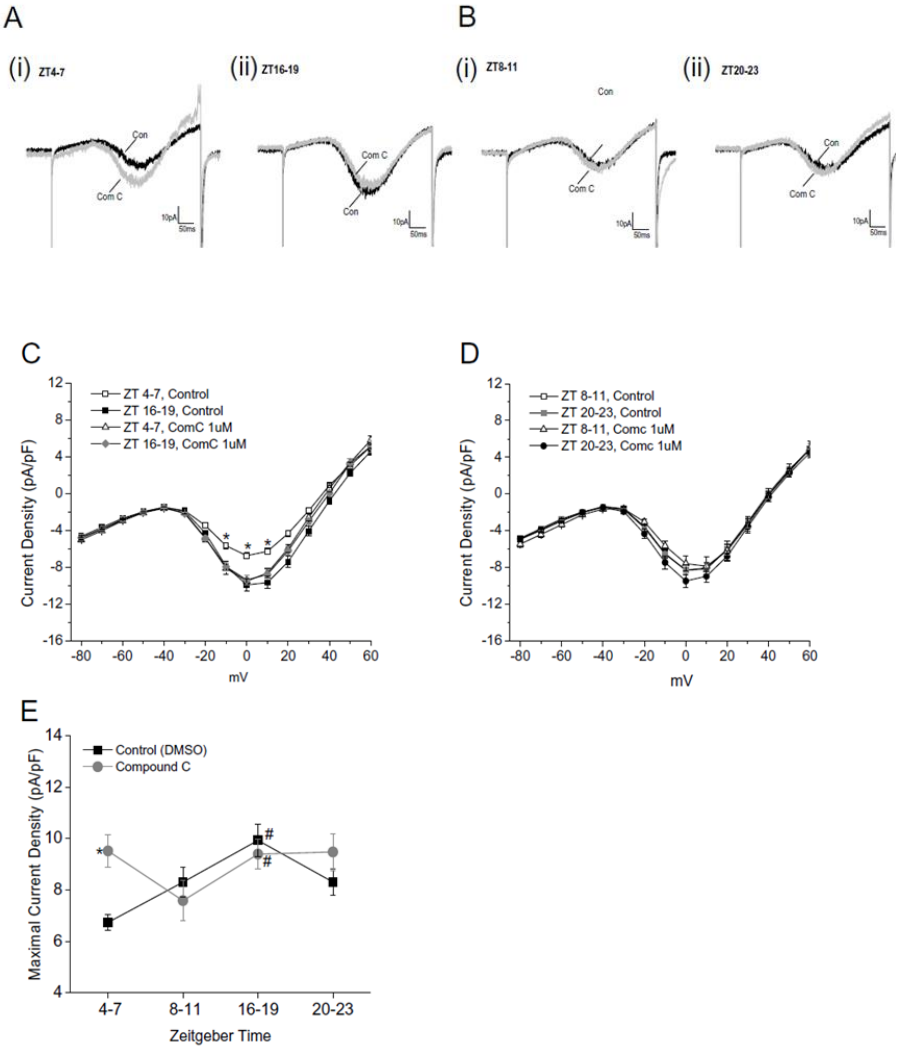

Figure 4.2 Inhibition of AMPK increases the L-VGCC current densities during the day.
(A and B) Representative L-VGCC current traces from 0.1% DMSO-treated cone photoreceptors (as control; black) and1 μM compound C-treated cells (gray) that were recoded either from (A) ZT 4-7 (i) and ZT 16-19(ii), or (B) ZT 8-11(i) and ZT 20-23(ii). (C) The average current-voltage relationships are 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 higher than the control recorded at night (ZT 16-19;black square), as well as compound C treated cells recorded both during the day (ZT 4-7; white triangle) and at night (ZT 16-19; gray diamond). (D) Average current-voltage relationships are shown in current density (pA/pF) and step-voltage (mV) from the cells recorded either from ZT 8-11 or ZT 20-23. Open square: control cells recorded at ZT 8-11; gray square: control cells recorded at ZT 20-23; white triangle: compound C-treated cells recorded at ZT 8-11; black circle: compound C-treated cells recorded at ZT 20-23. There was no statistical difference in these four groups. (E) The maximal current densities were elicited at 0 mV of the step command in different ZT phases. * indicates that 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.

4.4.2: AMPK modulates the protein expression of L-VGCCα1D

Since AMPK participated in the circadian phase-dependent modulation of L-VGCC currents in cone photoreceptors, we examined whether AMPK affected protein expression of L-VGCCs. In both mammalian retina and avian retina, the L-VGCCα1D abundant present in the inner segment, outer nuclear layer and outer plexiform layer ((Kersten et al., 2010) and Fig 4.3A). The avian cone photoreceptor can be identified with the characteristic oil droplets present in the inner segment at its distal most extremity just basal to the outer segment. The distribution of L-VGCCα1D is concentrated in the soma of cone photoreceptors (Fig 4.2 (Ko et al., 2007, Huang et al., 2013)), and the fluorescence intensity of L-VGCCα1D was significantly higher when retinal cells were fixed at CT 16 compared to cells fixed at CT 4 (Fig 4.2A left panel). Treatment with AICAR for 2 hr significantly decreased the fluorescence intensity of L-VGCCα1D in cone photoreceptors fixed at CT 16, while there was no fluorescence difference between controls and AICAR treated photoreceptor when cells were fixed at CT 4 (Fig 4.2 A right panel, 4.2B). Hence, activation of AMPK by AICAR reduced protein expression of L-VGCCα1D in cone photoreceptors.

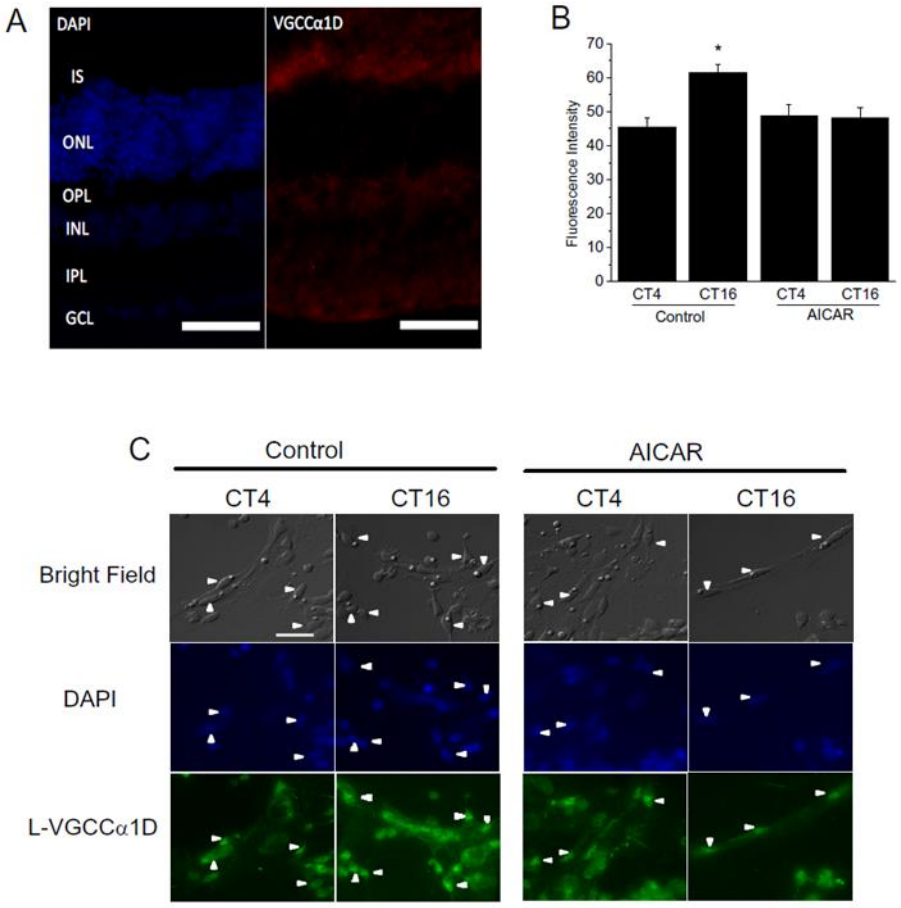

Figure 4.3 Protein expression of L-VGCCs decreases at night by the AMPK activator.
(A) The chicken eye section was fixed on E18. Protein expression of L-VGCCα1D is mainly distributed in the inner segment (IS), outer plexiform layer (OPL), inner plexiform layer (IPL) and ganglion cell layer (GNL). The scale bar is 50μm. (B) The fluorescence intensity of L-VGCCα1D is significantly higher at CT 16 of the control, compared to all other groups. Each group has at least 20 cells from 4 different trials. *$p<0.05$. (C) The reprehensive images from cultured retinal cells. Retinal cells were dissociated and cultured on glass coverslips at E12 and entrained to 12:12 LD cycles for five days *in vitro* and kept in DD. On the second day of DD, cells were treated with AICAR at CT 2 and CT 14 for 2hr followed by fixation at CT 4 and CT 16. The left panel shows the fluorescent images from the control and the right panel shows the images from AICAR treated cells. Arrowheads indicate the cone photoreceptors. The scale bar is 20 μm.

4.4.3: The AMPK activity is under circadian control

Since AMPK regulated the activities and protein expression of L-VGCCs in a circadian-phase-dependent manner, we investigated whether the activities of AMPK exhibit a circadian rhythm in the retina. Chick retinal samples were collected at six

different zetigeber time points throughout a day. The total ERK was used as the loading control since the total amount of ERK is constant throughout the course of a day (Ko et al., 2001). We found that the phosphorylation state of AMPK at Thr172 (p-AMPK), the major AMPK phosphorylation and activation site, peaked at ZT 4 when the chicken embryos were entrained under the LD cycles (Fig 4.4 A). To further confirm that the rhythmicity of AMPK activities was indeed governed by the retinal circadian clock, the retinas were collected on the second day of constant darkness (DD) after the chick embryos were entrained in LD and then kept in DD for free-running. On the second day of DD, the retinal samples were collected every four hours within a day. The p-AMPK showed the circadian rhythm with a peak during the subjective day, while the total amount of AMPK remained constant throughout the course of a day (Fig 4.4B). Hence, the activity of AMPK is under circadian regulation in the retina.

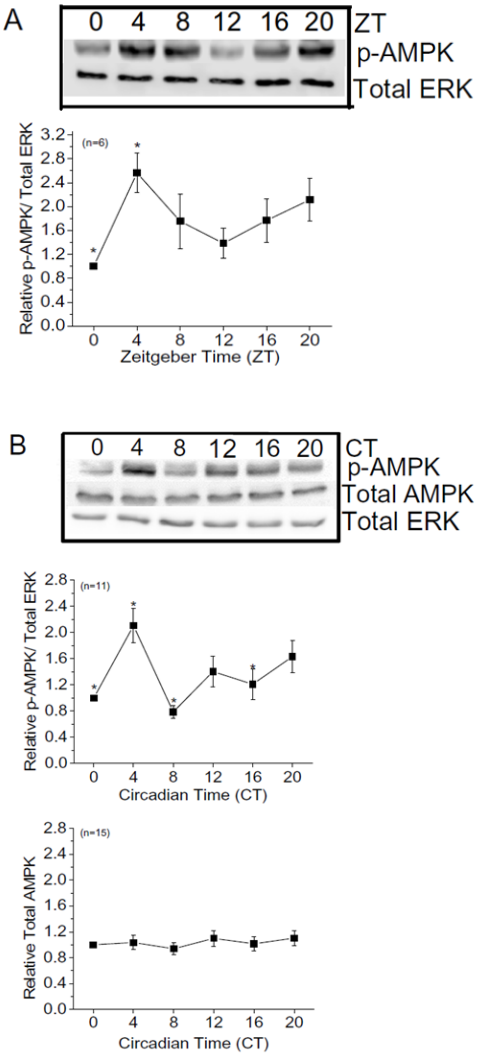

Figure 4.4 The AMPK activity is under circadian control.
(A) Retinas were collected at 6 zetigeber time points (0, 4, 8, 12, 16, 20) for immunoblotting analyses after entrainment to 12: 12 hr LD cycles for 8 days *in ovo*. Phosphorylation of AMPK at Thr-172 (p-AMPK) shows a diurnal rhythm with a peak at ZT 4. (B) After entrainment to 12: 12 hr LD cycles for 7 days *in ovo*, the eggs were moved to DD for one day. On the second day of DD, the retinas were collected at 6 circadian time points for immunoblotting analysis. p-AMPK exhibits a circadian rhythm with its peak at CT4 (upper panel), while the total amount of AMPK remains constant throughout a day (lower panel). * indicates statistical significance at ZT 4 compared to ZT0 or at CT4 compared to CT0, CT8, CT16. *p<0.05.

4.4.4: The cAMP-dependent signaling mediates AMPK activities

Although AMPK regulated the circadian rhythm of L-VGCCs, and with its own activation under the circadian control, how AMPK integrated with other cellular signaling to regulate L-VGCCs was not clear. Cyclic AMP 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 activity in a tissue-specific manner (Yin et al., 2003, Hurley et al., 2006, Omar et al., 2009, Djouder et al., 2010). Therefore, we investigated the relationship between cAMP signaling and AMPK in the retina. Activation of adenylyl cyclase by forskolin resulted in diminished phosphorylation of AMPK during the subjective day (Fig 4.5 A), while treatment with the adenylyl cyclase inhibitor MDL-12,330A significantly increased AMPK activity during subjective night (Fig 4.5B). Interestingly, we and other previously showed that the quantity of cAMP is under circadian control in the retina with its peak during the subjective night (Nikaido and Takahashi, 1998, Ivanova and Iuvone, 2003a, Huang et al., 2012). These data showed that not only was the cAMP signaling was upstream of AMPK in the retina, cAMP signaling served as a negative regulator of AMPK. When cAMP was high at night, the AMPK was relatively low.

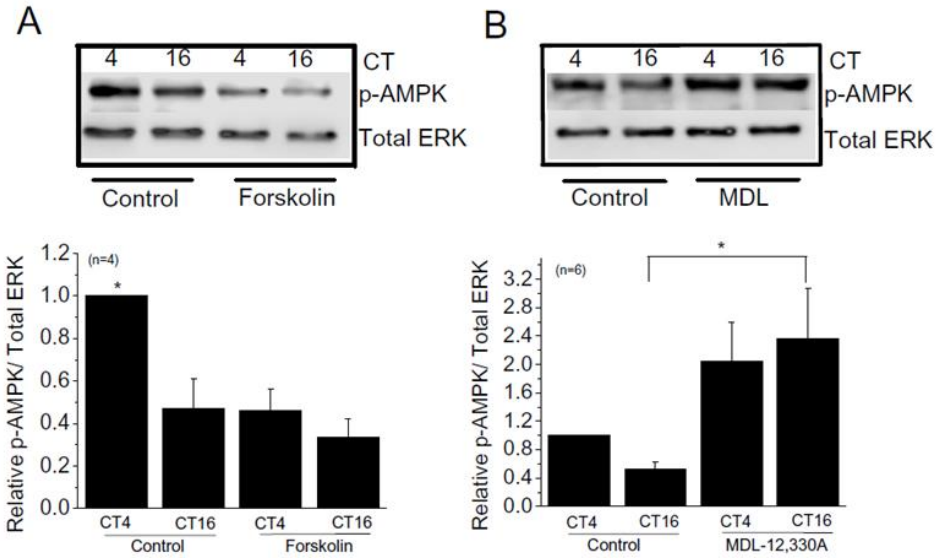

Figure 4.5 The cAMP signaling mediates AMPK activities.
Retinas were excised and cultured in DD for one day after chick embryos were entrained under LD cycles for 6 days. On the second day of DD, the cultured retinas were treated with DMSO (0.1%) or various inhibitors for 2 hr prior to harvest for immunoblotting at CT 4 and CT 16. (A) Treatment with forskolin (20μM) at CT 4 significantly decreased the phosphorylation of AMPK at Thr 172 (p-AMPK). * indicated that p-AMPK is significantly higher in the control at CT 4 than the other three groups. (B) Application with the adenylyl cyclase inhibitor, MDL-12,330A (50μM), significantly increased the AMPK phosphorylation at CT 16 compared to the control. There was no statistical difference between two CT 4 groups. *$p<0.05$.

4.4.5: The AMPK regulates mTORC1 signaling pathway

MAPK-ERK and PI3K-AKT signaling are involved in regulation of ion channel trafficking. These two signaling pathways are downstream of cAMP and parallel with each other in regulating the circadian rhythm of L-VGCCs in the retina (Ko et al., 2007, Ko et al., 2009). We previously showed that mTORC1 signaling is a downstream target of the PI3K-AKT and regulates the L-VGCC trafficking from cytosol onto plasma membrane (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 pERK (Fig 4.6A,B), and treatment with AICAR also significantly lowered the phosphorylation of S6 (pS6), a downstream target of mTORC1 signaling at night (Fig4.6A, D). Treatment with AICAR seemed to increase pAKT during the day (not statistically significant) (Fig 4.6A,C). Hence, AMPK integrated into the cell signaling network through inhibition of mTORC1 and pERK.

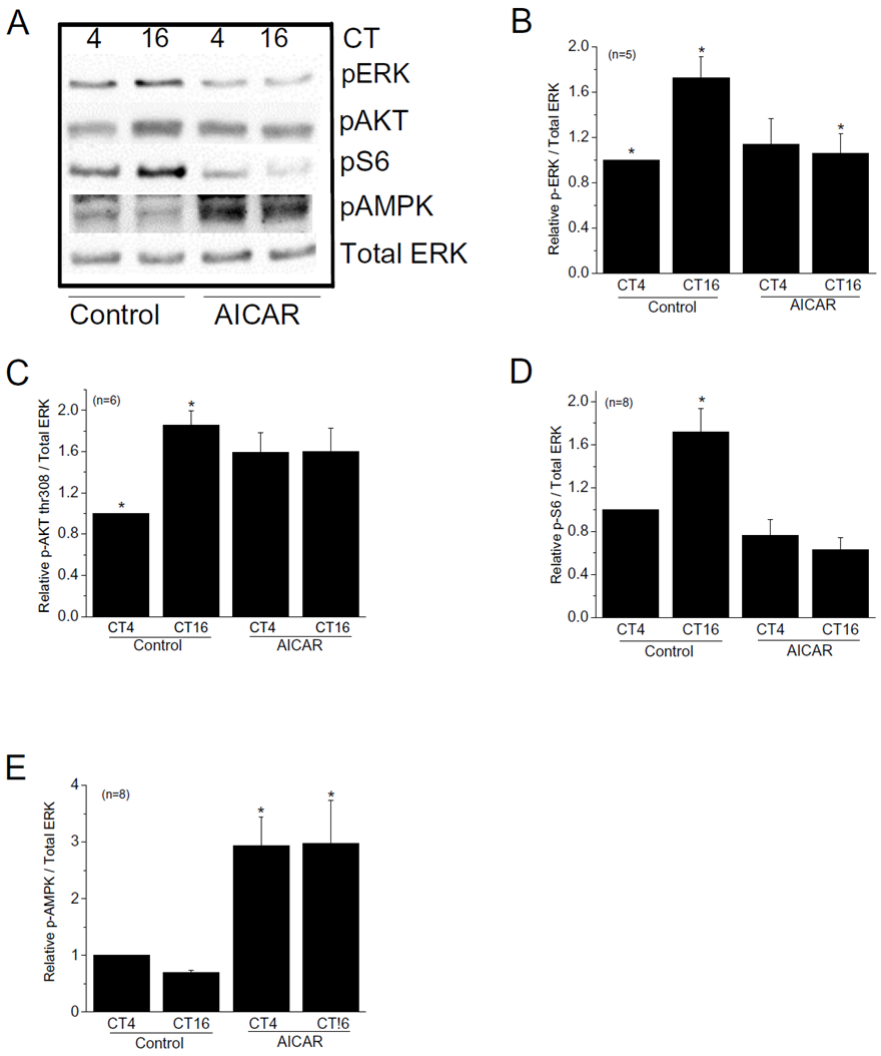

Figure 4.6 The AMPK is a negative regulator for mTORC1 signaling.
Chick embryos were entrained under LD cycles for 6 days. At E17, the retinas were dissected and cultured in DD for one day. On the second day of DD, the cultured retinas were treated with either AMPK activator AICAR for 2 hr. The retinal samples were collected at CT 4 and CT 16 for western blotting analyses. (A) The di-phospho-ERK (pERK), phosphor-AKT at Thr308 (pAKT), phospho-S6 (pS6) and phosphor-AMPK at Thr172 (pAMPK) were detected from samples treated with AICAR (500μM) or control. Since the total amounts of ERK, AKT or S6 remained constant throughout a day, the total amount of ERK (Total ERK) was used to serve as loading control in the experiment. (B) After treatment with AICAR, the pERK was slightly 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 does not significantly change the phosphorylation of AKT. * indicates the significant difference between CT 4 and CT 16 of the control groups. (D) The level of pS6 was 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 2hr increases AMPK activities. * indicated the significant difference between the AICAR groups and control groups at both CT 4 and CT 16, respectively.

4.5 Discussion

In this study, we demonstrated that AMPK participated in circadian regulation of L-VGCCs in the retina. Activation of AMPK led to decreased protein expression and current densities of L-VGCCs at night, while inhibition of AMPK resulted in enhancement of L-VGCC currents and protein expression during the day. In this regard, we observed that AMPK activity was rhythmic, with the peak of phosphorylated AMPK levels occurring during the subjective day. While AMPK is downstream of cAMP-signaling, it integrated into the signaling pathways that regulate L-VGCC trafficking, including MAPK-ERK, PI3K-AKT, and mTORC1-S6. 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 in multiple signaling pathways, which indicates that AMPK might have multiple functions other than serving as an energy sensor.

As a cellular energy sensor, the activity of AMPK is under influence of various factors, including intracellular Ca^{2+} concentration, cellular AMP/ATP ratio, nitric oxide formation and other metabolite signals (Viollet et al., 2010, Mihaylova and Shaw, 2011). AMPK regulates many aspects of cellular physiological processes, such as glucose, lipid and protein metabolism, and modulates ion channels and transporters (Steinberg and Kemp, 2009). How AMPK regulates ion channels is diverse, tissue/cell type-dependent, and sometimes controversial (Andersen and Rasmussen, 2012, Dermaku-Sopjani et al., 2014). For example, constitutively active AMPK slows the voltage-gated sodium

channel (Nav1.3) inactivation and shifts the voltage-activation curve toward a more hyperpolarized potential 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 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 in the regulation of sodium, potassium channels as well as various transporters, AMPK might be important for regulation of calcium channels, which are essential for synaptic transmission in the nervous system. Since photoreceptors are non-spiking neurons (Barnes and Kelly, 2002a), calcium influx through L-VGCCs at the synaptic terminals allows for the continuous release of neurotransmitters from ribbon synapses (Sterling and Matthews, 2005).

Here, we present evidence that AMPK regulated L-VGCCs in cone photoreceptors, which has not been presented to date. L-VGCCs in cone photoreceptors exhibit a circadian rhythm with their currents reaching maximum during the middle of night (Ko et al., 2007). Activation of AMPK decreased L-VGCC currents in cone photoreceptors in the middle of night (Fig 4.1) with a corresponding decrease in L-VGCCα1D protein expression (Fig 4.3), while inhibition of AMPK caused the opposite effect during mid-day (Fig4.2). These results give the first insight into the role of AMPK in circadian phase-dependent regulation of L-VGCCs, which is critical for photoreceptors to respond to various ambient light throughout the course of a day.

AMPK is also associated with degeneration and inflammation of photoreceptor cells. Mutation of the AMPK β subunit in Drosophila causes photoreceptor degeneration and defects in neurotransmitter release (Spasic et al., 2008). In a lipopolysaccharide-induced retinal inflammation model, activation of AMPK can rescue the ERG a-wave response and protect the length of outer segment of photoreceptors and expression of visual pigment rhodopsin (Kamoshita et al., 2014). AMPK has been found to be down-regulated in the retina in diabetic mice, which leads to increased inflammation by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB; (Kubota et al., 2011)). Moreover, activation of AMPK mediates retinal pigment epithelium phagocytosis of photoreceptors in oxidative stress (Qin and De Vries, 2008), and prevents apoptosis of retinal pericytes in diabetic retinopathy(Cacicedo et al., 2011). Hence, AMPK is crucial in maintaining the normal physiological function of photoreceptors, since these retinal neurons have a high energy demand in order to adjust to daily changes in illumination over twelve orders of magnitude. In retinal photoreceptors, energy consumption is highly compartmentalized (Linton et al., 2010, Wei et al., 2012). Most of the energy expenditure occurs at the outer segment 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 darkness, most energy consumption is in the inner segment and synaptic terminals to maintain the dark current and neurotransmitter release, respectively (Wong-Riley, 2010). We found that the overall energy state was fluctuated in chick retina throughout the course of a day, since AMPK activity peaked during mid-day (Fig 4.4).

Our result demonstrated that circadian rhythm of AMPK activities not only reflects the energy demands of the retina, indicating that rhythmicity might play an important role in regulating L-VGCCs and maintaining normal physiological functions of photoreceptors.

Circadian regulation of L-VGCCs occurs in part through two parallel pathways, the ERK and PI3K-AKT signaling pathways. Both signaling pathways are downstream of cAMP (Ko et al., 2007, Ko et al., 2009). Cyclic AMP has a negative action on AMPK, as we discovered that the activation of cAMP reduced AMPK activity (Fig 4.5). In a previous study, we found that mTORC1 signaling is a downstream target of the PI3K-AKT pathways that modulates L-VGCCα1D trafficking and translocation (Huang et al., 2013). Stimulation of AMPK significantly diminished phosphorylation of S6 and moderately decreased phosphorylation of ERK (Fig4.6). Because of the complexity of the cell signaling network, integration of AMPK into various signaling pathways in regulation of L-VGCCs indicates that AMPK has multiple roles other than serving as an energy sensor; but also the regulation of L-VGCCs is energy consuming. The circadian fluctuation of AMPK activities might be a way for cells to be more energy efficient. We postulate that the production of ATP from mitochondria in photoreceptors might also be under circadian control, which might have the opposite circadian phases from AMPK activities. In summary, we showed that the energy balance is associated with ion channel activity in the retina. The activity of AMPK is under circadian control. Activation of AMPK decreased the currents and protein expression of L-VGCCs in part through inhibition of mTORC1 signaling.

CHAPTER V

CONCLUSION

The circadian system in diverse species is an evolutionary advantage for organisms to anticipate environmental changes including light, temperature, food, and prepare for adaptation according to external time cues. Since the visual system has to anticipate large changes in daily ambient illumination, the circadian clocks in the retina provide a mechanism for the visual system to initiate more sustained adaptive changes throughout the course of a day (Cahill and Besharse, 1995, Green and Besharse, 2004). Indeed, many aspects of retinal physiology and function, including gene and protein expression, ion channel activities, retinal pH, outer segment shedding and renewal, and rod-cone photoreceptor coupling are under control of circadian clocks (Korenbrot and Fernald, 1989, Pierce et al., 1993, Dmitriev and Mangel, 2001, Ko et al., 2001, Ko et al., 2007, Storch et al., 2007, Holter et al., 2012, Liu et al., 2012). In addition, the retinal clocks also promote photoreceptor survival and growth in aging, and disruption of clock genes impacts visual processing as well as causes photoreceptor degeneration (Organisciak et al., 2000, Ogilvie and Speck, 2002, Grewal et al., 2006, Baba et al., 2009, Jun et al., 2011, Mollema et al., 2011, Ait-Hmyed et al., 2013, Bhatwadekar et al., 2013). Therefore, retinal circadian clocks are essential for general retinal function and health.

The work presented in this dissertation examined the cellular networking of various signaling pathways that participate in the circadian phase-dependent modulation

of L-VGCCs in the retina. The current densities of L-VGCCs are significantly larger at night than during the day, and both mRNA level and protein expression of α1D subunit of these channels are also under circadian control in chick photoreceptors (Ko et al., 2007). Thus far, two parallel pathways, cAMP-Ras-ERK and cAMP-Ras-PI3K-AKT, are known to serve as the circadian output pathways to regulate L-VGCCs in photoreceptors (Ko et al., 2007, Ko et al., 2009). Through combining biochemical, molecular, morphological, and electrophysiological analyses, we demonstrated that protein kinases (mTORC1, AMPK) and phosphatase (calcineurin) exhibit circadian rhythms in their activities, and they interact with other signaling pathways in the circadian regulation of L-VGCCs. The underlying cellular mechanisms of the circadian regulation of L-VGCCs were further dissected into three parts in this dissertation.

The activity of calcineurin, but not its protein expression, was under circadian control with the peak at the middle of the night. Calcineurin played a role in post-translational modification of L-VGCCs since inhibition of calcineurin did not disrupt the circadian rhythm of α1D subunit mRNA level, but decreased the protein expression of α1D subunit and L-VGCC current densities at night. Calcineurin acts as downstream of cAMP, but upstream of Ras-ERK and Ras-PI3K-AKT in the circadian regulation of L-VGCCs. Although results demonstrated in this dissertation indicate that calcineurin is upstream of Ras and downstream of cAMP, it is possible that there are missing links between cAMP and calcineurin, as well as between calcineurin and Ras. The cAMP signaling may indirectly regulate calcineurin activity in photoreceptors. Additionally, we showed calcineurin is upstream of Ras-ERK and Ras-PI3K-AKT pathways, but we

cannot rule out the possibility that calcineurin may interact with various other signaling pathways. Another signaling pathway, the NO-cGMP-PKG signaling, is upstream of Ras in the circadian regulation of L-VGCCs (Ko et al., 2013), and there is cross-talk between NO-cGMP-PKG and calcineurin in cardiac myocytes (Fiedler et al., 2002). Therefore, NO-cGMP-PKG signaling may inhibit calcineurin and lead to low the calcineurin activity during the daytime, which will need to be further investigated. Calcineurin is also involved in circadian phase-shifting in mammals (Katz et al., 2008). Although calcineurin serves as the circadian output pathway to regulation L-VGCCs, it is possible that calcineurin is also involved in the circadian input pathway to affect circadian core oscillation, which will require further investigation.

In the second part of dissertation, mTORC1 signaling was investigated since both the calcineurin inhibitor FK506 and the mTORC1 inhibitor rapamycin bind to FKBP12 (Schreiber, 1991). While the rapamycin-FKBP12 complex inhibits mTORC1 signaling, the FK506-FKBP12 complex targets calcineurin (Liu et al., 1991). We found that the activities of the mTORC1 signaling cascade display circadian oscillations, and mTORC1 is downstream of the PI3K-AKT pathway. Disruption of mTORC1 signaling decreases the plasma membrane-bound fraction of L-VGCCα1D at night, while there is no effect on the protein level of L-VGCCα1D found in cytosol compartment. The L-VGCCα1D protein reduction on the plasma membrane results in dampened L-VGCC current densities at night. Hence, mTORC1 signaling regulates the circadian rhythm of L-VGCCs in part through ion channel trafficking and translocation. Even though the mTORC1 signaling promotes L-VGCCα1D subunit trafficking from cytosolic onto the

plasma membrane at night, FKBP12 may potentially be involved in the circadian regulation of L-VGCCs, as we observed that FKBP12 showed circadian rhythmicity with its protein expression abundant at night in the retina (unpublished data). FKBP12 is physiologically associated with intracellular calcium channels including the ryanodine receptor (RyR) and the inositol 1,4,5-trisphosphate receptor (IP3R), and plays an important role in stabilization of these channels (Brillantes et al., 1994). Treatment with FK506 or rapamycin causes FKBP12 disassociation from intracellular calcium channels, which increase Ca^{2+} release from the ER intracellular Ca^{2+} stores (Timerman et al., 1993, Cameron et al., 1995, Kaftan et al., 1996). Thus, circadian phase- dependent modulation of L-VGCCs is likely through additional mechanisms, which could be FKBP12 - dependent, and calcium-dependent inactivation of L-VGCCs by elevated intracellular Ca^{2+} concentration.

Finally, we studied the role of AMPK in circadian regulation of L-VGCCs. AMPK is the cellular energy sensor that maintains the cellular energy balance in response to the environmental signals such as nutrition and stress. Interestingly, activity of AMPK peaked during the subjective day and lowered in the subjective night, exhibiting circadian phases opposite to activities of mTORC1 signaling pathway in the retina. The activation of AMPK led to attenuation of protein expression and current densities of L-VGCCs at night in part through inhibition of the downstream of mTORC1 target, S6. AMPK and its upstream kinase LKB1 are involved in the synapse transmission in the retina (Samuel et al., 2014). Deletion of either LKB1 or AMPK in young mice reduced ERG a- and b- waves and causes ectopic synapses in the outer

retina where photoreceptors present abnormal axonal retractions, and both bipolar cells and horizontal cells extend their dendrites into the outer nuclear layer (Samuel et al., 2014). These hallmarks caused by deletion of AMPK or LKB1 are also found in old mice occurring naturally as part of the aging process, but elevation of AMPK in old mice can rescue these aged-associated synaptic alternations (Samuel et al., 2014). We showed that AMPK plays a role in the circadian phase-dependent regulation of L-VGCCs. Since L-VGCCs are essential for neurotransmitter release in the synaptic terminals of retinal neurons, AMPK might be critical for maintaining morphological and functional synapses in the retina in addition to acting as an energy sensor. Another functional role of AMPK we discovered in the retina was that AMPK may also regulate sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA), an intracellular calcium transporter that transports Ca^{2+} from the cytosol into the endoplasmic reticulum. Protein expression of SERCA was significantly higher during the subjective night than during the subjective day (unpublished data). Activation of AMPK repressed SERCA expression at night. However, the underlying cellular mechanisms of how AMPK interacts with SERCA will need further investigation. It seems that AMPK is not only involved in regulation of L-VGCCs at the synaptic terminal, but it also regulates intracellular calcium homeostasis in the photoreceptors.

The circadian system in the retina provides the benefit for adjusting retina function in anticipation of daily scotopic and photic condition. The L-VGCCs in the retina are under circadian regulation, in which L-VGCCs govern a wide range of cellular processes including calcium homeostasis, neurotransmitter release and cell survival.

Calcium influx through L-VGCCs in the inner segment of photoreceptors regulate cellular metabolism and gene expression, while at the synaptic terminal of photoreceptors and bipolar cells, calcium enters into cells involving in sustained glutamate release. Mutation of the L-VGCCα1 subunit gene results in X-linked congenital stationary night blindness due to impairment of glutamate releases from photoreceptor synaptic terminal (Bech-Hansen et al., 1998). In mice, knockout of β2 subunit of L-VGCCs alters the thickness of OPL and ERG b-wave, which reflects the glutamate transmission is damaged in the OPL (Ball et al., 2002). Therefore, L-VGCCs are indispensable for synaptic transmission in photoreceptors. On the other hand, L-VGCCs are also involved in retinitis pigmentosa that is caused by the mutation of cGMP phosphodiesterase 6 β subunit gene (*Pde6b*) and followed by photoreceptor degeneration. Intracellular Ca^{2+} concentration is elevated in rd1 mice, a retinal degeneration mouse model with mutation of *Pde6b* gene, in part through Ca^{2+} entering into L-VGCCs. Modulation of L-VGCCs can delay photoreceptor degeneration in rd1 mice model (Frasson et al., 1999).

Even though, L-VGCCs play roles in visual processes and retinal diseases, the studies in this dissertation deliver different aspects that enhance our knowledge of the regulation of L-VGGCs. The circadian clocks in the retina provide a protective mechanism for maintaining the functions of the vision system in response to various daily illuminations, and the circadian regulation of L-VGCCs keeps the normal physiological functions in the photoreceptors. Disruption of circadian rhythm of L-VGCCs may impacts the health of photoreceptors and subsequently causes

photoreceptor degeneration. The significance of this dissertation is dedicated to the study of vision by understanding the circadian clock mechanism and ion channel activity in the retina for visual processing. Among these molecules studied in this dissertation, AMPK seems to play an important role in regulating circadian rhythm of L-VGCCs, since AMPK not only modulates mTORC1 signaling, but also acts as cellular energy sensor, and many factors, including nutrition, intracellular Ca^{2+}, oxygen and stress affect AMPK activation. Changes in these factors lead to altered-AMPK activities, and subsequently modulate the circadian rhythm of L-VGCCs in the retina. For example, oxidative stress results in AMPK activation (Lee et al., 2009). It is possible that light-induced and diabetic-induced oxidative stress (Wiegand et al., 1983, Du et al., 2013) at night activate AMPK activity in the retina, which subsequently decreases the activity of L-VGCCs. The reduced of L-VGCCs activity leads to decrease glutamate release, which impairs visual signal transmission and vision at night more than during the day. Overall, these cellular signaling pathways contribute to the circadian rhythm of L-VGCCs in cone photoreceptors, which may keep cone photoreceptors function normally.

Some interesting questions remain unanswered in the circadian regulation of L-VGCCs. Other post-transcriptional as well as post-translational modifications may also participate in the circadian regulation of L-VGCCs. For example, the microRNA26a is a post-transcriptional regulator that regulates the circadian rhythm of L-VGCCα1C subunit protein expression in chick photoreceptors (Shi et al., 2009b). In addition, mutation of retinoschisin, a protein encoded by X-linked juvenile retinoschisis gene, causes disruption of synaptic transmission between photoreceptors and bipolar cells and

disorganization of the retinal cell layer, which leads to the early onset of macular degeneration and loss of vision (Gehrig et al., 1999, Reid et al., 1999, Zeng et al., 2004). Our laboratory previously showed that retinoschisin interacts with L-VGCCs and plays important role in the L-VGCCα1 subunit retention on the plasma membrane in a circadian dependent action in the photoreceptors (Shi et al., 2009a), but whether other extracellular proteins contributing to the circadian regulation of L-VGCCs will require future investigations. Even though we showed that these cellular signaling pathways facilitate L-VGCCα1D subunit trafficking from cytosol onto the plasma membrane, little is known about whether the L-VGCCs insertion onto the plasma membrane, channel recycling, or the β subunit of L-VGCC are also under circadian control.

In summary, the research on cellular signaling pathways studied in this dissertation identified new information concerning the cellular mechanisms of circadian rhythm of L-VGCCs in the retina. The activities of calcineurin and mTORC1were predominately higher during the subjective night, while AMPK activity showed opposite actions with the phase significantly greater during the subjective day. We found that the diverse cellular signaling molecules are under circadian control, and they participate in circadian regulation of L-VGCCs in the retina in part through interaction with cAMP-Ras-ERK and cAMP-Ras-PI3K-AKTsignaling pathways (Fig 5.1). In the US, macular degeneration is the major cause of blindness for people of age 65 an above. Macula is a retinal area concentrated with cone photoreceptors that are prone to many environmental insults, such as bright light, hyperglycemia, hypoxia, and oxidative stress. Prevention or rescuing cone photoreceptors from age-related degeneration has been an important effort

in vision research. Investigation of molecular mechanism underlying the circadian regulation of L-VGCCs in cone photoreceptors is the first critical step toward understanding how cone photoreceptor health can be maintained, which will provide information on prevention or rescuing cone photoreceptors for human patients who suffer aged-related macular degeneration.

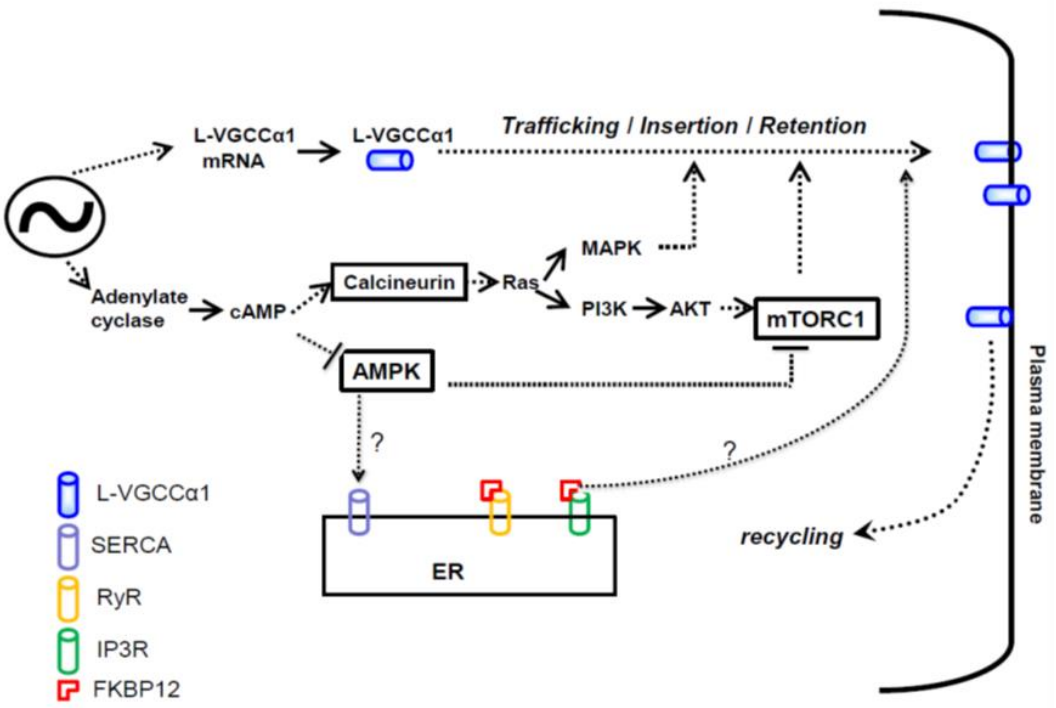

Figure 5.1 A schematic model summarizes the complex cell signaling in the circadian regulation of L-VGCCs in chicken photoreceptor.
The circadian clock in the photoreceptor regulates mRNA levels and protein expression of channel forming L-VGCCα1 subunits. The circadian clock also regulates the activities / phosphorylation states of cell signaling molecules. The complex cell signaling pathways involve the trafficking, translocation, plasma membrane insertion, and/or membrane retention of L-VGCCα1. In the current study, three molecules: calcineurin, mTORC1, as well as AMPK, in part contribute to the regulation of L-VGCCs in a circadian phase-dependent action. The molecule FKBP12 and the intracellular Ca^{2+} store (ER) may also affect the circadian rhythm of L-VGCCs.

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