Mammalian Target of Rapamycin Signaling and the Suprachiasmatic Circadian Clock

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of the Ohio State University

By
Ruifeng Cao, M.D., M.S.
Neuroscience Graduate Studies Program

The Ohio State University
2010

Dissertation Committee:
Karl Obrietan, Ph.D., Advisor
Randy J. Nelson, Ph.D.
John Oberdick, Ph.D.
Chien-liang Glenn Lin, Ph.D.
Copyright by
Ruifeng Cao
2010
ABSTRACT

Circadian (~24 hr) rhythmicity is a prevalent feature of virtually all the living organisms on the earth. This intrinsic property influences every aspect of life activities. In mammals, the master internal clock is localized in the suprachiasmatic nucleus (SCN) of the hypothalamus. Rhythmic clock gene expression constitutes the molecular basis of clock oscillation. The clock can be reset by signaling events that impinge on clock gene expression. Thus there has been significant interest in unraveling the intracellular signal transduction pathways that couple extracellular signals to clock gene expression. Along these lines, our lab has identified a pivotal role of the p42/44 mitogen-activated protein kinase (MAPK) signaling pathway in the clock resetting process. As a continuation of this line of research, my thesis project is focused on the role of two signaling pathways, the mammalian target of rapamycin (mTOR) and the mitogen and stress-activated protein kinase (MSK) pathways, both of which can be activated by MAPK signaling, in the circadian clock timing and entrainment.

In Chapter 1 I gave a brief introduction of the research background, including circadian rhythm, SCN, circadian clock entrainment and the mTOR signaling.

In Chapter 2 I analyzed the light-responsiveness of mTOR signaling cascade, a key regulator of inducible translation, in the SCN. Brief light exposure during the subjective night, but not during the subjective day, triggered rapid phosphorylation (a marker of catalytic activity) of the mTOR translation effectors p70 S6K, ribosomal S6 protein (S6) and 4E-BP1. In the absence of photic stimulation, marked S6 and 4E-BP1 phosphorylation was detected, indicating tonic mTOR activity in the SCN. Light stimulated the colocalized activation of p70 S6K and extracellular signal-regulated protein kinase (ERK), and pharmacological disruption of ERK
signaling abolished light-induced mTOR activity, revealing that the MAPK cascade is an essential intermediate that couples light to mTOR. Together these data identify a light-responsive mTOR cascade in the SCN, and thus, raise the possibility that inducible translation contributes to the clock entrainment process.

In Chapter 3, I used a combination of cellular, molecular, and behavioral assays to address the question whether mTOR-evoked mRNA translation contributes to clock entrainment. To this end, I show that the in vivo infusion of the mTOR inhibitor rapamycin led to a significant attenuation of the phase-delaying effect of early-night light. Conversely, disruption of mTOR during the late night augmented the phase-advancing effect of light. To assess the role of mTOR signaling within the context of molecular entrainment, the effects of rapamycin on light-induced expression of PERIOD1 and PERIOD2 were examined. At both the early- and late-night time points, abrogation of mTOR signaling led to a significant attenuation of light-evoked PERIOD protein expression. These results also reveal that light-induced mTOR activation leads to the translation of mRNAs with a 5'-terminal oligopyrimidine tract such as eukaryotic elongation factor 1A and the immediate early gene JunB. Together, these data indicate that the mTOR pathway functions as potent and selective regulator of light-evoked protein translation and SCN clock entrainment.

In Chapter 4, to begin to assess a role of mTOR signaling in autonomous clock function, I observed the mTOR activity in the SCN under free-running condition. Using phosphorylated S6 ribosomal protein (pS6) as a marker of mTOR activity, I found that mTOR cascade exhibited maximal activity during the subjective day, and minimal expression during the late night. Importantly, expression of S6 was not altered as a function of circadian time. Rhythmic S6 phosphorylation was detected throughout the dorsal-ventral axis of the SCN, thus suggesting that rhythmic mTOR activity was not restricted to a subset of SCN neurons. Rather, rhythmic pS6 expression appeared to parallel the expression pattern of the clock gene period1. Using a transgenic period1 reporter gene mouse strain, I found a statistically significant cellular level correlation between pS6 and period1 gene expression over
the circadian cycle. Further, photic stimulation triggered a similar coordinate upregulation of period1 and mTOR activation in a limited subset of SCN cells. Interestingly, this cellular level correlation between mTOR activity and period1 transcription appears to be specific, since a similar expression profile for pS6 and Period2 or c-fos expression was not detected. Finally, I revealed that rhythmic mTOR activity was downstream of the ERK/MAPK signal transduction pathway. Together these data suggest that the mTOR pathway is under the control of the SCN clock, and indicate that mTOR signaling may contribute to distinct aspects of the molecular clock timing process.

We have previously reported that light stimulates MSK1 activation in the SCN mediated by p42/44 MAPK pathway and that MSK1 stimulates mPeriod1 transcription via a cAMP response element-binding protein (CREB)-dependent mechanism in vitro. In Chapter 5, I utilized Mskl−/− and Mskl−/− mice to further investigate the potential roles of MSK (MSK1 and MSK2) in circadian clock timing and entrainment in vivo. I characterized their circadian phenotypes by monitoring their wheel-running behaviors and core temperature rhythms. The Msk null mice could be entrained to a 12h LD cycle and free-ran with a circadian rhythm, although there was larger variance in their activity onset, compared to wild-type mice. To further evaluate the clock entrainment capacity in these mice, I first employed a standard Aschoff type 1 light-stimulus paradigm at CT15 and CT22. Interestingly, the phase delay induced by short light pulse (100 lx, 15 min) at early night was significantly decreased in MSK null mice. In the “jet-lag” experiments, when the 12 h light period is abruptly advanced or delayed by 8 h, Msk knockout animals exhibited significant deficiency in synchronization with the new light cycle, as indicated by longer entrainment time either in wheel-running behavior or core body temperature recording. To gain mechanistic insights at the molecular level of the clock, I looked at light-induced immediate early gene expression, CREB phosphorylation and mPer1 transcription in these animals. Light-induced c-Fos expression and CREB phosphorylation in the SCN was significantly abrogated in Mskl−/− animals. By
crossing $Msk1^{-/-}$ mice with an $mPer1$-venus reporter line, I found $mPer1$ transcription was markedly decreased in these mice. Together, these data support a key role of MSK in coupling light to the circadian clock entrainment.
ACKNOWLEDGMENTS

At this moment, when I am finishing my dissertation and concluding my work as a graduate student, I would like to express my gratitude to all those who have kindly offered me their help and support throughout my graduate career.

First and foremost, I would like to thank my advisor, Dr. Karl Obrietan. It’s fortunate for me to be his student. I learned from him how to think as a scientist, and present as a scholar. I am influenced by his insights in identifying questions, his wisdom in solving problems and his optimism when facing difficulties. In his flexible lab environment, my passion and motivation to do science are well-conserved and developed. I appreciate all his support and help over these years.

I would also like to thank the members of my Dissertation Committee, Drs. Randy Nelson, John Oberdick and Glenn Lin. I benefit a lot from their advice and I appreciate their help in developing my professional career.

I am also grateful to Drs Chen Gu, Keri Hoyt, Georgia Bishop, Tom Boyd and Tony Brown, all of whom have helped me during my graduate studies.

I also thank my previous and current labmates: Drs. Boyoung Lee, Aiqing Li, Yeon-Joo Jung, Yun-Sik Choi, Mary Cheng, Kate Karelina and Kensuke Sakamoto, Ms. Heeyeon Cho, Heather Dziema, Saklayen Sanjida and Katelin Hansen, Mrs. Jim Stones and Victor Liu. Thank them for their help in the lab. They all make the lab an interesting place to work at.
VITA

03/2006~Present  Graduate Student and Graduate Research Associate  
Neuroscience Graduate Studies Program  
Ohio State University, Columbus, OH

10/2004~08/2005  Visiting Researcher  
Department of Physiology  
Kurume University School of Medicine, Kurume, Japan

07/2002~06/2004  Graduate Student  
Fourth Military Medical University, Xi’an, China

08/1997~06/2002  Medical Student  
Fourth Military Medical University, Xi’an, China

PUBLICATIONS


**FIELDS OF STUDY**

Major Field: Neuroscience

- Molecular and Cellular Neuroscience
- Chronobiology
TABLE OF CONTENTS

Abstract ........................................................................................................................................ ii
Acknowledgments ...................................................................................................................... vi
Vita ................................................................................................................................................ vii
List of Illustrations..................................................................................................................... xi
List of Tables............................................................................................................................... x iii
List of Abbreviations ................................................................................................................ x iv
Chapters
1. General Introduction ................................................................................................................ 1
   1.1 Circadian Rhythm .............................................................................................................. 1
   1.2 Master Circadian Clock in Mammals- the Suprachiasmatic Nuclei................................. 2
   1.3 Entrainment of the Suprachiasmatic Clock by Light......................................................... 3
   1.4 Mammalian Target of Rapamycin Signaling................................................................. 6
   1.5 Rational of Studying Mammalian Target of Rapamycin Signaling in the
       Suprachiasmatic Nuclei Circadian Clock...................................................................... 10
2. Photic Regulation of the Mammalian Target of Rapamycin Signaling Pathway in the
   Suprachiasmatic Circadian Clock.................................................................................... 12
   2.1 Introduction...................................................................................................................... 12
   2.2 Materials and Methods.................................................................................................. 14
   2.3 Results .......................................................................................................................... 18
   2.4 Discussion ..................................................................................................................... 28
3. Mammalian Target of Rapamycin Signaling Modulates Photic Entrainment of the
   Suprachiasmatic Circadian Clock.................................................................................. 36
   3.1 Introduction...................................................................................................................... 36
   3.2 Materials and Methods................................................................................................. 38
3.3 Results ........................................................................................................... 45
3.4 Discussion .................................................................................................... 62

4. Circadian Regulation of the Mammalian Target of Rapamycin Signaling in Mouse
   Suprachiasmatic Nuclei................................................................................. 67
   4.1 Introduction................................................................................................. 67
   4.2 Materials and Methods......................................................................... 69
   4.3 Results ...................................................................................................... 72
   4.4 Discussion ................................................................................................. 82

5. Mitogen and Stress-Activated Protein Kinase Modulates Photic Entrainment of the
   Suprachiasmatic Circadian Clock
   5.1 Introduction................................................................................................. 87
   5.2 Materials and Methods......................................................................... 89
   5.3 Results ...................................................................................................... 93
   5.4 Discussion ................................................................................................. 102

6. Conclusion.................................................................................................... 108

Bibliography..................................................................................................... 111
LIST OF ILLUSTRATIONS

Figure 2.1 Light-induced p70 S6K phosphorylation in the SCN…………………………..20
Figure 2.2 Light-induced p70 S6K activation is mTOR-dependant………………………..22
Figure 2.3. The MAPK pathway couples light to mTOR/p70 S6K……………………..25
Figure 2.4. Phosphorylated 4E-BP1 (Thr-37/46) in the SCN………………………….26
Figure 2.5. Light-induced S6 activation in the SCN………………………………………..29
Figure 2.6. Light-induced p70 S6K phosphorylation and CREB phosphorylation in the SCN…………………………………………………………………………30
Figure 2.7. Schematic of proposed mTOR signaling pathway in the SCN………………35
Figure 3.1. Light-induced mTOR activation in the SCN……………………………..46
Figure 3.2. Glutamatergic and PACAPergic neurotransmission couples light to mTOR activation in the SCN…………………………………………………….50
Figure 3.3. Disruption of mTOR signaling attenuates light-induced phase delaying of circadian locomotor activity……………………………………………….52
Figure 3.4. mTOR functions as a negative regulator of late-night light-evoked phase advancing of the circadian clock……………………………………………53
Figure 3.5. Disruption of mTOR signaling attenuates light-induced phase delaying but enhances light-induced phase advancing of the circadian core body temperature rhythm…………………………………………………………..54
Figure 3.6. mTOR facilitates early-night light-induced PER1 and PER2 expression in the SCN……………………………………………………………………56
Figure 3.7. mTOR facilitates light-induced PER1 expression in the SCN in the presence of actinomycin D…………………………………………………………..57
Figure 3.8. mTOR facilitates late-night light-induced PER1 expression in the SCN..58
Figure 3.9. Light induces mTOR-dependent eEF1A and JunB expression in the...
Figure 3.10. mTOR does not regulate light-induced c-Fos expression in the SCN.

Figure 4.1. Circadian rhythmic expression of phosphorylated S6 ribosomal protein in the SCN.

Figure 4.2. Colocalization of pS6 and vasopressin expressions in the SCN.

Figure 4.3. Colocalization of pS6 and mPer1-Venus expressions in the SCN over the 24 h cycle.

Figure 4.4. Colocalization of light-induced pS6 and mPer1-Venus expressions in the SCN.

Figure 4.5. Circadian p42/44 MAPK couples clock to mTOR activation.

Figure 4.6. Circadian p42/44 MAPK but not PI3K/Akt pathway couples clock to mTOR activation.

Figure 5.1. Genotyping and MSK1 expression in the Msknk1 knockout animals.

Figure 5.2. Msknk1+/− mice show compromised capability to be entrained by light in wheel-running activity.

Figure 5.3. Msknk1−/−/− mice show compromised capability to be entrained by light in core body temperature rhythm.

Figure 5.4. Light-induced c-Fos expression was decreased in Msknk1 knockout animals.

Figure 5.5. Light-induced phospho-CREB expression was decreased in Msknk1−/− animals.

Figure 5.6. Light-induced Per1-Venus expression was decreased in Msknk1−/− animals.

Figure 6.1. Schematic overview of MAPK-regulated processes that are thought to couple light to the SCN clock during the early night.
LIST OF TABLES

Table 1. Phenotypic Characteristics of $Msk^{-/-}$ Mice........................................96
LIST OF ABBREVIATIONS

3V              third ventricle
4E-BP           eukaryotic initiation factor 4E-binding protein
AANAT           arylalkylamine N-acetyltransferase
ACSF            artificial cerebral spinal fluid
AMPA            α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA           analysis of variance
AP-1            activator protein 1
APV             (2R)-amino-5-phosphonovaleric acid
Bmal1           brain and muscle Arnt-like protein 1
CaMK            calcium/calmodulin kinase
CBP             CREB binding protein
CK              casein kinase
CLOCK           circadian locomotor output cycle kaput
CNQX            6-cyano-7-nitroquinoxaline-2,3-dione
CNS             central nervous system
CRE             cyclic AMP response element
CREB            cyclic AMP response element binding protein
Cry             cryptochrome
CT              circadian time
DAB             diaminobenzidine
DD              dark/dark
DM              dissociation media
DMEM            Dulbecco’s modified Eagle medium
DMSO            dimethyl sulfoxide
EDTA            ethylenediaminetetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>eEF1A</td>
<td>eukaryotic elongation factor 1A</td>
</tr>
<tr>
<td>eEF2K</td>
<td>eukaryotic translational elongation factor 2K</td>
</tr>
<tr>
<td>EGR-1</td>
<td>early growth response factor 1</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>eIF4B</td>
<td>eukaryotic translational initiation factor 4B</td>
</tr>
<tr>
<td>eIF4E</td>
<td>eukaryotic initiation factor 4E</td>
</tr>
<tr>
<td>Elk-1</td>
<td>ETS like protein 1</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast-Fourier Transformation</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506-binding protein 12</td>
</tr>
<tr>
<td>FRAP</td>
<td>FKBP-12-rapamycin associated protein</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>hnRNP Q</td>
<td>heterogeneous nuclear ribonucleoprotein Q</td>
</tr>
<tr>
<td>HEAT</td>
<td>Huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase TOR</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early gene</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factors</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>LD</td>
<td>light/dark</td>
</tr>
<tr>
<td>LP / LF</td>
<td>light pulse / light flash</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>lx</td>
<td>lux</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/Erk kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MSK</td>
<td>mitogen- and stress-activated protein kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC</td>
<td>mTOR complex</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization sequence</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>OC</td>
<td>optic chiasm</td>
</tr>
<tr>
<td>OTS</td>
<td>oscillating tissue slicer</td>
</tr>
<tr>
<td>PAC1</td>
<td>PACAP receptor 1</td>
</tr>
<tr>
<td>PACAP</td>
<td>pituitary adenylate-cyclase activated polypeptide</td>
</tr>
<tr>
<td>Per</td>
<td>Period</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PIKK</td>
<td>phosphoinositide(PI)3–kinase-related kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol-4,5-phosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol-3,4,5-phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate-buffered saline + triton X-100</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDK</td>
<td>1,2-phosphoinositide-dependent protein kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>protein kinase G</td>
</tr>
<tr>
<td>pRGC</td>
<td>photoresponsive retina ganglion cell</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RHT</td>
<td>retinohypothalamic tract</td>
</tr>
<tr>
<td>RSK</td>
<td>p90 ribosomal S6 kinase</td>
</tr>
<tr>
<td>S6</td>
<td>ribosomal S6 protein</td>
</tr>
<tr>
<td>S6K</td>
<td>ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nuclei</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SNK test</td>
<td>Student–Newman–Keuls test</td>
</tr>
<tr>
<td>SRE</td>
<td>serum response element</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TOP mRNA</td>
<td>5′tract of oligopyrimidine mRNA</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis protein complex</td>
</tr>
<tr>
<td>U0126</td>
<td>1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenyl mercapto) butadiene</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>ZT</td>
<td>zeitgeber time</td>
</tr>
</tbody>
</table>
Chapter 1. General Introduction

1.1 Circadian rhythm

Circadian rhythm refers to the approximately 24-hour periodicity in the biochemical, physiological or behavioral processes of living organisms. As an evolutionary adaptation to the axial rotation of the earth, this rhythm is a prevalent property of almost all life forms, from single cell cyanobacteria to the most complicated form of life, mammals (Reppert and Weaver, 2002). The rhythm enables the living beings to organize their life activities in a temporally segmented manner, based on the day/night alteration and derivative light, temperature, moisture and air pressure cycle, so that optimal physiological and metabolic efficiency can be reached. Thus, plants and animals can predict and prepare for upcoming environment changes to meet their physiological needs.

Circadian rhythmicity is an intrinsic feature, which doesn’t depend on external cues. Under isolated conditions, the internal circadian clock still oscillates at an accurate speed with minimal daily variance (Reppert and Weaver, 2002). However, under natural conditions the clock is continuously adjusted (entrained) as it is receiving signal inputs (Zeigebers) from the environment (Cermakian and Sassone-Corsi, 2002; Hirota and Fukada, 2004). Within an organism, the rhythm is fundamental enough to override a variety of life activities, from the molecular level to the systemic and behavioral level (Van Someren and Riemersma-Van Der Lek, 2007). Importantly, disruption of the rhythms by extrinsic or intrinsic factors can cause malfunction in physiology and metabolism. For example, trans-time zone travel can cause jet lag. Point mutation of Period(Per)2 gene can lead to Familial Advanced Sleep Phase Syndrome in humans (Sack et al., 2007). Moreover, pathophysiology of a number of common human diseases has been related to...
circadian clock or clock genes, including hypertension, diabetes and cancer (Young and Bray, 2007; Kohsaka and Bass, 2007; Sahar and Sassone-Corsi, 2007).

Interestingly, in multi-cell organisms, every single cell can have an independent circadian rhythm, as identified in isolated cell culture (Welsh et al., 2010). Rhythms of individual cells or organs are orchestrated by the master pacemakers, which are usually a group of rhythm-generating neurons in animals. The central clock generates circadian rhythms and synchronizes peripheral clocks by a variety of output signals, including direct neural innervations and humoral secretion. In mammals, a pair of nuclei in the hypothalamus serves as the master circadian clock-the suprachiasmatic nuclei (SCN) (Reppert and Weaver, 2002).

1.2 Master circadian clock in mammals- the suprachiasmatic nuclei

The SCN are localized in the ventral surface of the brain, above the optic chiasm and adjacent to the third ventricle. They are part of the anterior hypothalamus and participate in the hypothalamic function of homeostatic regulation by generating circadian rhythms. This pair of wing-like structures is composed of only about 20,000 neurons but regulates various aspects of fundamental life activities such as body temperature, sleep/wake cycle and hormone secretion (Lowrey and Takahashi, 2000; Van Someren and Riemersma-Van Der Lek, 2007). Mechanistically the oscillation of SCN circadian clock is driven by several interlocking transcriptional/translational feedback loops. Over the past twenty years the main molecular components on the feedback loops in mice have been discovered by different groups (Reppert and Weaver, 2002; Ko and Takahashi, 2006). A general model of molecular circadian oscillation has been formed, although details are still being refined. Briefly, for the positive feedback loop, increasing REV-ERβ levels repress Bmal1 transcription. As a result, Bmal1 RNA levels fall, whereas mPer and mCry RNA levels rise. Translated PER1 and CRY proteins accumulates in the cytoplasm and translocate into the cell nucleus. By binding to CLOCK–BMAL1, they inhibit E-Box mediated transcription of mPer and mCry, forming a negative
feedback loop. They also inhibit Rev-Erbα transcription resulting in a de-repression (activation) of Bmal1 transcription. Thus, the positive and negative transcriptional feedback loops are co-regulated by CLOCK–BMAL1 heterodimers. Of special interest are the Per genes because of their key roles in the feedback loops, their temporal oscillation profiles and their sensitive photic responsibility. Three mouse Per (mPer) genes, termed mPer1, mPer2, and mPer3 are identified in mice (Sun et al., 1997; Tei et al., 1997; Shearman et al., 1997; Zylka et al., 1998). The high-amplitude rhythm of PER production controls the timing of the feedback (Takumi et al., 1998; Yan and Okamura, 2002; Yan and Silver, 2002). Disruption of the mPer1 and mPer2 genes causes immediate behavioral arrhythmicity (Albrecht et al., 2001). On the other hand, mPER1 and mPER2 expressions are highly light-inducible, which may be the mechanism of photic resetting of the clock (Shigeyoshi et al., 1997; Akiyama et al., 1999; Wakamatsu et al., 2001; Yan and Silver, 2004). mPer3 does not have a critical role in the maintenance of the core clock feedback loops. Instead, mPER3 may function as an output signal.

1.3 Entrainment of the SCN clock by light
1.3.1 Neural pathway

As an adaptation to the ever-changing external environment, the circadian clock can be reset by entrainment cues such as light (Cermakian and Sassone-Corsi, 2002; Challet et al., 2003; Hirota and Fukada, 2004). Importantly, mPER1 and mPER2 are induced by light and their levels correlates closely with light-induced phase resetting and previous work has suggested that their induction by light is a key component of the clock resetting process (Reppert and Weaver, 2002). Photic input is relayed from the retina to the SCN via the retinohypothalamic tract (RHT). The pathway is distinct from the image-forming visual pathway in that the reception of light is mediated by melonopsin-expressing photoresponsive retina ganglion cells (pRGCs). The axons of these cells (RHT) form synaptic connection with SCN neurons (Golombek and Rosenstein, 2010). In response to a light stimulus, the RHT axon terminals release
the excitatory neurotransmitter glutamate and the neurohormone pituitary adenylate cyclase-activating peptide (PACAP) (Hannibal, 2002), which in turn bind to postsynaptic receptors on SCN neurons and evoke a series of intracellular signal transduction events that trigger clock resetting (Golombek and Rosenstein, 2010).

1.3.2 Signal transduction in the SCN

However, the signal transduction events that couple light to clock gene transcription are not completely understood (Meijer and Schwartz, 2003). The second messenger signaling pathways implicated thus far include MAPK (Obrietan et al., 1998; Butcher et al., 2002; Coogan and Piggins, 2003), PKA (Lee et al., 1999; Tischkau et al., 2000; Meyer-Spasche and Piggins, 2004), PKG (Weber et al., 1995; Mathur et al., 1996; Tischkau et al., 2003; Oster et al., 2003) and PKC (Schak and Harrington, 1999; Lee et al., 2007; Jakubcakova et al., 2007).

Previous studies have revealed that the MAPK pathway plays a central role in circadian timing and entrainment. Transient light entrainment cues trigger a concordant increase in ERK activation (Obrietan et al., 1998; Coogan and Piggins, 2003, 2004). Light-induced ERK activation only occurs during the subjective nighttime, corresponding to the time period when light functions as an entrainment cue. Importantly, disruption of light-induced MAPK activation by MEK inhibitor U0126 or SL-327 significantly attenuates light-induced phase delay during the early subjective night (Butcher et al., 2002). MAPK functions through activation of multiple downstream targets. Along these lines, photic stimulation has been shown to trigger activation of the ERK/MAPK effectors p90 ribosomal S6 kinase (RSK) and mitogen- and stress-activated protein kinase (MSK) (Butcher et al., 2004, 2005). The light-activated kinases lead to the phosphorylation of cAMP response element-binding proteins (CREB) at Ser-133. The phospho-CREB interacts with its transcriptional co-activator CREB binding protein (CBP) and induces target gene expression. Importantly, the promoters of many clock-related genes contain cAMP response element (CRE), including mPer1 and mPer2. It has been shown that light
exposure at night lead to CRE-mediated gene transcription in the SCN of CRE-B-galactosidase mice (Obrietan et al., 1999). And CRE activation appears to be necessary for the light entrainment process (Tischkau et al., 2003). In addition to CREB, the transcription factor Elk-1 is a substrate of ERK. Elk binds to SRE, which are also present on some clock gene promoter sequences. Moreover, many photically-inducible immediate early genes (c-fos, egr-1, jun-b) contain CREs and SREs on their promoters (Coogan and Piggins, 2004) and the disruption of light-induced MAPK activity attenuates their induction (Dziema et al., 2003), although their role in light induced phase resetting is not known. Despite all the evidence indicating the role of MAPK in clock gene transcription, it is less known, however, whether MAPK signaling can regulate mRNA translation in the SCN. Recently, it is reported that microRNAs can also be induced by photic entrainment cues via a MAPK/CREB-dependent mechanism (Cheng et al., 2007). Beside the significant role in clock entrainment, ERK MAPK has recently been shown to be indispensable for the robust autonomous oscillation of the clock. In a SCN slice culture system, disruption of MAPK by MEK inhibitor U0126 markedly dampened the rhythms and basal levels of clock gene expressions and the spontaneous firing of single SCN neurons (Akashi et al., 2008).

1.3.3 Light-induced clock gene expression

As aforementioned, the mouse Per genes, mPer1 and mPer2, are photo-inducible in the SCN with phase-dependence similar to that in behavioral rhythmicity (Albrecht et al., 1997; Zylka et al., 1998). The mRNA levels begin to rise 10 to 15 min after light onset during subjective night, peak at about 1 to 2 h and return to baseline after about 3 h. The protein immunoreactivities also rise following the mRNA level increase, but technically difficult to detect until the basal levels decrease to a very low level (e.g. 4 hours after light at CT15) (Yan and Silver, 2004). It has been suggested that light-induced PER protein expression may be the key events in the photic entrainment process. The increased levels of PER1 and PER2 proteins early in the
night could extend the period of transcriptional inhibition by the PER/CRY complex while the increase late in the night could lead to a earlier initiation of the PER/CRY mediated inhibition. This may be why light in the early night causes a phase delay while light in the late night causes a phase advance (Reppert and Weaver, 2002). Using Per1 antisense, Akiyama et al. (1999) inhibited light- and glutamate-induced phase delays of the locomotor rhythm in mice and Wakamatsu et al. (2001) eliminated the delays in vivo by injecting antisense oligos to both mPer1 and mPer2. However, three lines of mice genetically deficient in Per1 (all lack PER1 protein expression) have been reported but not all mice exhibited impaired phase shift by light (Cermakian et al., 2001; Zheng et al., 2001; Bae et al., 2001). Further investigation is still needed to clarify the key molecular events in the photic entrainment process.

Besides Pers, Bmal expression is also modulated by light. BMAL1 protein expression is reduced by light at night. And Bmal1 mutant mice exhibit significant deficits in photic entrainment (Golombek and Rosenstein, 2010).

1.4 mTOR signaling

Mammalian target of rapamycin (mTOR, also known as FRAP) is a conserved serine/threonine protein kinase. Its N-terminus contains several HEAT (Huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase Tor) domains (modules for protein–protein interactions) and its C-terminus includes a kinase domain similar to the phosphatidylinositol-3-kinases (PI3Ks), thus belonging to the family of phosphoinositide(PI)3–kinase-related kinases (PIKKs). Immediately N-terminal to its kinase domain is a region that binds the immunophilin FKBP12 (FK506-binding protein 12) when that protein is bound to rapamycin. It forms two multiprotein complexes involving distinct protein components. One complex, mTORC (mTOR complex) 1 contains raptor (regulatory associated protein of mTOR) while the other one, mTORC2, contains rictor (rapamycin-insensitive companion of mTOR). mTORC1 mediates effects of mTOR that are sensitive to rapamycin, whereas effects mediated through mTORC2 are insensitive to this
compound. mTORC1 is linked to the control of components of the translational machinery while mTORC2 regulates cytoskeleton organization (Hay and Sonenberg, 2004; Tee and Blenis, 2005).

1.4.1 upstream of mTOR

Several upstream signals regulate mTORC1 signaling, including growth factors, energy, stress, and amino acids. Growth factors, including insulin, signal to mTOR through a PI3K/AKT-dependent process. Binding of insulin or insulin-like growth factors (IGFs) to their receptors leads to recruitment and phosphorylation of the insulin receptor substrate (IRS), and subsequent recruitment of PI3K. PI3K bound to IRS converts phosphatidylinositol-4,5-phosphate (PIP2) in the cell membrane to phosphatidylinositol-3,4,5-phosphate (PIP3). PIP3 accumulation is antagonized by the lipid phosphatase PTEN. PIP3 corecruits PDK1 and AKT to the membrane, resulting in the phosphorylation and activation of AKT by PDK1. mTOR is wired to the PI3K pathway through tuberous sclerosis protein complex (TSC)1 and TSC2. TSC1 and TSC2 act as a heterodimer that negatively regulates mTOR signaling. TSC2 is phosphorylated and functionally inactivated by AKT. AKT activation leads to phosphorylation and inactivation of TSC2, which, in turn, blocks its GTPase-activating protein (GAP) activity for the small GTPase Rheb, thus allowing the GTP-loaded form of Rheb to activate mTORC1 in a GTP-dependent manner (Gao et al., 2002; Inoki et al., 2002; Long et al., 2005; Li et al., 2004).

The Ras/MAPK pathway targets TSC2, as evidenced by the observation that expression of an activated allele of ras induces phosphorylation of TSC2 (Roux et al., 2004). Furthermore, loss-of-function mutations in the tumor suppressor neurofibromin, a RasGAP, deregulate TSC2 and ultimately cause constitutive mTORC1 signaling (Johannessen et al., 2005). Ras activates the Raf-MEK1/2-Erk1/2 cascade. Activated Erk1/2 directly phosphorylates TSC2 at sites that differ from the AKT target sites, thereby causing functional inactivation of the TSC1-TSC2 complex (Ma et al., 2005). The ERK-activated kinase RSKs (or
p90<sup>RSk</sup>s) also phosphorylates TSC2 at a C-terminal site, Ser<sup>1798</sup> in the most widely used numbering system. They may provide another link between Ras/Raf/MEK (MAPK/ERK kinase)/ERK signalling and the activation of mTOR. RSK-mediated phosphorylation impairs TSC2's Rheb-GAP and therefore leads to activation of mTOR. This would allow growth factors and mitogens to turn on mTORC1 signalling. In addition, a PKC-dependent TSC2 regulatory pathway has been proposed (Ballif et al., 2005). Thus, the PI3K/AKT and Ras/Erk signaling pathways converge on TSC1-TSC2, resulting in TSC2 inactivation and ultimately mTORC1 activation. This implies that both PI3K/AKT and Ras/Erk can regulate translation via mTORC1 signaling.

mTORC1 can also be regulated by cellular energy status through AMP-activated protein kinase (AMPK). AMPK is activated in response to low cellular energy (high AMP/ATP ratio). Activated AMPK downregulates energetically demanding processes like protein synthesis and stimulates ATP-generating processes such as fatty acid oxidation. Activation of AMPK by AICAR, an AMP analog, inhibits mTORC1 activity. Activated AMPK directly phosphorylates TSC2 and thereby enhances its GAP activity, leading to the inhibition of mTORC1 signaling (Inoki et al., 2002).

1.4.2 mTOR and mRNA translation

Protein synthesis is important for cell physiology and therefore highly regulated through multiple signaling pathways. The regulation is conducted on both a short (minutes) and long term (hours to days) basis. The mechanisms of short-term regulation primarily include rapid changes in the functional states of translational machinery components (translation factors and specific RNA-binding proteins), mainly mediated by phosphorylation or dephosphorylation. For a long-term control, the capacity for protein synthesis is regulated, requiring changed levels of translation factors and ribosomes. The mammalian target of rapamycin (mTOR, also known as FRAP) signaling pathway is a key regulator of mRNA translation in cell growth and metabolism (Sarbassov et al., 2005; Wullschleger et al., 2006; Proud, 2007).
mTORC1 exhibits protein kinase activity and its activation leads to phosphorylation of downstream translation effectors. Raptor interacts with the TOS (target of rapamycin signalling) motifs in targets of mTOR in a rapamycin-sensitive manner. The mTORC1 pathway regulates ribosome biogenesis and mRNA translation through two main effectors: S6 kinase1 (S6K1, including two isoforms, p70 S6K and p85 S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Both S6K1 and 4E-BP1 contain a TOS motif which binds the mTORC1 component raptor. Each of them is subject to phosphorylation at multiple sites. S6K phosphorylation at Thr-389 is associated with mTOR-dependent activation of p70 S6K, and thus can be used to monitor the relative level of pathway activity (Pearson et al., 1995; Jefferies et al., 1997; Weng Q, 1998; Burnett et al., 1998). Activated (phosphorylated) S6K regulates the activity of a number of proteins involved in regulation of mRNA translation and processing, such as ribosomal protein S6 (Ser240/244), eIF4B(Ser322), eEF2K(Ser366) and Pdcd4(Ser67)(Proud C, 2007) by phosphorylation. In addition to the regulation of the translational efficiency, S6K1 can also stimulate translation capacity by actuating the synthesis of new ribosomes. Besides S6K1, 4E-BP1 is another direct downstream effector of mTOR that has been shown to regulate mRNA translation. The phosphorylation of 4E-BP1 at Thr37/46 is required for priming the phosphorylation on other phosphorylation sites(Ser65 and Thr70) that directly regulates the binding of 4E-BP1 to eIF4E. In the absence of stimulation, 4E-BP1 binds to eIF4E, thereby blocking its ability to stimulate cap-dependent mRNA translation initiation (Gingras et al., 1999; Haghighat et al., 1995; Lin et al., 1995). mTORC1 phosphorylates 4E-BP1 and inhibit its association with eIF4E, thereby allowing cap-dependent mRNA translation to occur (Lin et al., 1994; Pause et al., 1994; Gingras et al., 1999; 2001).

1.4.3 mTOR activity in the central nervous system

In the developing central nervous system (CNS), mTOR regulates neuronal
survival and differentiation, as well as axon growth and navigation, dendritic arborization, and synaptogenesis. In the adult CNS, mTOR is crucial for many forms of synaptic plasticity, such as long-term potentiation in hippocampus, and thereby plays an important role in the process of learning and memory. In the hypothalamus, mTOR functions as an energy sensor to control animal food intake and regulate body energy balance. Recently, mTOR signaling has been found robustly expressed in the SCN circadian clock and regulated by photic signal and the clock (Cao et al., 2008, 2010). Interestingly, recent work has shown that neuronal activity triggers rapid mRNA translation via an mTOR-dependent mechanism (Gong et al., 2006; Gelinas et al., 2007; Tsokas et al., 2007; Di Nardo et al., 2007) and that disruption of mTORC1 inhibits late phase long-term potentiation (LTP) in the hippocampus (Tang et al., 2002; Cammalleri et al., 2003). Furthermore, in the hypothalamus, mTOR activity has been shown to play a central role in food intake and energy balance (Cota et al., 2006). Together, these data raise the prospect that TOR signaling plays a key role in shaping activity-dependent neuronal physiology.

1.5 Rationale of studying mTOR signaling in the SCN circadian clock

The mechanisms of photic clock entrainment are not well understood. Current models theorize that clock resetting is closely related to light-induced clock protein production because application of translation inhibitors suppresses the capacity of light to entrain the clock (Johnson and Nakashima, 1990; Raju et al., 1990; Murakami et al., 1995; Zhang et al., 1996). mPer1 and mPer2 genes and their protein products are highly responsive to light during the night. Light induces significant increase of mPer1 and mPer2 transcription followed by elevated mPER1 and mPER2 protein expression in the SCN (Albrecht et al., 1997, 2001; Zylka et al., 1998; Yan and Silver, 2004). mRNA translation is an energy-consuming process. It is not likely that cells maintain the maximum translation efficiency all the time. Rather, coordinate control of signaling pathways which regulate translation capacity is required for meeting the variable physiological needs. Cells can upregulate their rates of protein synthesis
either via increasing the translation efficiency of existing ribosomes and/or by increasing the capacity for translation through the production of new ribosomes (ribosome biogenesis). Along these lines, when light induces massive production of \textit{mPer1} and \textit{mPer2} transcripts in the SCN neurons, there may be a coordinate mechanism that upregulates the translation capacity in the SCN neurons, so that the new transcripts can be efficiently translated. mTOR signaling is known to be key regulators of mRNA translation during cell metabolism and growth (Sarbassov et al., 2005; Wullschleger et al., 2006; Proud, 2007). Preliminary data show that mTOR signaling maintains a relative high basal activity in the SCN. All this information makes it intriguing to examine the presence, activity and function of the mTOR signaling in SCN circadian clock.

For this purpose, this dissertation includes the following sections: in Chapter 2, I studied the presence and light-regulated mTOR activity in the SCN. In Chapter 3, I studied the function of mTOR signaling during photic entrainment of the SCN clock and its mechanisms. In Chapter 4, I looked at the circadian expression pattern of mTOR signaling and studied its relevance with clock gene expressions. In Chapter 5, I switched the gear from mTOR signaling and studied the role of MSK, a downstream kinase of MAPK, in regulating clock entrainment by using MSK knockout animals.
Chapter 2. Photic Regulation of the mTOR Signaling Pathway in the Suprachiasmatic Circadian Clock

2.1 Introduction

The inherent pacemaker activity of the mammalian circadian clock located in the suprachiasmatic nuclei (SCN) drives a vast array of biochemical, physiological and behavioral processes with 24 h periodicity ([Reppert and Weaver, 2002] and [Lowrey and Takahashi, 2000]). As an adaptation to the ever-changing external environment, the circadian clock can be reset by multiple entrainment cues such as light ([Cermakian and Sassone-Corsi, 2002], [Challet et al., 2003] and [Hirota and Fukada, 2004]). Photic input is relayed from the retina to the SCN via the retinohypothalamic tract (RHT). In response to a light stimulus, RHT axon terminals release the excitatory neurotransmitter glutamate and the neurohormone pituitary adenylate cyclase-activating peptide (PACAP) (Hannibal, 2002), which in turn bind to postsynaptic receptors on SCN neurons and evoke a series of intracellular signal transduction events that trigger clock resetting.

Transcriptional activation appears to be a key event in the entrainment process. Disruption of photically induced clock gene expression has been shown to abrogate clock entrainment ([Akiyama et al., 1999] and [Albrecht et al., 2001]). In addition, mRNA translation is also a critical event for light-entrainment of the clock. Along these lines, work performed in a wide range of clock model systems has shown that the application of translation inhibitors suppresses light-entrainment ([Johnson and Nakashima, 1990], [Raju et al., 1990], [Murakami et al., 1995] and [Zhang et al., 1996]). Several timing- and entrainment-relevant mRNA translation mechanisms have been reported. For example, in pinealocytes, heterogeneous nuclear ribonucleoprotein Q (hnRNP Q) mediates rhythmic translational regulation of arylalkylamine
N-acetyltransferase (AANAT: Kim et al., 2007). In addition, nocturnin, which regulates mRNA stability through the deadenylation of the mRNA polyA tail, is rhythmically expressed in retinal photoreceptor cells and can be upregulated in response to mitogenic signals ([Baggs and Green, 2003] and [Garbarino-Pico et al., 2007]). Furthermore, our lab has recently shown that both light and the circadian clock regulate microRNA expression in the SCN (Cheng et al., 2007). microRNAs are potent negative regulators of mRNA translation, and as such may play a pivotal role in sculpting both the light- and clock-regulated gene expression profile.

Given these findings, additional efforts to identify translation regulatory processes may be a key to our understanding of the entrainment process. Within this context, one potential route by which mRNA translation may be regulated is the mammalian target of rapamycin (mTOR) signaling pathway ([Sarbassov et al., 2005], [Wullschleger et al., 2006] and [Proud, 2007]). mTOR is a Ser/Thr kinase which is at the core of two distinct multiprotein complexes, the rapamycin-sensitive mTOR complex 1 (mTORC1) and rapamycin-insensitive mTORC2. In response to increased nutrient levels and mitogens mTORC1 activates the downstream targets p70 S6 kinase (p70 S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which in turn lead to actuation of mRNA translation machinery ([Hay and Sonenberg, 2004] and [Tee and Blenis, 2005]). Interestingly, recent work has shown that neuronal activity triggers rapid mRNA translation via an mTOR-dependent mechanism ([Gong et al., 2006], [Gelinas et al., 2007], [Tsokas et al., 2007] and [Di Nardo et al., 2007]) and that disruption of mTORC1 inhibits late phase long-term potentiation (LTP) in the hippocampus ([Tang et al., 2002] and [Cammalleri et al., 2003]). Furthermore, in the hypothalamus, mTOR activity has been shown to play a central role in food intake and energy balance (Cota et al., 2006). Together, these data raise the prospect that TOR signaling plays a key role in shaping activity-dependent neuronal physiology.

Here we examined the regulation of mTOR-dependent signaling in the SCN clock. The data reveal that photic stimulation triggers a phase-dependent increase in
the activation state of p70 S6K and 4E-BP1. Moreover, the data reveal that mTOR is 
a downstream target of the p42/44 mitogen-activated protein kinase (MAPK) 
signaling pathway, and that light triggers coordinate cyclic AMP-response element 
binding protein (CREB) and mTOR activation in SCN neurons. Together these data 
identify mTOR as a light-activated signaling pathway.

2.2 Materials and methods
2.2.1 Photic stimulation and tissue processing

Initially, adult (8–10-week-old) C57BL/6 mice were entrained to a 12 h LD 
cycle for at least 2 weeks and then transferred to total darkness for two consecutive 
24 h cycles. After dark adaptation, animals received a single light exposure (400 lx, 
15 min) at one of three time points: the middle of the subjective day (CT 6), early 
subjective night (CT 15), or late subjective night (CT 22). CTs were calculated based 
on Zeitgeber time (ZT) and the tau of C57BL/6 mice (approximately 23 h 45 min) 
under free running conditions, with ZT 0 denoting light on and ZT 12 denoting light 
off. Immediately after light treatment, animals were anesthetized via an 
intraperitoneal (ip) injection of ketamine (95.2 mg/kg) and xylazine (30.8 mg/kg) 
under dim red light (Kodak series 2 filter < 10 lx at cage level; Eastman Kodak, 
Rochester, NY) and both eyes were covered with opaque black tape. The mice were 
then transcardially perfused with cold saline followed by perfusion with 4% 
paraformaldehyde (w/v in 10 mM phosphate-buffered saline, PBS, pH 7.4). From the 
end of light exposure, it took 5–8 min to begin paraformaldehyde perfusion.

Next, brains were harvested, cut into 1.5 mm coronal slices with an oscillating tissue 
slicer (OTS 2000; Electron Microscopy Sciences, Fort Washington, PA), post-fixed in 
4% paraformaldehyde for 4–6 h at room temperature and then transferred into 30% 
sucrose (w/v, with 2 mM sodium azide and 3 mM NaF) overnight at 4 °C. The “no 
light” control animal groups underwent the same handling conditions at the same time 
points. All procedures were in accordance with Ohio State University animal welfare 
guidelines and approved by the Institutional Animal Care and Use Committee.
2.2.2 Cannulation and infusion paradigms

Mice were anesthetized via an ip injection of ketamine (95.2 mg/kg) and xylazine (30.8 mg/kg) and placed in a stereotaxic apparatus (Cartesian Research). The coordinates (posterior, 0.34 mm from bregma; lateral, 0.90 mm from the midline; and dorsoventral, −2.15 mm from bregma) were used to place the tip of a 24-gauge guide cannula into the lateral ventricle. Cannulae were held in place with dental cement and a 30-gauge stylus was secured in the cannula to ensure patency. After surgery animals were housed individually and allowed to recover for at least 2 weeks under a standard 12 h LD cycle. For the infusion, animals were restrained by hand under dim red light, and the infusate was delivered at a rate of 1 µl/min. To disrupt the MAPK cascade, 2 µl of 1,4-diamino-2,3-dicyano-1,4-bis o-aminophenylmercapto butadiene (U0126, 10 mM; Calbiochem, La Jolla, CA) was infused 30 min before photic stimulation. To inhibit the activity of mTOR, 2 µl of rapamycin (100 µM, Cell Signaling Technology: [Kunz et al., 1993] and [Brown et al., 1994]) was infused 30 min before light treatment. Control animals were infused with an equivalent volume of vehicle (DMSO).

2.2.3 Immunohistochemistry

Coronal brain sections (1.5 mm) containing the SCN were thin cut (40 µm) using a freezing microtome and placed in PBS containing 2 mM sodium azide and 3 mM NaF, pH 7.4. For the immunohistochemical staining, sections were first treated with 0.3% H₂O₂ and 20% methanol in PBS for 10 min to deactivate endogenous peroxidases and permeabilize the tissue and then blocked for 1 h in 10% goat serum/PBS and incubated (overnight, 4 °C) in mouse monoclonal anti-phospho-p70 S6 kinase (Thr-389) antibody (1:1000 final dilution; Cell Signaling Technology, Beverly, MA) or rabbit monoclonal anti-phospho-4E-BP1 (Thr-37/46)(236B4) antibody (1:1000 final dilution; Cell Signaling Technology). Next, tissue was incubated for 1.5 h at room temperature in biotinylated
anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) and then placed in an avidin/biotin HRP complex for 1 h (prepared according to instructions of the manufacturer; Vector Laboratories). Sections were washed in PBS (three times, 10 min/wash) between each labeling step. The signal was visualized using nickel-intensified DAB substrate (Vector Laboratories) and sections were mounted on gelatin-coated slides with Permount media (Fisher Scientific, Houston, TX).

For immunofluorescent labeling, tissue was permeabilized with PBST (PBS with 1% Triton X-100) for 30 min, blocked as described above and then incubated (overnight, 4 °C) in 5% goat serum/PBS with one or more of the following antibodies: mouse monoclonal anti-phospho-p70 S6 kinase (Thr-389) (1:300; Cell Signaling Technology), rabbit monoclonal anti-mTOR (1:300; Cell Signaling Technology), rabbit polyclonal anti-phosphorylated ERK (Thr-202, Tyr-204) (1:300; Cell Signaling Technology), rabbit polyclonal anti-phospho-S6 ribosomal protein (Ser-240/244) (1:300; Cell Signaling Technology) or rabbit polyclonal phospho-CREB (Ser-133) (1:300; Cell Signaling Technology). The following day, sections were incubated (3 h, room temperature) in Alexa Fluor-594-conjugated goat anti-rabbit IgG antibody (1:500; Molecular Probes, Eugene, OR) and/or Alexa Fluor-488-conjugated goat anti-mouse IgG antibody (1:500; Molecular Probes). Brain sections were washed in PBS (three times, 10 min/wash) between each labeling step. Sections were mounted on slides with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

Bright-field or dark-field fluorescent photomicrographs were captured using a 16 b digital camera (Micromax YHS 1300; Princeton Instruments, Trenton, NJ) mounted on an inverted Leica microscope (DM IRB; Nussloch, Germany). Images were acquired with Metamoph software (Molecular Devices, Sunnyvale CA). Confocal fluorescent images were captured using a Zeiss 510 Meta confocal microscope (Oberkochen, Germany). All confocal parameters (pinhole, contrast, brightness, etc.) were held constant for all data sets from the same experiment.
2.2.4 Western blotting

Light-treated mice were transcardially perfused (as described above) with cold saline for 1–2 min and their brains were removed rapidly. Brains were then immersed in chilled, oxygenated artificial cerebrospinal fluid (ACSF, in mM: NaCl 117, KCl 4.7, NaH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 2.5, NaHCO₃ 25, and d-glucose 11: 299 ± 4 mOsm), blocked and cut into 500 µm coronal sections with the oscillating tissue slicer. The tissue sections were then frozen on dry ice, placed on a dissecting microscope and SCN were isolated using a razor blade. Tissue was lysed in 100 µl RIPA buffer [50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 mM sodium vanadate, 1 mM NaF and 1 × protease inhibitor cocktail (Roche)]. Extracts (24 µl/lane) were electrophoresed into an 8% SDS-PAGE gel, then transblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Membranes were blocked in 10% bovine serum albumin (BSA, Fisher scientific, Fair Lawn, NJ) and then incubated (overnight, 4 °C) in PBST (with 5% BSA) with mouse monoclonal anti-phospho-p70 S6 kinase (Thr-389, Cell Signaling Technology) antibody (1:1000). Next, membranes were incubated in PBST (with 5% milk) with a goat anti-mouse IgG alkaline phosphatase-conjugated antibody (1:4000, PerkinElmer Life Sciences). Immunoreactivity was developed using the Western-star alkaline phosphatase detection system (Tropix). As a protein loading control, membranes were probed for total ERK expression using a goat polyclonal anti-ERK antibody (1:1000 final dilution, Santa Cruz Biotechnology) followed by a donkey anti-goat IgG antibody conjugated to horseradish peroxidase. The signal was visualized using Renaissance chemiluminescent horseradish peroxidase substrate (PerkinElmer Life Sciences). Between each antibody treatment, membranes were washed a minimum of three times (10 min/wash) in PBST. SCN tissue was pooled from three mice for each condition and the experiment was repeated three times.
2.2.5 Materials

Unless otherwise indicated, all reagents were obtained from Sigma.

2.2.6 Data analysis

All photomicrographic data sets were statistically analyzed using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA). For cell counting, an intensity threshold filter was initially applied to eliminate nonspecific background labeling, and then the number of detectable signals above threshold (now defined as positive cells) were counted for each SCN. A mean value for each animal was generated from 3 central SCN sections per animal; this value was then used to generate the group mean. For the pS6 intensity analysis, SCN were digitally outlined and the mean pixel values determined. Next, a digital oval (150 × 200 pixels) was placed on the adjacent lateral hypothalamus and this mean value was subtracted from the adjacent SCN signal to provide a normalized SCN intensity value. For the p4E-BP1 intensity analysis, a digital circle (Φ1000 pixels) was placed in the ventral SCN and the mean labeling intensity was determined. A digital oval (150 × 200 pixels) was then placed over the adjacent lateral hypothalamus and the ratio of the SCN signal to mean lateral hypothalamic signal was generated and used to normalize SCN intensity values. The lateral hypothalamic 4E-BP1 values were not altered by light or rapamycin infusion. Mean data from different animals were pooled into treatment groups and compared by one-way ANOVA followed by SNK post-tests. \( p < 0.05 \) was accepted as statistically significant. The values are presented as the mean ± standard error of mean (SEM). All statistical analysis were performed using SPSS software (SPSS Inc, Chicago, IL).

2.3 Results

2.3.1 Light induces p70 S6K phosphorylation in the SCN in a phase-restricted manner
To begin to characterize the mTOR/p70 S6K pathway in the SCN, mice were entrained to a 12 h light/dark (LD) cycle, dark-adapted for 48 h, and then exposed to light (400 lx; 15 min) during either the subjective day or the subjective night. Animals were killed immediately after cessation of the light stimulus and the SCN-containing tissue was processed for the Thr-389 phosphorylated form of p70 S6K (p-p70 S6K). Phosphorylation at Thr-389 is associated with mTOR-dependent activation of p70 S6K, and thus can be used to monitor the relative level of pathway activity ([Pearson et al., 1995], [Jefferies et al., 1997], [Weng et al., 1998] and [Burnett et al., 1998]). Photic stimulation during either the early [(circadian time (CT) 15)] or late (CT 22) subjective night triggered robust p70 S6K phosphorylation in the SCN (Figures 2.1A and B). p-p70 S6K expression was highest in the central SCN, with limited expression in the rostral and caudal regions of the SCN (Figure 1D). In contrast to the nighttime, p70 S6K activity was not stimulated by light exposure during the middle of the subjective daytime (CT 6: Figures 2.1A and B). The phase-restricted induction of p70 S6K activity parallels the phase-restricted light-dependent activation pattern of a number of kinases and immediate early genes as well as the phase-restricted capacity of light to entrain the clock (Meijer and Schwartz, 2003). In the absence of photic input, significant expression of the activated form of p70 S6K was not detected in the SCN at any of the three circadian time points examined (Figures 2.1A and B).

To complement the immunohistochemical analysis of p70 S6K activity, SCN tissue from control and light-treated (400 lx, 15 min, CT 15) animals was probed for p-p70 S6K expression using Western analysis. Photic stimulation triggered a modest increase in the antigenicity of an approximate 70-kDa band (Figure 2.1C). This small increase in band intensity, relative to a robust increase detected using immunohistochemical labeling, is the likely result of the different experimental methods. Hence, Western blotting combines responsive and none-responsive cells, thus generating an averaged response, whereas immunolabeling allows for identification of limited numbers of highly antigenic cells. Importantly, the size of the
main band corresponds with the size of p70 S6K, thus supporting the results obtained using immunohistochemical detection procedures. Of note, the antibody also detected

![Image of figure 2.1](image.png)

**Figure 2.1 Light-induced p70 S6K phosphorylation in the SCN.**

Animals were initially dark-adapted for 2 days and then exposed to light (400 lx, 15 min) during the mid-subjective day [circadian time (CT) 6], early night (CT 15), or late night (CT 22). A, Representative immunohistochemical images of p-p70 S6K in the SCN. Relative to control animals (No Light, top row), light exposure (middle and bottom rows) triggered a marked increase in p-p70 S6K expression at the two night time points. The highest level of antigenicity was observed in the ventral region of the SCN. Photic stimulation during the subjective day did not increase p70 S6K phosphorylation, indicating that the capacity of light to couple to p70 S6K is
phase-restricted. Boxed regions are magnified below. Scale bars: 100 µm; 3V, third ventricle; OC, optic chiasm. B, Quantification of p-p70 S6K-positive cells per SCN. The number of animals used for each condition is shown above each (Continued) (Figure 2.1 Continued) histogram. Error bars denote the SEM. \( **p < 0.001 \). C, Western blotting analysis of p-p70 S6K expression in the SCN. Mice received a single light pulse (15 min, 400 lx) at CT 15, and SCN tissue was dissected, pooled (n = 3 for each condition) and probed for p-p70 S6K expression. Compared with the “no light” control, a modest increase in the density of the 70 kDa band was detected in the light-treated condition. As a protein loading control, the blot was also probed for total ERK (erk 1 and erk 2) expression. D, Distribution of light-induced p-p70 S6K expression within the rostrocaudal axis of the SCN. A 15-minute light treatment (400 lx) at CT 15 triggered limited p-p70 S6K expression in the rostral and caudal regions of the SCN; the highest level of expression was observed within the central SCN. Scale bars: 100 µm.

a weak band at 85 kDa. This band is likely to be p85 S6 Kinase (p85 S6K), an isoform of p70 S6K. According to the manufacturer, this antibody detects p85 S6K when phosphorylated at Thr-412, a site analogous to Thr-389 in p70 S6K. For the sake of clarity, we will only refer to p70 S6K when describing results collected with this antibody. As a protein loading control, the blot was stripped and probed for total ERK expression. Together, these data support the observation that p70 S6K is tightly regulated by photic input to the SCN.

2.3.2 Light-induced p70 S6K phosphorylation is mTOR-dependent

To determine whether light-induced p70 S6K phosphorylation in the SCN is mediated by mTOR signaling, we initially examined the expression of mTOR and activated p70 S6K. Immunofluorescent labeling revealed marked cytoplasm expression of mTOR in the SCN (Figure 2.2A). As expected, relative to control animals (Figure 2.2A top), a 15-minute light treatment (400 lx) at CT15 elicited p70 S6K activation (Figure2.2A bottom). Merging the mTOR and p-p70 S6K signals revealed cellular but not spatial colocalization of the two kinases (Figure 2.2A) in the SCN. Along these lines, p-p70 S6K appeared to be largely nuclear, whereas mTOR expression was largely cytoplasmic.
Figure 2.2 Light-induced p70 S6K activation is mTOR-dependant.
A, Representative confocal images showing colocalization of phosphorylated p70 S6K (p-p70 S6K, green) and mTOR (red) in the SCN. Compared with “no light” control animals (top), a 15-minute light treatment (400 lx) at CT 15 elicited robust p70 S6K activation in the SCN. The merged immunolabeling images (right column) reveal a largely nuclear expression pattern for p-p70 S6K and a cytoplasmic expression pattern for mTOR. OC, optic chiasm. Boxed regions are magnified below each image. Scale bars: 20 µm. B, Quantification of light-induced p-p70 S6K expression and its colocalization with mTOR in the SCN. Cellular coexpression of p-p70 S6K with mTOR was detected in 95% p-p70 S6K positive cells (315 out of 330 cells counted from five mice). Quantitation was performed on 5 control (no light) mice. Error bars denote SEM. C, Representative fluorescent micrographs showing that infusion of the mTOR inhibitor rapamycin blocks light-induced p-p70 S6K expression. Two µl of rapamycin (100 µM) or dimethylsulfoxide (DMSO, vehicle) was infused into the lateral ventricle 30 min before a 15-minute light treatment at CT 15. The boxed regions are shown below. Scale bar, low magnification: 100 µm, high magnification: 75 µm; OC, optic chiasm; 3V, third ventricle. Statistical analysis is presented in Figure 2.5C.

To test whether mTOR activity stimulated light-induced p70 S6K phosphorylation, mice were infused in the lateral ventricle with the mTORC1
inhibitor rapamycin (100 µM, 2 µl) 30 min prior to light exposure (400 lx, 15 min) at CT 15. Relative to the robust light-induced p70 S6K phosphorylation observed in vehicle- (Dimethyl sulfoxide, DMSO) infused mice, infusion of rapamycin totally blocked light-activated p-p70 S6K expression (Figure 2.2 and Figure 2.5). Together these data reveal that photic stimulation drives activation of the canonical mTOR/70 S6K signaling cassette in the SCN.

2.3.3 The MAPK pathway couples light to mTOR/p70 S6K activation

Recent reports indicate that the p42/44 MAPK pathway can function as an upstream regulator of the mTOR/p70 S6K pathway ([Ma et al., 2005], [Roux et al., 2004], [Tsokas et al., 2007] and [Gelinas et al., 2007]). These findings and the prominent role that MAPK signaling plays in light-entrainment of the circadian clock ([Butcher et al., 2002] and [Coogan and Piggins, 2003]), led us to examine a possible connection between MAPK signaling and the mTOR/p70 S6K pathway in the SCN. To begin to address this question, we tested whether light stimulates coordinate p70 S6K and MAPK activation. SCN tissue from control (no light) and light-treated (400 lx, 15 min, CT 15) mice was processed using immunofluorescent labeling for the phosphorylated form of p70 S6K and the dual phosphorylated (Thr-202/Tyr-204) forms of extracellular signal-regulated kinase 1 (erk 1) and erk 2 (collectively referred to as ERK). This dual phosphorylation event is required for ERK enzymatic activity and thus can be used to monitor MAPK pathway activation. Under control conditions, low levels of activated ERK and p-p70 S6K were observed (Figure 2.3A). However, photic stimulation led to an increase in the phosphorylated forms of both kinases and merging the two images revealed colocalized expression of activated ERK and p70 S6K in a subset of SCN cells (Figures 2.3A and B). Together these results identify a light-induced spatial and temporal colocalization of activated ERK and p70 S6K in the SCN.

To test the causal connection between light-induced MAPK activation and p70 S6K phosphorylation, we employed the ventricular infusion technique to
disrupt MAPK signaling in the SCN. To this end, mice were infused with the
specific MEK 1/2 inhibitor U0126 (10 mM, 2 µl) 30 min before photic stimulation
(400 lx, 15 min) at CT 15 and tissue was immunolabeled for ERK and p70 S6K
activation. Consistent with our prior work ([Butcher et al., 2002] and [Dziema et al.,
2003]), infusion of U0126 completely blocked light-induced ERK activation (Figure
2.3C). Importantly, disruption of MAPK activation effectively suppressed
light-induced p70 S6K activation (Figure 2.3C). Representative data for activated
ERK (pERK) and p70 S6K are from the same mouse and quantitative data are
presented in Figure 2.5C. In conclusion, these results reveal the presence of a
light-responsive MAPK–mTOR/p70 S6K signaling cassette in the SCN.

2.3.4 Light-induced 4E-BP1 phosphorylation

In addition to p70 S6K, 4E-BP1 is another direct downstream effector of mTOR
that has been shown to regulate mRNA translation ([Sarbassov et al.,
2005], [Wullschleger et al., 2006] and [Proud, 2007]). In the absence of
stimulation, 4E-BP1 binds to eIF4E, thereby blocking its ability to stimulate
cap-dependent mRNA translation initiation ([Gingras et al., 1999], [Haghighat et al.,
1995] and [Lin et al., 1995]). A number of studies have shown that this association
can be disrupted by mTOR-dependent phosphorylation of 4E-BP1 ([Lin et al., 1994]
and [Pause et al., 1994]). To gain insight into the functional status of 4E-BP1, SCN
tissue was probed with an antibody against 4E-BP1 phosphorylated at Thr-37 and
Thr-46, two sites that regulate 4E-BP1 binding to eIF4E ([Burnett et al.,
1998], [Gingras et al., 1999] and [Mothe-Satney et al., 2000]). Interestingly, under
control conditions (CT 15-no light) marked 4E-BP1 phosphorylation was detected
throughout the SCN (Figure 2.4A), whereas little immunoreactivity was detected
outside of the SCN. To test whether 4E-BP1 phosphorylation was dependent
on mTOR, mice were sacrificed 30 min after rapamycin (100 µM, 2 µl) infusion.
Disruption of mTOR signaling led to a dramatic decrease in 4E-BP1 phosphorylation,
indicating that tonic mTOR activity drives a relatively high basal level of 4E-BP1 phosphorylation in the SCN. To test whether

![Figure 2.3](image)

**Figure 2.3. The MAPK pathway couples light to mTOR/p70 S6K.**

A, Compared with “no light” control animals, a 15-minute light treatment (400 lx) at CT15 elicited robust p-p70 (S6K: green) and phospho-ERK (pERK: red) expression. Merged confocal images revealed that light-induced p70 S6K and ERK activation occurred in the same subset of cells (arrows). Boxed regions are magnified below each image. Scale bars: 20 µm. B, Quantification of colocalized, light-induced, p-p70 S6K expression and ERK activation in the SCN. Of note, after the light flash
81% p-p70 S6K positive cells were also pERK-positive (338 out of 416 cells counted from six mice); quantitation was performed on 7 control (no light) mice. Error bars denote SEM. C, Representative fluorescent micrographs showing that light-(Continued) (Figure 2.3 Continued) induced p-p70 S6K is dependent on the MAPK pathway. Mice received a ventricular infusion of U0126 (10 mM, 2 µl) or DMSO (2 µl) vehicle 30 min before a 15-minute light treatment at CT 15. SCN sections were double-labeled for pERK (red) and p-p70S6K (green) expression. The boxed regions are magnified and shown below. Scale bars, high magnification: 75 µm, low magnification: 100 µm; OC, optic chiasm; 3V, third ventricle. Statistical analysis is presented in Figure 2.5C.

4E-BP1 is stimulated by photic input, mice were exposed to light (400 lx, 15 min) at CT 15, and SCN tissue was processed for 4E-BP1 phosphorylation. Representative data and quantitative analysis revealed that photic stimulation led to a modest, but significant, increase in 4E-BP1 phosphorylation (Figures 2.4A and B).
Figure 2.4. Phosphorylated 4E-BP1 (Thr-37/46) in the SCN.

A, Representative immunohistochemical images of phosphorylated 4E-BP1 (p4E-BP1) expression in the SCN. The treatment conditions are shown above the (Continued) (Figure 2.4 Continued) images. Under control conditions (no light) marked p4E-BP1 expression was detected in the SCN at CT15. Pretreatment with rapamycin (100 µM, 2 µl) 30 min prior to sacrificing triggered a significant reduction in p4E-BP1 expression. A 15-minute light treatment (400 lx) at CT 15 stimulated a moderate increase of p4E-BP1 expression in the SCN. Rapamycin (100 µM, 2 µl) infusion (30 min before light) blocked light-induced p4E-BP1 expression. Mice not infused with rapamycin were infused with DMSO (2 µl). Scale bars: low magnification 250 µm, high magnification 150 µm. B, Quantitative analysis of 4E-BP1 phosphorylation under the four experimental conditions. The treatment conditions are shown below the histograms. Values are normalized against p4E-BP1 levels in the lateral hypothalamus. Please see the Experimental methods section for a description of the quantitation procedures. Error bars denote SEM. The numbers above the bars indicate the number of animals tested for each condition. ***p < 0.001; **p < 0.01.

2.3.5 p-p70 S6K phosphorylates ribosomal S6 protein in the SCN

Next, we turned to potential downstream targets of p70 S6K in the SCN. To this end, we examined the regulation of the ribosomal S6 protein (S6). S6 is a downstream target of p70 S6K and has been suggested to play a role in the regulation of TOP-dependent translation (Meyuhas, 2000). To monitor the activation state of S6, tissue was immunolabeled for phosphorylation of S6 (pS6) at Ser-240 and Ser-244. Importantly, p70 S6K targets these sites, and thus, changes in its phosphorylation state can be used to infer levels of both S6 and p70 S6K activity. At CT 15, low levels of S6 phosphorylation were detected in the SCN (Figure 2.5A). However, photic stimulation (400 lx, 15 min) triggered a robust increase of S6 phosphorylation (Figure 2.5A) and double labeling revealed colocalized expression of activated p70 S6K and S6 (Figure 2.5A). To confirm that light-induced S6 phosphorylation was dependent on the mTOR/p70 S6K pathway, mice were infused with rapamycin (100 µm, 2 µl) 30 min prior to photic stimulation. Light-induced S6 phosphorylation was markedly attenuated by rapamycin (Figures 2.5B and D). Interestingly, the low magnification images shown in Figure 4B reveal that rapamycin infusion decreased S6 phosphorylation throughout the periventricular hypothalamic region, which
includes the SCN. As expected, the infusion of U0126 also led to a significant
decrease in light-induced S6 phosphorylation within the SCN (Figures 2.5B and D).
Together, these data indicate that light triggers p70 S6K enzymatic activity (as
assessed by S6 phosphorylation).

2.3.6 Light-induced p-p70 S6K and pCREB colocalize in the SCN

Finally, to gain insight into whether mTOR/p70 S6K activity occurs in neurons
in which rapid transcriptional activation occurs, we examined the spatial and temporal
correlation between p-p70 S6K expression and expression of the
Ser-133 phosphorylated form of CREB (pCREB). Ser-133 phosphorylation is
necessary for CREB to stimulate transcription activation (Gonzalez and Montminy,
1989). CREB activity was the focus of this examination, since a large number of
studies have shown that CREB is a light-responsive transcription factor ([Ginty et al.,
1993], [Obrietan et al., 1998] and [Dziema et al., 2003]) that stimulates clock gene
expression ([Travnickova-Bendova et al., 2002] and [Butcher et al., 2005]). To study
the relationship between p70 S6K and CREB activation, SCN tissue from control (no
light) and light-treated (400 lx, 15 min, CT 15) mice was double-labeled for p-p70
S6K and pCREB. Representative confocal images show a spatial and temporal
correlation between light-induced p-p70 S6K and pCREB (Figure 2.6). Along these
lines, nearly all pCREB immunoreactive SCN cells within the central SCN were also
immunopositive for p-p70 S6K. These data raise the possibility that light stimulates
inducible transcription (as assessed by pCREB immunolabeling) and translation (as
assessed by p-p70 S6K immunolabeling) in the same light-responsive SCN cells.

2.4 Discussion

The goal of this line of inquiry was to begin to identify inducible translation
control pathways in the SCN. To this end, we focused on the mTOR signaling cassette.
We report that light evokes phase-dependent activation of the mTOR pathway and
that mTOR is a downstream target of the MAPK cascade. Together, these data reveal a new light-actuated signaling cassette in the SCN.

Figure 2.5. Light-induced S6 activation in the SCN.
A, Confocal images showing immunolabeling for S6 phosphorylation (pS6, red) and p-p70 S6K (green) in the SCN. Compared with control animals (no light), a 15-minute light treatment (400 lx) at CT 15 elicited an increase in pS6 and p-p70 S6K. Merging the two signals revealed colocalization of light-induced p-p70 S6K and pS6 expression in a subset of the cells. OC, optic chiasm. Boxed regions are magnified below each image. Scale bars: 20 µm. B, Infusion of U0126 and rapamycin blocked light-induced p-p70 S6K expression in the SCN. U0126 (10 mM, 2 µl), rapamycin (100 µM, 2 µl) or DMSO (2 µl) was infused into the lateral ventricle 30 min before a 15-minute light treatment at CT 15. Also, note that infusion of U0126 and rapamycin led to a decrease in pS6 expression within the brain regions surrounding the third ventricle. Scale bar: 750 µm; 3V, third ventricle. C and D, Quantitative analysis of light-induced p-p70 S6K (C) and pS6 (D) expression in the SCN. For (D), the Y-axis
denotes normalized fluorescent intensity (0–255 scale). Please see the Experimental methods section for a description of the quantitation procedure. The treatment conditions are shown below the histograms. Error bars denote SEM. The numbers above the bars indicate the number of animals used for each condition. ***p < 0.001.

Figure 2.6. Light-induced p70 S6K phosphorylation and CREB phosphorylation in the SCN.
Compared with the control mice (No Light), photic stimulation (400 lx, 15 min) at CT 15 increased the number of p-p70 S6K-positive (green) and pCREB-positive (red) cells. Merging of p-p70 S6K and pCREB signals (right) revealed that the expression of the activated kinase and transcription factor were colocalized in a subset of the cells (arrows); arrowhead denotes a cell where colocalization was not detected. The boxed regions are magnified and shown below. Scale bar: 20 µm.

mTOR signaling is a key regulator of inducible gene transcription, ribosome biogenesis, and mRNA translation ([Sarbassov et al., 2005], [Wullschleger et al., 2006] and [Proud, 2007]). In one of the best-characterized routes to its activation, growth factors, including insulin, signal to mTOR through a phosphatidylinositol-3-kinase (PI3K)/AKT-dependent process. AKT activation leads to phosphorylation of tuberous sclerosis protein2 (TSC2), which, in turn, blocks its GTPase-activating protein (GAP) activity for the small G protein Rheb, thus allowing the GTP-loaded form of Rheb to activate mTOR (as part of the mTORC1 complex) ([Gao et al., 2002], [Inoki et al., 2002], [Long et al., 2005] and [Li et al.,
In this study, we examined two principal downstream effectors of mTORC1 which have been implicated in inducible translation control: p70 S6K and 4E-BP1.

The activation of p70 S6K is coordinately regulated by a complex series of phosphorylation steps. A key step in this process is mTOR-mediated phosphorylation of Thr-389 (Weng et al., 1998). In turn, this event creates a docking site for phosphoinositide-dependent kinase 1 (PDK1), which then phosphorylates p70 S6K within the catalytic domain at Thr-229 ([Pullen et al., 1998] and [Frödin et al., 2002]), thus leading to enzymatic activation of p70 S6K (Pullen and Thomas, 1997). In our study, we found that a brief photic stimulus triggered p70 S6K phosphorylation at Thr-389. Interestingly, in the absence of photic stimulation, immunohistochemical labeling was not able to detect the Thr-389 phosphorylated form of p70 S6K in the SCN. This lack of a signal raised the possibility that the SCN exhibits low basal levels of mTOR activity. However, Western analysis was able to detect phosphorylated p70 S6K under control conditions, thus suggesting that our immunohistochemical staining approach lacked the necessary sensitivity to detect a baseline level of phosphorylation. Furthermore, data from S6 and 4E-BP1 (discussed below) supports the idea that the SCN exhibits a relatively high level of mTOR activity.

Although most SCN neurons express mTOR1, light-induced p-p70 S6K expression was only detected in a subset of cells. There are a number of potential explanations for this. Along these lines, limited p-p70 S6K expression may be due to the low sensitivity of the antibody (noted above). Another possibility is that we may be identifying only a subset of the “activated cells”; hence activation may be rapid and transient in some cells whereas others cells may show delayed activation and rapid inactivation. Thus, data collected from a single time point may only capture a subset of the responsive cells. Finally, at a cellular level, the degree of RHT innervation may also contribute to the efficiency of activation.

To test whether light-induced phosphorylation of p70 S6K and 4E-BP1 were dependent on mTOR, we employed an intraventricular infusion method to deliver the
mTORC1 inhibitor rapamycin to the ventricular system. Similar rapamycin infusion approaches have been used by other groups to study the effects of mTOR in the brain ([Tischmeyer et al., 2003] and [Narita et al., 2005]). The infusion of rapamycin led to a complete inhibition of p70 S6K activity, its downstream target, S6, as well as 4E-BP1. Together, these data strongly support the idea that light triggers activation of mTOR-dependent signaling. It should be noted that rapamycin does not affect mTOR as part of the mTORC2 complex (Wullschleger et al., 2006). Interestingly, mTORC2 is regulated by the same upstream TSC2/Rheb2 signaling cassette, but actuates a distinct set of signaling events from mTORC1, such as actin polymerization (Jacinto et al., 2004). Additional work will be required to assess inducible activation and function of mTORC2 in the SCN.

Interestingly, in contrast to p70 S6K, we detected relatively high levels of 4E-BP1 phosphorylation under control conditions and light treatment led to a relatively modest increase in 4E-BP1 phosphorylation. The activation of 4E-BP1 is mediated by a sequential set of phosphorylation events. Activation appears to be initiated by the phosphorylation of Thr-37 and Thr-46 (the two sites examined in this study). This dual phosphorylation is a priming event that allows for phosphorylation at Ser-65 and Thr-70 to occur ([Gingras et al., 1999] and [Gingras et al., 2001]). Ser-65 and Thr-70 phosphorylation are thought to be the trigger that initiates dissociation of 4E-BP1 from eIF4E, thereby allowing cap-dependent mRNA translation to occur ([Lin et al., 1994] and [Pause et al., 1994]). A number of studies have shown that the phosphorylation of Thr-37 and Thr-46 is mediated by mTOR, whereas Ser-65 and Thr-70 phosphorylation is regulated by both mTOR-dependent and -independent signaling ([Gingras et al., 1999] and [Nojima et al., 2003]). Of note, the finding that rapamycin infusion triggered a decrease in 4E-BP1 phosphorylation supports the idea that the SCN exhibits a relatively high tonic level of mTOR activity, thus raising the possibility that the mTOR pathway plays a broader role in the SCN than simply conveying photic information.
Next, our attention turned to potential effectors of p70 S6K in the SCN. To this end, we focused on S6. The rationale for examining S6 was two-fold: first, its phosphorylation can be used as a functional read-out for p70 S6K activity, and second, several studies have shown that it regulates the translation of a subset of mRNAs which contain a 5′ tract of oligopyrimidine (TOP) ([Jefferies et al., 1994], [Terada et al., 1994], [Jefferies et al., 1997], [Kawasome et al., 1998] and [Schwab et al., 1999]). Interestingly, a number of 5′TOP mRNAs are components of the translation machinery and thus may influence the overall translational potential of the cell. However, the precise role of S6 in this process is not clear, and several studies have reported that S6 does not directly regulate translation of 5′TOP mRNAs ([Tang et al., 2001] and [Ruvinsky et al., 2005]). Our results show that light induced a marked increase in S6 phosphorylation at Ser-240 and Ser-244 and that rapamycin potently blocked this process. Interestingly, pS6 was detected in the absence of photic input, further supporting the idea that the SCN exhibits tonic mTOR activity.

The phase-restricted capacity of light to activate mTOR-dependent signaling is consistent with a large body of work showing that photic stimulation exerts a dominant effect during the night time domain. Along these lines, phase-restricted light responses have been characterized at both a behavioral and molecular level. For example, light exposure during the early night causes a phase delay in clock timing, whereas light exposure during the late night causes a phase advance in clock timing (Daan and Pittendrigh, 1976). At the molecular level, the capacity of light to trigger the expression of the immediate early genes such as Fos, JunB and early growth response factor 1 (EGR-1) and core clock timing genes period-1 and period-2 is restricted to the night ([Aronin et al., 1990], [Kornhauser et al., 1990], [Kornhauser et al., 1992], [Rusak et al., 1990], [Albrecht et al., 1997], [Zylka et al., 1998] and [Mendoza et al., 2007]). Likewise, light activation of the CREB/CRE transcription pathway and kinases such as protein kinase C and the MAPK cascade is phase-restricted to the night time domain ([Ginty et al., 1993], [Obrietan et al., 1993].
One key question for this study relates to the upstream signaling pathway to this phase-restricted photic response network. The data reported here add the mTOR signaling pathway to this phase-restricted photic response network.

One key question for this study relates to the upstream signaling cascade that couples light to rapid activation of mTOR. To this end, we examined the MAPK cascade, a key signaling intermediate in light-mediated SCN entrainment ([Obrietan et al., 1998], [Butcher et al., 2002] and [Coogan and Piggins, 2003]). As an initial assessment of the potential role of MAPK signaling in light-induced mTOR activity, SCN sections were double-labeled for activated ERK and p70 S6K. These assays detected a light-induced temporal and cellular correlation between MAPK pathway activity and p70 S6K phosphorylation. Furthermore, infusion of the MEK1/2 inhibitor U0126 potently repressed p70 S6K activity, thus indicating that light-induced MAPK signaling is an upstream activator of mTOR in the SCN.

There are several potential mechanisms by which the MAPK pathway could stimulate mTOR activity. First, recent work has revealed that ERK triggers phosphorylation-dependent inactivation of TSC2 GAP activity ([Ma et al., 2005] and [Ma et al., 2007]). Second, ERK-regulated 90 kDa ribosomal kinase 1 (RSK1) has been shown to block TSC2 activity (Roux et al., 2004), and thereby stimulate mTOR1 signaling. Interestingly, we recently reported that RSK1 is activated by light in the SCN (Butcher et al., 2004). Additional work will be required to delineate the precise mechanism by which MAPK signaling regulates mTOR activity in the SCN.

In the SCN, MAPK signaling is thought to couple light to the core clock timing mechanism via a process that elicits transcription factor activation and, in turn, clock gene expression. Along these lines, the MAPK cascade has been shown to facilitate CRE-dependent transcription and period-1 transcription ([Obrietan et al., 1999], [Dziema et al., 2003], [Travnickova-Bendova et al., 2002] and [Butcher et al., 2005]). Interestingly, the data presented here reveal that light triggers coordinate activation of CREB and mTOR-mediated signaling in the SCN. Given that MAPK signaling regulates both of these processes, these data raise the possibility that
the MAPK cascade serves coordinate roles as a regulator of gene transcription and mRNA translation (Figure 2.7). Further studies aimed at addressing inducible translation regulation will shed new light on the key cellular signaling events that shape circadian clock timing and entrainment.

![Figure 2.7. Schematic of proposed mTOR signaling pathway in the SCN.](image)

Photic input from the retina induces the release of glutamate and PACAP from RHT terminals. Transmitter binding to postsynaptic receptors on SCN neurons evokes a series of intracellular signal transduction events, including MAPK pathway activation. One downstream target of the MAPK cascade is CREB, which, when (Continued) (Figure 2.7 Continued) phosphorylated on Ser-133, drives clock gene transcription. Light-induced activation of the MAPK cascade also stimulates mTORC1, which in turn targets the translation regulators p70 S6K and 4E-BP1. Activated p70 S6K stimulates S6 phosphorylation, whereas 4E-BP1 phosphorylation has been shown to lead to its dissociation from eIF4E, thereby allowing cap-dependent mRNA translation to occur. U0126 is a specific MEK 1/2 inhibitor and rapamycin inhibits mTORC1.
3.1 Introduction

In mammals, the master circadian clock is localized in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. Through both synaptic and paracrine mechanisms, the SCN pacemaker imparts rhythms to an array of biochemical, physiological, and behavioral processes. At a molecular level, the SCN clock timing mechanism is derived from the workings of several interlocking transcription/translation feedback loops (for review, see Reppert and Weaver, 2002; Ko and Takahashi, 2006). A key feature of the SCN clock is that its phasing is tightly regulated by the 12 h light/dark (LD) cycle. Light entrainment of the clock is mediated via a photic input pathway from the retina. Thus, in response to light, melanopsin-expressing retinal ganglion cells release glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) (for review, see Hannibal, 2002). During the night time domain, these two transmitters work in combination to elicit a rapid and irreversible resetting of the clock. Although the precise molecular mechanism by which photic input drives clock entrainment have not been resolved, a number of studies support a model in which the rapid induction of gene expression, and in particular, the expression of the circadian clock genes Period1 (Per1) and Period2 (Per2), drive the resetting process (Akiyama et al., 1999; Albrecht et al., 2001; Wakamatsu et al., 2001).

With respect to light-evoked gene expression, studies examining the transregulatory events have revealed a number of light-activated kinase pathways and transcription factors (Golombek et al., 2003). Along these lines, the 42/44 mitogen-activated protein kinase (MAPK) pathway is activated by light in a
phase-restricted manner, and couples photic information to activation of cAMP response element-binding protein (CREB), a key effector of light-actuated clock gene transcription (Obrietan et al., 1998, 1999; Coogan and Piggins, 2003; Tischkau et al., 2003; Butcher et al., 2005). Given that gene expression is also regulated at a translational level, there has been significant interest in examining the role inducible translation plays in clock entrainment. Initial studies using a variety of model systems have shown that clock entrainment requires protein synthesis (Johnson and Nakashima, 1990; Raju et al., 1990; Murakami et al., 1995; Zhang et al., 1996). More recently, specific translation regulatory mechanisms have been identified. For example, we reported that light-evoked microRNA expression functions as a feedback regulator of clock entrainment (Cheng et al., 2007). In addition, the clock-regulated immediate early gene nocturnin, a deadenylase, which regulates mRNA stability, has been shown to regulate rhythmic gene expression (Baggs and Green, 2003; Garbarino-Pico et al., 2007). Furthermore, we recently reported on another potential mechanism of inducible gene expression in the SCN: the mammalian target of rapamycin (mTOR) signaling pathway (Cao et al., 2008).

mTOR is a Ser/Thr kinase that serves as the central component of the multiprotein mTOR complex 1 (mTORC1). A large number of studies have revealed a key role for mTORC1 as an integration center, regulating inducible mRNA translation to match changes in the metabolic/physiologic state of the cell (for review, see Sarbassov et al., 2005; Wullschleger et al., 2006; Proud, 2007). Actuation of mTORC1 triggers bifurcating signal transduction pathways. One arm, which is activated by p70 S6 kinase (p70 S6K) has been implicated in the translation of mRNAs with a 5'-terminal oligopyrimidine tract (5'-TOP mRNAs), a subset of which encode for translation factors that increase ribosomal processivity (Jastrzebski et al., 2007). The second arm is activated by eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which stimulates cap-dependent translation initiation (Hay and Sonenberg, 2004; Tee and Blenis, 2005). Our previous work revealed that photic stimulation
triggers robust activation of both arms of the mTOR-dependent signaling pathway in
the SCN (Cao et al., 2008). Here we further these studies by examining the
potential contribution of the mTOR-dependent signaling pathway to SCN clock
entrainment. The data presented here reveal that the disruption of mTOR signaling
leads to an attenuation of the early-night light-evoked phase delay, whereas late night
mTOR abrogation enhances the phase-advancing effect of light. The
modulatory effects of mTOR were paralleled by data showing that mTOR alters the
capacity of light to couple to the core clock timing mechanism. Together, these data
reveal a key role for the mTOR-signaling pathway in sculpting the phase-specific
entraining effects of lights.

3.2 Materials and methods
3.2.1 Light-treatment paradigm and tissue processing.

Adult (8- to 10-week-old) C57BL/6 mice were entrained to a 12 h light/dark (LD)
cycle (100 lux) for at least 2 weeks and then transferred to total darkness for two
consecutive 24 h cycles. After dark adaptation, animals received a single light
exposure (400 lux, 15 min) either during the early subjective night, circadian time (CT)
15, or the late subjective night (CT20). CT was calculated based on Zeitgeber time
(ZT) and the tau for C57BL/6 mice under free-running conditions (23.77 h)
(Schwartz and Zimmerman, 1990), with ZT 0 denoting lights on and ZT 12 denoting
lights off. At the indicated time points after light exposure, animals were anesthetized
with an intraperitoneal injection of ketamine hydrochloride (140 mg/ml) and
xylazine (13 mg/ml) under dim red light (<10 lux). Opaque black tape was placed over
the eyes, and mice were transeardially perfused with cold PBS, pH 7.4, followed by
4% paraformaldehyde in PBS. Next, brains were postfixied in 4% paraformaldehyde
for 4 h at 4°C and cryoprotected with 30% sucrose in PBS. The "no light" control
animal groups underwent the same handling procedures (except for light exposure)
and were killed at the same time points. All procedures were in accordance with Ohio
State University animal welfare guidelines and approved by the Institutional Animal Care and Use Committee.

3.3.2 Cannulation and infusion.

Mice were cannulated in the lateral ventricles using the techniques described by Cao et al. (2008). Briefly, animals were anesthetized with ketamine hydrochloride (91 mg/ml) and xylazine (9 mg/ml) and placed in a stereotaxic apparatus (Cartesian Research). The coordinates (posterior, 0.34 mm from bregma; lateral, 0.90 mm from the midline; dorsoventral, –2.15 mm from bregma) were used to place the tip of a 24 gauge guide cannula into the lateral ventricle. Cannulae were held in place with dental cement and a 30 gauge stylus was secured in the cannula to ensure patency. After surgery, animals were housed individually and allowed to recover for at least 2 weeks under a standard 12 h LD cycle. For the infusion, animals were restrained by hand under dim red light, and the infusate was delivered at a rate of 1 µl/min. To disrupt light-evoked PACAPergic and ionotropic glutamatergic neurotransmission, mice were infused with the PAC1 receptor antagonist, PACAP 6-38 (Bachem) and the ionotropic glutamate antagonists CNQX (Sigma) and APV (Sigma) in a total volume of 4 µl (diluted in physiological saline) 15 min before photic stimulation (400 lux, 15 min) at CT15. Inhibitor concentrations are noted in Figure 2. To activate mTOR signaling, PACAP (200 µM; Bachem) and glutamate (2.5 mM) were diluted in physiological saline (4 µl) and infused at CT14.5. Control animals were infused with the same volume of physiological saline at CT14.5, and mice were killed 30 min later. To inhibit mTOR signaling, rapamycin (2 µl, 100 µM; Cell Signaling Technology) was infused 30 min before light treatment. Control animals were infused with an equivalent volume of vehicle (DMSO). To inhibit gene transcription, 1 µl of actinomycin D (2 µg/µl; Sigma) was infused 30 min before light treatment.

3.2.3 SCN neuron culture and stimulation
For neuronal culture, SCN-enriched tissue was collected from embryonic day 19–21 Sprague Dawley rat pups. Brains were placed in a sterile Petri dish containing cold oxygenated dissociation media (DM) [containing the following (in mM): 90 \( \text{Na}_2\text{SO}_4 \), 30 \( \text{K}_2\text{SO}_4 \), 16 \( \text{MgCl}_2 \), 0.25 \( \text{CaCl}_2 \), and 32 HEPES, and 0.01% phenol red (Sigma), pH 7.7], and using the optic chiasm and optic nerves as landmarks, the region containing the SCN was microdissected from the ventral surface of the brain. SCN tissue was washed in DM and incubated in digestion solution [100 U/ml papain latex (Worthington) and 4.5 mg of cysteine (Sigma) in DM] for 30 min at 37°C. Tissue was then transferred to standard culture medium (Minimal Essential Medium; Invitrogen) containing 1% fetal bovine serum (Invitrogen), 2% B27 (Invitrogen), and 100 U/ml penicillin/streptomycin (Invitrogen), and triturated into a single-cell suspension. Cells were plated onto poly-D-lysine-coated 9 mm\(^2\) glass coverslips at a density of 50,000 cells/cm\(^2\). Media was changed 1 h after plating, and cells were maintained in a Napco 6100 incubator (37°C, 5.5% CO\(_2\)) for 10 d. Media was replenished every third day. Four hours before experimental stimulation, tissue culture media was replaced with artificial CSF (ACSF) buffer [containing the following (in mM): 137 \( \text{NaCl} \), 25 glucose, 10 HEPES, 5 KCl, 1 \( \text{MgCl}_2 \), and 3 \( \text{CaCl}_2 \), pH 7.4]. Cells were treated for 30 min with glutamate (10 \( \mu \text{M} \)) and/or PACAP (200 nM). Stimulated cells were washed with ACSF and fixed (15 min) with paraformaldehyde (4% w/v, 25°C) followed by cold methanol (−20°C). Phospho-p70 (p-p70) S6K immunofluorescent labeling was performed as described below.

3.2.4 Behavioral rhythm recording

Cannulated mice were individually housed in polycarbonate cages equipped with running wheels. Wheel rotation was detected via the closure of a magnetic switch and recorded using ActiView software (MiniMitter). Mice were entrained to a 12 h LD cycle (100 lux) for at least 10 d, and then transferred to continuous darkness (DD). Rapamycin (100 \( \mu \text{M}, 2 \mu \text{l} \)) or vehicle (DMSO, 2 \( \mu \text{l} \)) was infused 30 min before light treatment (100 lux, 15 min) at CT15 or CT22 using the technique described above.
Mice were then returned to their home cages for at least 14 d. After this period, mice received a second infusion and light pulse: half of the mice were infused with rapamycin first; the other half received DMSO first. Control "no light" animals received the same two-infusion paradigm (rapamycin and DMSO at CT14.5 or CT21.5) but no light treatment.

For core body temperature recording, animals were anesthetized with ketamine hydrochloride (91 mg/ml) and xylazine (9 mg/ml) and implanted with transmitters (E-Mitters; MiniMitter) in the intraperitoneal cavity. After surgery, animals were housed singly in cages placed on receiving platforms (ER-4000 receiver) that communicated with the E-Mitter: data were collected every 60 s. The signal was acquired via the VitalView software program (MiniMitter) and analyzed via the ActiView software program. To clearly visualize the circadian rhythm of body temperature in an actograph format, a threshold was set to 2–2.5°C below the maximum recorded temperature during the entire experimental period, and only signals above this threshold temperature were displayed.

3.2.5 Assessment of light-induced phase shifts

The linear regression method described by Daan and Pittendrigh (1976) was used to assess light-induced phase shifts. Specifically, the difference in activity onset before and after the day of light exposure was determined by a least-squares method. Thus, a line calculating the activity onset for a period of at least 6 d preceding light treatment was calculated. This line was extended to project to when activity onset should occur during the period after light exposure. A second regression line was generated to determine activity onset after light administration. Wheel running activity 3–10 d after light treatment was used to generate this line. The difference in the projected versus the actual activity onset after light treatment was the phase shift. Group data (DMSO, DMSO plus light, rapamycin, and rapamycin plus light) are expressed as mean phase shift ± SEM. Significance was assessed using one-way
ANOVA analysis followed by the Student–Newman–Keuls (SNK) test. A value of $p < 0.05$ was accepted as statistically significant.

### 3.2.6 Immunohistochemistry

For immunohistochemical staining, brains sections (500 µm thick) were prepared using a vibratome, and SCN-containing sections were then thin-cut (40 µm) on a sliding microtome. Sections containing the central SCN were washed in PBS containing 2 mM sodium azide and 3 mM NaF, pH 7.4, and then treated with 0.3% H$_2$O$_2$ and 20% methanol in PBS for 10 min to deactivate endogenous peroxidases and permeabilize the tissue. Sections were then blocked for 1 h in 10% goat serum/PBS and incubated (overnight, 4°C) in one of the following antibodies: rabbit monoclonal anti-phospho-mTOR (p-mTOR, Thr-2448, 1:500; Cell Signaling Technology); mouse monoclonal anti-p-p70 S6 kinase (Thr-389, 1:1000; Cell Signaling Technology); rabbit polyclonal anti-phospho-S6 ribosomal protein (p-S6; Ser-240/244, 1:1000; Cell Signaling Technology); mouse monoclonal anti-S6 ribosomal protein (S6; 1:100; Cell Signaling Technology); rabbit anti-phospho-MSK1 (Ser-360, 1:500; Cell Signaling Technology); rabbit anti-mPER1 (1:3000; a generous gift from Dr. Steven Reppert, University of Massachusetts, Worcester, MA); mouse anti-mPER2 (1:500; Alpha Diagnostic); mouse eukaryotic elongation factor 1A (eEF1A) monoclonal antibody (1:1000; Millipore); mouse monoclonal anti-JunB (1:2000; Santa Cruz Biotechnology); or rabbit polyclonal anti-c-Fos (1:3000; Calbiochem). Next, tissue was incubated for 1.5 h at room temperature in biotinylated anti-mouse or rabbit IgG (1:200; Vector Laboratories) and then placed in an avidin/biotin HRP complex (Vector Laboratories) for 1 h, following the manufacturer's instructions. The signal was visualized using nickel-intensified DAB substrate (Vector Laboratories), and sections were mounted on gelatin-coated slides with Permount media (Fisher Scientific). Sections were washed in PBS (three times, 10 min per wash) between each labeling step.
For immunofluorescent labeling, both tissue sections and cultured neurons were permeabilized with PBS with 1% Triton X-100 (PBST) for 30 min, blocked as described above and then incubated (overnight, 4°C) in 5% goat serum/PBS with the following antibodies: mouse monoclonal anti-p-p70 S6 kinase (Thr-389, 1:300; Cell Signaling Technology) and/or rabbit polyclonal anti-p-S6 (Ser-240/244, 1:300; Cell Signaling Technology). The following day, sections were incubated (3 h, room temperature) in Alexa Fluor-594-conjugated goat anti-rabbit IgG antibody (1:500; Invitrogen) and Alexa Fluor-488-conjugated goat anti-mouse IgG antibody (1:500; Invitrogen). Sections were mounted on slides with Cytoseal 60 (Richard-Allan Scientific). Tissues were washed in PBS (three times, 10 min per wash) between each labeling step.

Bright-field photomicrographs were captured using a 16 bit digital camera (Micromax YHS 1300; Princeton Instruments) mounted on an inverted Leica microscope (DM IRB). Images were acquired with MetaMorph software (Molecular Devices). Confocal fluorescence images were captured using a Zeiss 510 Meta confocal microscope. All confocal parameters (pinhole, contrast, brightness, etc.) were held constant for all data sets from the same experiment.

3.2.7 Western blot analysis

Mice were dark adapted for 2 d and then exposed to light at CT15 (400 lux, 15 min). Brains were isolated, and 500 µm sections were cut with a vibratome. The SCN was excised using a 700 µm tissue punch and frozen on dry ice. Tissue was pooled from six animals per condition, and then lysed in 100 µl radioimmunoprecipitation assay buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 mM sodium vanadate, 1 mM NaF, and 1x protease inhibitor cocktail (Roche)]. Protein extracts (24 µl/lane) were electrophoresed into an 8% SDS-PAGE gel and then transblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were blocked in 10% bovine serum albumin (BSA) (Fisher Scientific) and then incubated (overnight, 4°C)
in PBST (with 5% BSA) with the p-p70 S6K (Thr-389, 1:1000 dilution), p-mTOR (Ser-2448, 1:500 dilution), or p-S6 (Ser-240/244, 1:1000 dilution) antibodies. These are the same antibodies used for our immunolabeling analysis. Next, membranes were incubated in PBST (with 5% milk) with a goat anti-mouse or anti-rabbit IgG horseradish peroxidase or alkaline phosphatase-conjugated antibodies (1:2000 dilution; PerkinElmer Life Sciences). As a protein loading control, membranes were probed for total ERK expression using a goat polyclonal anti-ERK antibody (1:1000 dilution; Santa Cruz Biotechnology) followed by a donkey anti-goat IgG antibody conjugated to horseradish peroxidase (1:2000 dilution; Rockland Immunochemicals). The signal was visualized using the Western Lighting Chemiluminescence light-emitting system (PerkinElmer Life Sciences) and a Blue Lite Autorad film (ISC BioExpress). Between each antibody treatment, membranes were washed a minimum of three times (10 min per wash) in PBST.

3.2.8 Data analysis

All data were quantified using Adobe Photoshop software (Adobe Systems). For the p-S6 and PER2 intensity analysis, images of the SCN were acquired (10x) and digitally outlined, and the mean pixel values were determined. Next, a digital oval (150 x 200 pixels) was placed on the adjacent lateral hypothalamus, and this mean value was subtracted from the adjacent SCN signal to provide a normalized SCN intensity value. For PER1 and eEF1A immunolabeling, SCN images were acquired at 40x, a digital circle (1000 pixels) was placed in the ventral SCN, and the mean labeling intensity was determined. A digital oval (150 x 200 pixels) was then placed over the adjacent lateral hypothalamus, and the mean value was subtracted from the SCN value to generate a normalized SCN intensity value. Of note, the lateral hypothalamic immunolabeling values were not altered by light or rapamycin infusion. For p-p70 S6K-, p-MSK1-, c-Fos-, and JunB-positive cell counting, SCN images were acquired at 40x and a digital circle (1000 pixels) was placed in the ventral SCN. An intensity threshold filter was applied to eliminate nonspecific background labeling.
Then, the number of detectable signals above threshold (now defined as positive cells) was tabulated. For all experiments, data were averaged from three central SCN sections per animal, and these values were pooled to generate mean values for treatment groups. For p-p70 S6K expression analysis in SCN cell culture, neuronal images were acquired at 40x, the cell soma were digitally outlined, and the mean pixel values were determined. Next, a digital oval (150 x 200 pixels) was placed in an adjacent empty area, and the mean value within this region was subtracted from the adjacent cellular signal to provide a background-corrected p-p70 S6K intensity value. For statistical analysis, the p-p70 S6K expression level of all cells within a visual field (mean, six cells per field) was averaged. Data from 10 visual fields were statistically analyzed for each treatment group. Group statistical analysis was performed via one-way ANOVA followed by an SNK test. A value of p < 0.05 was accepted as statistically significant. The values are presented as mean ± SEM. All statistical analysis was performed using SPSS software.

3.3 Results

3.3.1 Light-evoked mTOR activation in the SCN

We begin our study with an examination of the light-evoked activation and temporal regulation of mTOR signaling. As an initial validation of our light-stimulus paradigm, we monitored mTOR phosphorylation (p-mTOR) at Ser-2448, a widely used marker of mTOR activity (Hou and Klann, 2004; Tsokas et al., 2005; Hoeffer et al., 2008). For these studies, mice were dark adapted for 2 d, exposed to light (400 lux, 15 min) at CT15, and then killed 5 min after light cessation. Immunohistochemical labeling and Western blot analysis of SCN extracts revealed that light triggered an increase in p-mTOR within the SCN (Figure 3.1A,C,E). Using the same stimulus conditions, immunofluorescent labeling detected a marked increase in the Thr-389 phosphorylated form of p70 S6K (Figure 3.1A). Phosphorylation of p70 S6K is mediated by mTOR, and thus can be used to validate mTOR activation (Jefferies et al., 1997; Burnett et al., 1998; Weng et al., 1998). Of note, this antibody also
recognizes p85 S6K when phosphorylated at Thr-412, a site analogous to Thr-389 in p70 S6K. The specificity of the antibody was tested in our previous study (Cao et al., 2008) and in the published studies of several other groups (Tsokas et al., 2005; Cota et al., 2006; Hoeffer et al., 2008). Western blot analysis presented here detected a light-induced increase in an ~70 kDa band, which is likely to be p70 S6K (Figure 3.1D). To avoid confusion, we will specifically refer to p70 S6K when describing results generated using this antibody. Next, double labeling revealed a light-evoked increase in the active (i.e., Ser-240 and Ser-244 phosphorylated) form of S6 (p-S6) (Figure 3.1A,C). The two phosphorylation sites examined here are specifically targeted by p70 S6K. Thus, given that S6 catalytic activity is regulated by p70 S6K, S6 phosphorylation can be used to monitor the relative activation of the p70 S6K/S6 arm of the mTOR pathway. Along these lines, merging the p-p70 S6K and p-S6 signals revealed colocalized expression of the antigens (Figure 3.1A). A light-evoked increase in p-mTOR and p-S6 was also detected using Western blot analysis of SCN extracts (Figure 3.1E). Together, these data support and extend our prior findings (Cao et al., 2008) that light triggers activation of the mTOR/p70 S6K/S6 signaling cassette in the SCN.
Figure 3.1. Light-induced mTOR activation in the SCN.

A, Top, Representative micrographs of tissue labeled for the Thr-2448-phosphorylated form of mTOR. Relative to the control condition (Continued) (Figure 3.1 Continued) (No Light), photic stimulation (400 lux, 15 min, CT15) triggered an increase in mTOR phosphorylation. Mice were killed 5 min after cessation of the light pulse. Middle, Representative confocal images of SCN tissue double labeled for the expression of Thr-389-phosphorylated p70 S6K (p-p70 S6K; green) and Ser-240/244 phosphorylated S6 (p-S6; red). Relative to the control condition (No Light), light triggered a marked increase in the phosphorylated forms of both proteins. Bottom, Merging of the two signals revealed cellular colocalization of the two antigens. The framed area is magnified to the right. 3V, Third ventricle; OC, optic chiasm. Scale bars, 100 µm. B, Top, Middle rows, Temporal profile of light-induced p70 S6K and S6 phosphorylation. Mice were killed at the noted times after light exposure (400 lux, 15 min) at CT15. Control mice (No Light) were killed at CT15. Bottom, Immunohistochemical profile of total S6 expression after light exposure. Scale bars, 100 µm. C, Quantification of light-induced p-mTOR, p-p70 S6K, p-S6, and S6 expression. *p < 0.05 compared to the "no light" control value, which was normalized to a value of 1. Data were collected from four or five animals for each group. D, E, Control and light-pulsed animals (CT15, 400 lux, 15 min) were killed at CT15.25, and SCN tissue was examined via Western blot analysis. Probing with a p-p70 S6K antibody (D) revealed that light treatment (L1 and L2; biological replicates) stimulated an increase in the phosphorylation of an ~70 kDa band relative to the control "no light" condition (NL1, NL2; biological replicates). Relative to the
"no light" condition, an increase in p-mTOR and pS6 expression (E) was also detected. The molecular weights of these bands are consistent with the sizes of mTOR (~289 kDa) and S6 (~32 kDa). The arrow indicates a nonspecific band. As loading controls, blots were probed for total ERK expression.

To gain insight into the regulation of mTOR signaling, we examined the temporal profile of light-evoked mTOR, p70 S6K, and S6 phosphorylation (Figure 3.1B,C). For these experiments, mice were treated to the same light-stimulus paradigm described above, and then returned to darkness for 5 min to 4 h before being killed. Immunolabeling revealed that mTOR and p70 S6K phosphorylation was rapidly activated, reaching a peak phosphorylation level by 30 min after light exposure and declining quickly thereafter. However, light led to a robust and persistent increase in p-S6: maximal expression was detected at 120 min after light, and a significant increase in expression could still be detected at 4 h after light treatment. In contrast, the level of total S6 within the SCN was not affected by light (Figure 3.1B,C).

Next, we examined the proximal transmitter systems that actuate light-induced mTOR activation in the SCN. Toward this end, we focused on the two principal neurotransmitters of the retinohypothalamic tract (RHT): PACAP and glutamate. PACAP has been shown to regulate the clock via the PACAP type 1 (PAC1) receptor subtype (Bergström et al., 2003), whereas glutamate functions at both NMDA and AMPA/kainate subclasses of ionotropic glutamate receptors to affect clock timing (Colwell and Menaker, 1992; Rea et al., 1993). Thus, to antagonize signaling through both pathways, we infused a mixture of the PAC1 receptor antagonist PACAP 6-38 (1 mM), the NMDA receptor antagonist APV (25 mM), and the AMPA/kainate receptor antagonist CNQX (2.5 mM) 15 min before photic stimulation (400 lux, 15 min) at CT15. Abrogation of PACAP and ionotropic glutamate receptor activation potently suppressed light-induced p70 S6K and S6 phosphorylation (Figure 3.2A–D). These effects were dose dependent (Figure 3.2B). Interestingly, infusion of either PACAP 6-38 or CNQX and APV also significantly decreased light-induced p70 S6K and S6 phosphorylation (Figure 3.2C,D), indicating that coactivation of PACAPergic and glutamatergic signaling is required for robust mTOR activation. In the absence of light,
the infusion of PACAP 6-38, CNQX, and APV did not markedly affect basal p70 S6K and S6 phosphorylation (data not shown). To confirm these results, we attempted to activate mTOR signaling in the SCN via the exogenous administration of glutamate (2.5 mM) and PACAP (200 µM). Animals were killed at 30 or 120 min after neurotransmitter microinjection at CT14.5. Relative to the injection of drug vehicle (saline), the infusion of glutamate and PACAP led to an increase in p-S6 expression at both time points (Figure 3.2E,F). We also used cultured SCN neurons to test the capacity of glutamate (10 µM) and PACAP (200 nM) to evoke p70 S6K activation. Application of glutamate or PACAP induced relatively modest p70 S6K phosphorylation. However, a combined application of the two transmitters induced robust p70 S6K phosphorylation (Figure 3.2G,H). In conclusion, these data indicate that coordinate PACAPergic and glutamatergic neurotransmission is required to drive marked mTOR activation in the SCN.

3.3.2 mTOR signaling couples light to SCN clock entrainment

Light exposure during the early subjective night induces a phase delay of the circadian clock, whereas late-night light induces a phase advance of the SCN clock timing process. Our prior work showing that mTOR signaling is activated by both early- and late-night light (Cao et al., 2008) led us to investigate the potential role of this pathway in clock entrainment.

To test this idea, we used a ventricular infusion approach to transiently disrupt mTOR signaling and assess its effect on clock entrainment via an examination of overt wheel-running activity. For these experiments, cannulated mice were dark adapted for at least 10 d and then infused with the mTOR inhibitor rapamycin (100 µM) or vehicle (DMSO) 30 min before light (100 lux, 15 min) exposure at CT15 or CT22. We showed recently (Cao et al., 2008) that this rapamycin-infusion paradigm results in potent repression of light-evoked mTOR kinase activity in the SCN; furthermore, the 100 lux photic stimulation paradigm used here led to a statistically
significant (p < 0.01, Student's t test) increase in p70 S6K activation (light, 89 ± 14 SEM immunopositive cells vs control, 7 ± 2 immunopositive cells; n = 4 animals for each condition) (Figure 3.3E). After light treatment, animals were returned to darkness for ~14 d and then received a counterbalanced infusion of rapamycin or vehicle: half of the animals received rapamycin first, and the other half received vehicle first. Additionally, to test for the effects of rapamycin and drug vehicle on clock phasing or period, control "no light" animals received counterbalanced infusions of rapamycin and DMSO vehicle. As expected, in vehicle-infused mice, light exposure at CT15 triggered a significant phase delay in activity onset. Interestingly, pretreatment with rapamycin significantly attenuated (~50 min or 36%) this phase-delaying effect of light (Figure 3.3A,B,D). Importantly, in the absence of light, the early-night infusion of rapamycin did not significantly affect clock phasing (Figure 3.3C,D), indicating that the transient disruption of mTOR signaling specifically affected the capacity of light to entrain the clock.

Next, we examined whether mTOR signaling affects the phase-advancing effects of late-night light. As expected (Schwartz and Zimmerman, 1990), in vehicle-treated mice, light exposure at CT22 triggered a relatively modest phase advance (Figure 3.4A,B,D). Interestingly, disruption of mTOR signaling led to a significant lengthening of the light-induced phase advance. Thus, relative to vehicle infusion, the infusion of rapamycin triggered an ~2.2-fold increase in the phase advance. Given that rapamycin infusion in the absence of light did not significantly alter clock phase (Figure 3.4C,D), these data indicate that light-actuated mTOR signaling represses the phase-advancing effect of late-night light.
Figure 3.2. Glutamatergic and PACAPergic neurotransmission couples light to mTOR activation in the SCN.

A, Representative immunohistochemical labeling for control (Saline) and light-evoked (400 lux, 15 min, CT15) p-p70S6K and p-S6 after infusion (Continued) (Figure 3.2 Continued) of physiological saline (Saline+Light) or a mixture of CNQX (2.5 mM), AP5 (25 mM), and PACAP 6-38 (P6-38, 1 mM; CNQX+AP5+P6–38+Light). Compared to the Saline+Light condition (middle), the infusion of PACAP and glutamate receptor antagonists led to an attenuation of p70S6K and S6 phosphorylation (right). Scale bars, 100 µm. B, Quantitative analysis of p-S6 expression under three dosages of the inhibitor cocktail. C1, CNQX (150 µM), AP5 (1.6 mM) and PACAP 6-38 (60 µM) plus light; C2, CNQX (600 µM), AP5 (6 mM), and PACAP 6-38 (240 µM) plus light; C3, CNQX (2.5 mM), AP5 (25 mM), and PACAP 6-38 (1 mM) plus light. The light-stimulus paradigm is described in A. C, D, Quantitative analysis of p-p70S6K (C) and p-S6 (D) expression under the five treatment conditions indicated below each panel. Mice were infused with PACAP 6-38 (P6-38, 1 mM) and/or CNQX (2.5 mM) and AP5 (25 mM) at CT14.5. The light-stimulus paradigm is described in A. E, Representative immunohistochemical
labeling for p-S6 after the ventricular infusion of saline or PACAP (200 μM) and glutamate (2.5 mM) at CT14.5. Animals were killed 30 or 120 min after infusion. Under the control condition, low p-S6 levels were detected in the SCN. PACAP and glutamate infusion increased S6 phosphorylation. Scale bar, 100 μm. F, Histogram quantifying p-S6 under the three conditions outlined in D. S, Saline; G+P30′, killed 30 min after glutamate and PACAP infusion; G+P120′, killed 120 min after glutamate and PACAP infusion. G, Representative confocal images of p70 S6K phosphorylation in cultured SCN neurons. Cultures were stimulated (30 min) with glutamate (10 μM), PACAP (200 nM), or glutamate and PACAP (10 μM and 200 nM, respectively) and then fixed and immunolabeled. Relative to mock stimulation (ACSF), glutamate or PACAP alone induced a moderate increase in p-p70 S6K expression. However, the coadministration of glutamate and PACAP induced robust p70 S6K activation. H, Quantitative analysis of p-70S6K expression in SCN neurons under the conditions described in G. For all histograms, data were normalized to the saline (or ACSF) condition, which was set to a value of 1. Please see Materials and Methods for a detailed description of the quantitation methods.

To complement the wheel-running experiments, we analyzed the effects of rapamycin on light-evoked entrainment of core body temperature rhythms. To this end, mice were implanted with radio transmitters, entrained to a 12 h LD cycle, and then transferred to DD. After ~10 d under DD, mice were infused with DMSO vehicle or the mTOR inhibitor rapamycin (100 μM) 30 min before light exposure (100 lux, 15 min) at CT15 or CT22. By applying a threshold, which was set to be 2–2.5°C below the maximum recorded temperature during the entire experiment, a circadian temperature oscillation could be clearly displayed in the actogram (Figure 3.5A,B).
Figure 3.3. Disruption of mTOR signaling attenuates light-induced phase delaying of circadian locomotor activity.

A, B, Representative double-plotted actographs of wheel-running activity. Initially, mice were entrained on a 12 h LD cycle and then transferred to total darkness. After ~10 d under DD, mice were infused with DMSO vehicle (A) or the mTOR inhibitor rapamycin (100 µM; B) 30 min before light exposure (100 lux, 15 min) at CT15 (red asterisks). Animals free ran for 14 d and then received a second infusion of rapamycin (A) or DMSO (B) followed by light treatment. Regression lines approximate the phase-delaying effects of light. The small horizontal red bars in the activity records denote an "off-line" period when wheel-running activity was not recorded. C, Representative actograph shows that rapamycin (100 µM) infusion at CT15 did not markedly affect clock timing or phasing. D, Statistical representation of the early-night phase-shifting data. Of note, the light-evoked phase delay was significantly attenuated by rapamycin. Numbers above the bars denote the number of animals examined for each condition. E, Light-evoked p70 S6K phosphorylation. To test whether the light intensity (100 lux, 15 min) used in the behavioral experiments evokes mTOR activation, mice were exposed to light (100 lux, 15 min) at CT22 and killed immediately thereafter. Immunohistochemical labeling revealed a light-evoked increase in p-p70 S6K, relative to control mice (no light). Scale bar, 100 µm.
Figure 3.4. mTOR functions as a negative regulator of late-night light-evoked phase advancing of the circadian clock.

A, B, Representative double-plotted actographs of wheel-running activity. Mice were dark adapted and infused with DMSO vehicle (A) or rapamycin (100 µM; B) 30 min before light (100 lux, 15 min) exposure at CT22 (red asterisks). Mice were allowed to free run under DD for 19 d, and then they received a second infusion of rapamycin (A) or DMSO (B) followed by light exposure. Regression lines approximate the light-evoked phase shifts. C, A representative actograph shows that rapamycin infusion at CT22 did not markedly affect clock timing or phasing. D, Statistical analysis of late-night phase shifting. Of interest, the phase-advancing effect of light was significantly enhanced by rapamycin infusion. Numbers above the bars denote the number of animals analyzed for each condition.

Similar to the results of the wheel-running experiments, pretreatment with rapamycin significantly attenuated (~69 min or 53%) the phase-delaying effect of light at CT15 (Figure 3.5A,C) but led to a significant lengthening (~32 min or 114%) of the light-induced phase advance at CT22 (Figure 3.5B,C). Together, these behavioral rhythms studies suggest that mTOR functions as a time-of-day-specific regulator of the clock, facilitating early-night light-induced phase delays and repressing late-night phase advances.
Figure 3.5. Disruption of mTOR signaling attenuates light-induced phase delaying but enhances light-induced phase advancing of the circadian core body temperature rhythm.

A, B, Representative double-plotted actographs of core body temperature recordings. Initially, cannulated mice were entrained on a 12 h LD cycle and then transferred to total darkness. After ~10 d under DD, mice were infused with DMSO vehicle or the mTOR inhibitor rapamycin (Rapa; 100 µM) 30 min before light exposure (100 lux, 15 min) at CT15 (A) or CT22 (red asterisks) (B). Animals free ran for 7–14 d and then received a second infusion of rapamycin or DMSO followed by light treatment. Regression lines approximate the phase-delaying (A) or phase-advancing (B) effect of light. C, Statistical representation of the early-night (CT15) and late-night (CT22) data sets. Of note, the light-evoked phase delay was significantly attenuated by rapamycin. However, the light-evoked phase advance was significantly enhanced by rapamycin. Numbers above the bars denote the number of animals examined for each condition.

3.3.3 mTOR modulates light-induced PERIOD protein expression

A good number of studies support the idea that light-induced Period1 and Period2 gene expression are key events in the clock entrainment process (Akiyama et al., 1999; Albrecht et al., 2001; Wakamatsu et al., 2001). These findings, coupled with our work showing that mTOR regulates clock entrainment, led us to examine whether mTOR influences light inducible PERIOD (PER) protein expression. To this end, we used the ventricular infusion technique described above to deliver rapamycin (100 µM) or vehicle (DMSO) 30 min before a light pulse (400 lux, 15 min) at CT15.
Animals were then returned to darkness and killed 4 h later. Sampling schedules were based on a recent study (Yan and Silver, 2004) showing that maximum PER protein induction within the core region of the SCN was observed 4–6 h after photic stimulation. Immunohistochemical labeling revealed that photic stimulation triggered an increase in PER1 and PER2 protein expression in the SCN, relative to control animals (no light) (Figure 3.6). Similar to the expression pattern reported by Yan and Silver (2004), light-evoked PER1 was mainly detected within the central SCN (Figure 3.6A), whereas the increase in PER2 expression was predominately located in the dorsal and lateral regions of the SCN (Figure 3.6C). Interestingly, light induction of both PER1 (Figure 3.6A,B) and PER2 (Figure 3.6C,D) was significantly attenuated by rapamycin infusion. In the absence of light, rapamycin did not significantly alter basal PER1 and PER2 expression, thus indicating that mTOR facilitates light-evoked clock protein expression. Control experiments revealed that rapamycin infusion did not significantly alter the capacity of light to trigger MSK1 phosphorylation (data not shown), thus suggesting that rapamycin does not affect kinase signaling events that have been shown to stimulate CREB-dependent Per1 transcription (Butcher et al., 2005). To test whether this repression of PER1 protein expression is specifically mediated at the level of mRNA translation, we performed a parallel set of experiments, where the transcription inhibitor actinomycin D (2 µg/µl, 1 µl) was infused before light treatment. Previous work has shown that the infusion of this concentration of actinomycin D effectively inhibits transcription (Daniels D, 1971; Frey et al., 1996). Interestingly, even in the absence of transcriptional activation (i.e., actinomycin D treatment), light still induced moderate PER1 expression in a subset of SCN cells (Figure 3.7A,B). Under this condition, the infusion of rapamycin significantly attenuated light-evoked PER1 expression (Figure 3.7A,B). Together, these data indicate that photic input stimulates PER1 induction via mTOR-mediated mRNA translation.
Figure 3.6. mTOR facilitates early-night light-induced PER1 and PER2 expression in the SCN.

A, C, Representative immunohistochemical labeling for PER1 (A) and PER2 (C) protein expression in the SCN. Cannulated mice were dark adapted for 2 d and then infused (CT14.5) with rapamycin (100 µM) or DMSO vehicle and exposed to light (400 lux, 15 min) at CT15. After light exposure, animals were returned to darkness for 4 h and then killed at CT19. In addition, two "no light" control groups were infused with DMSO or rapamycin at CT14.5 and killed at CT19. Immunolabeling revealed that light (DMSO+Light) evoked an increase in PER1 and PER2 expression relative to the control condition (DMSO). PER1 induction was mainly located in the ventral SCN (A), whereas the increase in PER2 expression was predominantly located in the lateral and dorsal regions of the SCN (C). For each representative section, the boxed area is magnified and shown below. Scale bars, 100 µm. B, D, Quantification of light-induced PER1 (B) and PER2 (D) expression in the SCN. Of note, the light-induced increase in PER1 and PER2 expression was significantly attenuated by rapamycin. Under basal conditions, rapamycin led to a modest but statistically insignificant decrease in PER1 and PER2 protein expression. Numbers in the bars denote the number of animals analyzed for each condition. PER1 and PER2 expression data were normalized to the DMSO infusion condition, which was set to a value of 1.
Figure 3.7. mTOR facilitates light-induced PER1 expression in the SCN in the presence of actinomycin D.

A, Cannulated mice were dark adapted for 2 d and then infused (CT14.5) with actinomycin D (Acti D; 2 µg/µl, 1 µl) and rapamycin (Rapa; 100 µM, 2 µl) or DMSO vehicle and exposed to light (400 lux, 15 min) at CT15. After light exposure, animals were returned to darkness for 4 h and then killed (CT19). In addition, two "no light" control groups were infused with actinomycin D and DMSO or rapamycin at CT14.5 and killed at CT19. Immunolabeling revealed that in the presence of actinomycin D, light (Acti D+Light) evoked a moderate increase of PER1 expression relative to the control condition (Acti D). Infusion of rapamycin attenuated the light-evoked increase (Acti D+Rapa+Light) in PER1 expression. For each representative section, the boxed area is magnified and shown below. Scale bars, 100 µm. B, Quantification of the data set depicted in A. Of note, the light-evoked, actinomycin D-insensitive increase in PER1 expression was significantly attenuated by rapamycin. Numbers on the bars denote the number of animals analyzed for each condition. The PER1 expression data were normalized to the actinomycin/no light infusion condition, which was set to a value of 1. See Materials and Methods for a detailed description of the quantitation methods.

Finally, similar to the early-night data, infusion of rapamycin 30 min before a late night (CT20) light pulse (400 lux, 15 min) triggered a significant reduction in light-induced PER1 expression relative to vehicle-infused animals (Figure 3.8A,B). Given its limited light inducibility during the late night (Albrecht et al. 1997; Zylka et al., 1998; Yan and Silver, 2002, 2004), PER2 expression was not examined.
Figure 3.8. mTOR facilitates late-night light-induced PER1 expression in the SCN.

A, Representative immunohistochemical labeling for PER1 protein expression. Cannulated animals were dark adapted for 2 d and then infused at CT19.5 with rapamycin (100 µM) or DMSO vehicle. Mice were exposed to light (400 lux, 15 min) at CT20, returned to darkness, and killed 6 h later (CT2). Control mice were infused with DMSO as described above, and killed at CT2. Immunolabeling revealed that light induced a moderate increase in PER1 expression in the SCN (DMSO+Light vs DMSO). The light-evoked increase in PER1 was attenuated by rapamycin infusion (Rapa+Light). The presentation of high-magnification images of a single SCN was necessitated by the relatively modest light-evoked PER1 induction pattern. Scale bar, 100 µm. B, Quantification of PER1 expression. Numbers above the bars denote the number of animals analyzed for each condition. PER1 expression data were normalized to the DMSO infusion condition, which was set to a value of 1. See Materials and Methods for a detailed description of the quantitation methods.

3.3.4 mTOR and 5'-TOP mRNA translation in the SCN

Next, we turned to possible mechanisms by which mTOR affects PERIOD protein expression. Along these lines, the p70 S6K arm of the mTOR pathways has been shown to stimulate the translation of 5'-TOP mRNA transcripts. A subset of these mRNAs encode for translational machinery, including ribosomal proteins and elongation factors (e.g., eEF1A and PABP1) (Jefferies et al., 1994, 1997; Peterson and Schreiber, 1998), and the increased expression of these proteins has been shown to
correlate with enhanced mRNA translation (Giustetto et al., 2003; Huang et al., 2005; Tsokas et al., 2005; Antion et al., 2008). To examine whether light-activation of mTOR results in increased elongation factor expression, we profiled eEF1A protein levels in the SCN. Using an immunohistochemical labeling approach, we detected a marked increase in eEF1A expression after a brief light pulse (400 lux, 15 min) at CT15 (Figure 3.9A,B). Induction was observed within 5 min of light treatment and peaked 2 h after light treatment (Figure 3.9B). Induction appeared be specific to the SCN, since light did not significantly alter eEF1A expression within the piriform cortex (Figure 3.9A,B). To test the idea that eEF1A expression in the SCN is dependent on mTOR, we used the ventricular infusion approach described above to transiently disrupt mTOR signaling. Analysis of mice killed 120 min after light exposure (CT15) revealed that the disruption of mTOR activity completely repressed EF1A expression (Figure 3.9C).

Figure 3.9. Light induces mTOR-dependent eEF1A and JunB expression in the SCN.

A, Representative immunohistochemical images of eEF1A expression in the SCN. Mice were dark adapted for 2 d and then exposed to light (400 lux, 15 min) at CT15. Animals were killed at CT17. Control animals (No Light) were also killed at CT17. Relative to control animals, light exposure triggered a moderate increase (Continued)
(Figure 3.9 Continued) in eEF1A expression in the SCN; the boxed regions of the SCN are magnified and shown to the right. In contrast to the SCN, light did not affect eEF1A expression in the piriform cortex (Cortex). Scale bars: Low magnification, 100 µm; high magnification, 50 µm. B, Quantification of light-induced eEF1A expression in the SCN and piriform cortex (Cortex). Animals were killed 5, 30, 60, 120, and 240 min after light exposure at CT15. Control animals (No Light) were killed at CT15. The eEF1A expression in the SCN increased as a function of time after light exposure (up to 2 h after light). Numbers on the bars denote the number of animals analyzed for each condition. *p < 0.05 versus the "no light" control. eEF1A expression data were normalized to the no light conditions, which were set to a value of 1. C, Rapamycin infusion abolished light-induced eEF1A expression in the SCN. Rapamycin (100 µM) was infused 30 min before light treatment at CT15. Animals were killed 2 h after light exposure. Data are presented as described in B. D, Representative immunohistochemical labeling for JunB protein expression in the SCN. Mice were dark adapted for 2 d and then infused with rapamycin (100 µM) or DMSO vehicle 30 min before light (400 lux, 15 min) exposure at CT15. Animals were killed 0–10 min after termination of the light stimulus. Immunolabeling revealed that the light-induced increase in JunB expression (DMSO+Light) was attenuated by rapamycin (Rapa+Light). Scale bars, 100 µm. E, Quantification of light-induced JunB expression. Numbers above the bars denote the number of animals analyzed for each condition. See Materials and Methods for a detailed presentation of the cell-counting method.

In addition to eEF1A, the mRNA of AP-1 binding protein JunB also belongs to the 5'-TOP mRNA family (Staber et al., 2007; Vesely et al., 2009). Given its marked light-inducibility, we examined whether JunB protein expression is modulated by mTOR. In these experiments, mice were killed 0–10 min after termination of a brief light pulse (400 lux, 15 min) at CT15. The goal of this short time course was to examine rapid mRNA translation (e.g., translation of the mRNA pool that was transcribed before the light pulse), and to minimize the contribution of light-evoked junB transcription to the evoked JunB signal. The data presented in Figure 3.9, D and E, reveal that light-evoked JunB protein expression was significantly repressed by rapamycin. Together, these data indicate that 5'-TOP mRNA expression within the SCN is regulated by light in an mTOR-dependent manner.

Finally, the expression of the immediate early gene c-fos has been shown to be translated via an mTOR-independent mechanism. Along these lines, c-fos mRNA does not belong to the 5'-TOP mRNA family, and it can be translated via a
cap-independent mechanism (Vesely et al., 2009). Thus, to test the specificity of the protein expression findings described above, we examined whether rapamycin infusion affects light-evoked c-Fos protein expression. Using the same infusion and photic stimulation paradigm described above for JunB, we found that c-Fos expression was not significantly altered by disruption of mTOR (Figure 3.10A,B). Together, these data indicate that the mTOR pathway functions as selective regulator of light-evoked protein translation and, in turn, SCN clock entrainment.

![Image of Figure 3.10](image_url)

**Figure 3.10. mTOR does not regulate light-induced c-Fos expression in the SCN.**

A. Representative immunohistochemical labeling for c-Fos protein. Mice were dark-adapted for 2 d and then infused with rapamycin (100 µM) or DMSO vehicle 30 min before light (400 lux, 15 min) at CT15. Animals were killed 0–10 min after termination of the light stimulus. The robust light-evoked increase in c-Fos expression (DMSO+Light) was not affected by rapamycin treatment (Rapa+Light). Framed areas of the SCN are magnified and shown below. Scale bars, 100 µm. B. Quantification of c-Fos protein expression in the SCN. Numbers above the bars denote the number of animals analyzed for each condition. See Materials and Methods for a detailed presentation of the cell-counting method.
3.4 Discussion

Our previous work identified a light-responsive mTOR cascade in the SCN (Cao et al., 2008). In this study, we examined the role of mTOR as a potential regulator of clock entrainment. The results reported here reveal that the mTOR cascade modulates the responsiveness of the clock to light, facilitating early-night phase delays and attenuating late night phase advances. These effects were paralleled by data showing that mTOR alters the capacity of light to couple to PER1 and PER2 expression. The data also show that light-induced 5'-TOP mRNA translation is dependent on mTOR signaling. Together, these results indicate that mTOR-regulated mRNA translation plays a role shaping the phase-specific entraining effects of light.

The mTOR signal transduction pathway is a key effector of translational machinery (e.g., ribosomes and translation factors) biogenesis and mRNA translation (Sarbassov et al., 2005; Wullschleger et al., 2006; Proud, 2007, 2009). Within the CNS, a number of recent studies have revealed a key role for mTOR in neuronal physiology. Central to this are studies showing that mTOR plays an essential role in translational control of long-term synaptic plasticity and memory (Costa-Mattioli et al., 2009; Richter and Klann, 2009). Furthermore, hypothalamic mTOR functions as a cellular energy sensor, which plays a critical role in regulating food intake and energy balance (Cota et al., 2006; Mori et al., 2009). These findings, coupled with our prior work showing that photic stimulation leads to mTOR activation in the SCN, led us to test the contribution of mTOR to clock entrainment.

As a starting point for this examination, we monitored the time course of light-induced mTOR, p70 S6K, and S6 activation. These data indicate that a short light pulse (15 min) led to a rapid (within 5 min) and long-lasting (up to 4 h) activation in mTOR-dependent signaling events. As noted, p70 S6K and S6 have been implicated in regulating the translation of 5'-TOP mRNAs, a subclass of which code for translation factors and ribosomal proteins. The increased expression of translation machinery would, in turn, increase the translational capacity of the cell (Proud, 2007,
To assess the ability of light to elicit the induction of 5'-TOP mRNAs, we monitored the expression of eEF1A, an elongation factor that drives the movement of charged aminoacyl-tRNA to the ribosome A site, a key event in mRNA translation/peptide elongation (Sonenberg, 1993; Proud, 1994). Our data revealed that light induced a rapid (within 5 min) and sustained (>2 h) upregulation of eEF1A in the SCN. This relatively long time course paralleled the profile of light-induced S6 phosphorylation, and thus raises the interesting prospect that light actuates a long-lasting period of enhanced mRNA translation. Rapid inducible expression (within minutes) of eEF1A has also been reported in other model systems (Tsokas et al., 2005). Interestingly, eEF1A mRNA has been shown to be docked to activated synapses, and primed for rapid translation in response to increased neuronal activity (Moon et al., 2008). In addition to eEF1A, junB also belongs to the 5'-TOP mRNA family (Staber et al., 2007; Vesely et al., 2009). Here, we also showed that the mTOR pathway contributes to the well-characterized light induction of JunB expression in the SCN (Kornhauser et al., 1992). The specificity of these findings is supported by work showing that c-Fos protein expression was not affected by rapamycin infusion. Importantly, previous studies have shown that rapamycin did not affect stimulus-induced junB (Staber et al., 2007) or eEF1A gene transcription (Huang et al. 2005; Tsokas et al., 2005). Together, these studies support the idea that rapamycin affects 5'-TOP mRNA expression via a translational-dependent mechanism within the SCN.

As part of this study, we also examined the transmitter events that couple light to mTOR activation. To this end, the infusion of a mixture of ionotopic glutamate receptor and PACAP receptor antagonists led to a marked suppression of light-evoked mTOR activation. This observation indicates that the canonical glutamatergic/PACAPergic photic input pathway drives mTOR activation. Simultaneous activation of glutamate and PACAP receptors appears to synergize at the level of mTOR activation. One possible explanation for this effect is that PACAP augments glutamate receptor-evoked neuronal excitation. Along these lines, recent
work has shown that PACAP receptor activation increases the magnitude of both NMDA and AMPA-evoked currents, and enhances L-type Ca\(^{2+}\) calcium channel transients (Dziema and Obrietan, 2002; Michel et al., 2006). Downstream of glutamate and PACAP, the MAPK pathway appears to be a key intermediate that couples receptor activation to mTOR in the SCN. Along these lines, we recently reported that light triggers the colocalized cellular expression of activated ERK (a component of the MAPK signaling cassette) and p-p70 S6K, and that the in vivo disruption of MAPK signaling blocks light-induced mTOR activity in the SCN (Cao et al., 2008). Thus, these data, coupled with the work presented here, provide another route by which the MAPK pathway affects the clock timing process.

To gain insight into the potential role of mTOR in clock physiology, we used a pharmacological approach (i.e., rapamycin) to acutely disrupt mTOR signaling in the SCN. Rapamycin specifically inhibits mTOR as part of mTORC1, a multiprotein complex that drives mRNA translation via p70 S6K and 4E-BP1 (Sarbassov et al., 2005; Wullschleger et al., 2006; Proud, 2007). This drug delivery method proved to be very effective, completely blocking mTOR signaling in the periventricular region for at least 90 min (data not shown), which is a sufficient period of time to uncouple a 15 min light pulse from mTOR activation. Using a standard Ashoff type 1 light-stimulus paradigm, we found that the early-night phase-delaying effect of light was depressed by rapamycin. In contrast to the effects during the early night, late-night phase advances were significantly lengthened by disruption of mTOR. Of note, in the absence of light, infusion of rapamycin did not significantly affect the free-running rhythm, indicating that transient suppression of mTOR-dependent mRNA translation does not significantly alter the inherent pacemaker capacity of the SCN. Collectively, these data support the idea that protein expression mediated by both inducible transcription and mRNA translation are key components of clock entrainment. In some respects, these data are analogous to work showing that long-lasting forms of LTP are dependent on a bipartite gene transcription and mTOR-dependent mRNA translation process.
A good deal of work has implicated inducible gene expression as a key event in the actuation of clock entrainment. Here, we addressed whether the mTOR pathway may be contributing to this process. For these studies, we focused on Per gene expression, which is thought to be a key event in the light entrainment process (Shigeyoshi et al., 1997; Akiyama et al., 1999; Albrecht et al., 2001). Our data reveal that the infusion of rapamycin led to a significant decrease in early- and late-night light-evoked PER expression, indicating that PER protein synthesis is under the influence of the mTOR pathway. Of note, relative to control conditions, significant PER induction was still detected after rapamycin infusion, thus indicating that Per mRNA translation occurs in the absence of light-actuated mTOR. One possible reason for PER expression in the absence of functional mTOR (within the mTORC1 complex) is the presence of residual 4E-BP1 and p70 S6K activity, which was actuated before the pharmacological inhibition of mTOR. Additionally, it should be noted that mTOR functions as an auxiliary signaling pathway, augmenting constitutive mRNA translation, and thereby allowing the translation capacity of the cell to match increased physiological demand. Thus, in the absence of mTOR activity, the basal translational capacity of SCN neurons may be sufficient to drive moderate levels of light-evoked PER protein expression.

Several issues regarding mTOR regulation of light-evoked PER expression and clock entrainment should be noted. With respect to the early-night time point, the results showing a rapamycin-induced reduction in PERIOD protein expression are consistent with the data showing a reduction in the light-induced phase shift in behavioral rhythms. Consistency is based on work showing that PER proteins (i.e., PER1 and PER2) are state variables of the clock, and as such, attenuated expression would be expected to decrease the light-evoked phase shift (Albrecht et al., 1997; Akiyama et al., 1999). Conversely, at the late-night time point, the rapamycin-induced attenuation of PERIOD expression is not mechanistically consistent with the observed augmentation of the light-induced phase advance. There may be a number of reasons for this apparently discordant result. One potential explanation is that mTOR regulates
the expression of proteins that enhance the phase-advancing effects of light. These effects would likely be independent of the effects on PERIOD proteins, and work in opposition to the decrease in PERIOD expression. Conceivably, late night disruption of mTOR translation could give rise to a transient light-evoked alteration in clock speed, which could result in an amplification of the phase shift. This explanation is based on work showing that clock speed contributes to the amplitude of the light-evoked phase shift (Daan and Pittendrigh, 1976). Clearly, additional work will be required to reveal how mTOR shapes the light-evoked proteomic profile and, in turn, clock entrainment.

In conclusion, these research findings reveal a key role for the mTOR-signaling pathway in regulating the entraining effects of light. These effects appear to be mediated by a rapid and sustained increase mTOR-dependent mRNA translation within the SCN. With these data, we can now add mTOR-dependent translation to the list of cellular signaling events that drive clock entrainment.
Chapter 4. Circadian Regulation of mTOR Signaling in the Mouse SCN

4.1 Introduction

Circadian timing refers to the 24 h rhythms of biochemical, physiological and behavioral processes (Reppert and Weaver, 2002) that are intrinsic to a phylogenetically diverse array of organisms. For mammals, the dominant rhythm is generated by the suprachiasmatic nuclei (SCN), a relatively small brain region (~20,000 neurons/nucleus) located within the ventral hypothalamus (Ukai and Ueda, 2010). The rhythm-generating capacity of the SCN arises from a network of autonomous SCN oscillator neurons, which, through synaptic and paracrine-driven processes, form a coherent and stable oscillator network (Welsh et al., 2010).

At the molecular level, the cellular clock is driven by several interlocking transcriptional/translational feedback loops (Ko and Takahashi, 2006). One major negative feedback loop is formed by the interactive transcription and translation of period (Per) and cryptochrome (Cry) genes. Briefly, CLOCK and BMAL1 form heterodimers and activate transcription of the Per and Cry genes by binding to their E-box enhancers. As the levels of PER proteins increase, they form complexes with CRY, translocate into cell nucleus and associate with CLOCK–BMAL1 heterodimers which in turn leads to transcriptional repression of Per and Cry genes. Clock gene transcriptional inhibition is relieved via the posttranslational-component of the feedback loop. Along these lines, phosphorylated by casein kinase Iε (CKIε) plays a key role in targeting in the PER/CRY complex for ubiquitin-mediated degradation, which relieves transcription repression, thereby allowing a new cycle of clock gene expression to occur (Gallego and Virshup, 2007).

Interestingly, a relatively new piece of this rhythm generating puzzle has begun to emerge: mRNA translation. In support of this concept, several studies have shown that microRNAs, which function as potent negative regulators of mRNA translation,
influence clock timing (Cheng et al., 2007; Shi et al., 2009; Na et al., 2009; Nagel et al., 2009). Likewise, the rhythmically-expressed gene nocturnin regulates mRNA stability through the deadenylation of the mRNA polyA tail. The RNA-binding protein LARK regulates period gene expression and clock function in Drosophila melanogaster (Kojima et al., 2007). Of particular relevance here, recent work in Drosophila has indicated a role for the mammalian target of rapamycin (mTOR) in clock physiology.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that is inhibited by the antifungal metabolite rapamycin (Hay and Sonenberg, 2004). mTOR is a phylogenetically-conserved protein kinase that plays fundamental roles in regulating cell growth and metabolism. It executes its function by forming two distinct multi-protein complexes: the rapamycin-sensitive mTOR Complex 1 (mTORC1) containing Raptor and rapamycin insensitive mTORC2 containing Rictor (Wullschleger et al., 2006). mTOR functioning within mTORC1 regulates translational control by direct phosphorylation of two distinct translation effectors: S6 kinase1 (S6K1, including two isoforms, p70 S6K and p85 S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Along these lines, S6K regulates the phosphorylation-dependent activation of a number of proteins involved in regulation of mRNA translation and processing, including ribosomal protein S6 (Ser240/244), eIF4B (Ser322), eEF2K (Ser366) and Pdcd4 (Ser67) (Proud CG, 2009).

Our recent work has identified a light-regulated mTOR signal cascade in the SCN and that mTOR signaling couples light to PER1 expression (Cao et al., 2008, 2010). The robust basal expression (without light induction) of mTOR signaling in the SCN leads us to further characterize its activity as a function of circadian time. Here, we report a robust circadian rhythmicity of mTOR activity in the SCN. Further, we found a linear correlation between circadian *Per1* gene expression and rhythmic mTOR activity at a cellular level. These data connect mTOR signaling to circadian clock gene expression, and as such, raise the prospect to mTOR contributes to clock physiology.
4.2 Materials and methods

4.2.1 Photic entrainment and tissue processing

Initially, adult (8~10-week-old) wild-type C57BL/6 or mPer1-Venus transgenic mice (Cheng et al., 2009) were entrained to a 12h/12 h light/dark (LD) cycle for at least 2 weeks and then transferred to total darkness for two consecutive 24 h cycles. After dark-adaptation, animals were sacrificed under dim red light (Kodak series 2 filter <5 lux at cage level; Eastman Kodak, Rochester, NY) at one of six time points: Circadian time (CT) 0, CT4, CT8, CT12, CT16, or CT20. CTs were calculated based on Zeitgeber time (ZT) and the period length (tau value) of C57BL/6 mice (approximately 23 h 45 min) (Schwartz and Zimmerman, 1990) and mPer1-Venus mice (approximately 23 h 54 min) (Cheng et al., 2009) under free running conditions, with ZT 0 subjective daytime and ZT 12 denoting the beginning of the subjective nighttime. For the light flash experiment, after dark adaptation mPer1-Venus animals received a single light exposure (100 lux, 15 min) at CT 15 and then were sacrificed 4 hr later at CT19. Control mice (no light exposure) were also sacrificed at CT19. Animals were killed via cervical dislocation, and brains were removed under red light (Kodak series 2 filter <10 lux at cage level; Eastman Kodak, Rochester, NY). Brains were then placed in chilled, oxygenated physiological saline, cut into 1.5 mm coronal slices with a vibrotome (OTS 2000; Electron Microscopy Sciences, Fort Washington, PA), fixed in 4 % paraformaldehyde for 6 hr at room temperature and then transferred into 30% sucrose (w/v, with 2 mM sodium azide and 3 mM NaF) overnight at 4 °C. All procedures were in accordance with Ohio State University animal welfare guidelines and approved by the Institutional Animal Care and Use Committee.

4.2.2 Cannulation and infusion

Cannulation and infusion procedures were conducted as previously described (Butcher et al., 2005). Briefly, adult (8~10-week-old) wildtype C57BL/6 mice were
anesthetized and placed in a stereotaxic apparatus (Cartesian Research). The coordinates (posterior, 0.34 mm from bregma; lateral, 0.90 mm from the midline; and dorsoventral, −2.15 mm from bregma) were used to place the tip of a 24-gauge guide cannula into the lateral ventricle. After surgery animals were housed individually and allowed to recover for at least 2 weeks under a standard 12 h/12h L/D cycle. To disrupt the MAPK cascade, 2 µl of 1,4-diamino-2,3-dicyano-1,4-bis o-aminophenylmercapto butadiene (U0126, 10 mM; Calbiochem, La Jolla, CA) was infused 30 min before sacrifice. To inhibit the activity of mTOR, 2 µl of rapamycin (100 µM, Cell Signaling Technology) was infused 30 min before sacrifice. To inhibit the activity of PI3K, 2 µl of LY294002 (30 mM, Calbiochem) was infused 30 min before sacrifice. To inhibit the activity of AKT, 2 µl of AKTi-1/2 (20 mM, Chemdea, Ridgewood, NJ) was infused 30 min before sacrifice. Control animals were infused with an equivalent volume of vehicle (DMSO).

4.2.3 Immunohistochemistry

Coronal brain slices (1.5 mm) containing the SCN were cut into thin sections (40 µm) using a freezing microtome and placed in PBS containing 2 mM sodium azide and 3 mM NaF, pH 7.4. For the immunohistochemical staining, sections were first treated with 0.3 % H₂O₂ and 20 % methanol in PBS for 10 min to deactivate endogenous peroxidases and to permeabilize the tissue. The tissue was then blocked for 1 h in 10% goat serum/PBS and incubated in rabbit anti-phospho-S6 ribosomal protein (pS6, Ser-240/244) (1:1000; Cell Signaling Technology, Danvers, MA), mouse anti-S6 ribosomal protein (1:500; Cell Signaling Technology), rabbit anti-phosphorylated AKT (pAKT, Thr308) (1:1000; Cell Signaling Technology) or rabbit anti-phosphorylated ERK (pERK, Thr-202, Tyr-204) (1:2000; Cell Signaling Technology) antibody overnight at 4°C. Next, tissue was incubated for 1.5 h in biotinylated anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) at room temperature and then placed in an avidin/biotin/HRP complex for 1 h (Vector Laboratories). Sections were washed in PBS (three times, 10 min per wash) between
each labeling step. The signal was visualized using nickel-intensified DAB substrate (Vector Laboratories) and sections were mounted on slides with Permount media (Fisher Scientific, Houston, TX).

For immunofluorescent labeling, tissue was permeabilized with PBST (PBS with 1% Triton X-100) for 30 min, blocked as described above and then incubated (overnight, 4°C) in 5% goat serum/PBS with a combination of two of the following antibodies: mouse anti-pERK (Thr-202, Tyr-204) (1:300; Cell Signaling Technology), rabbit polyclonal anti-pS6 (Ser-240/244) (1:300; Cell Signaling Technology), chicken anti-GFP antibody (1:2,000; Abcam, Cambridge, MA), or guinea pig anti-vasopressin (1:500; Abcam). The following day, sections were incubated (3 h, room temperature) in Alexa Fluor-594-conjugated goat anti-rabbit IgG antibody (1:500; Molecular Probes, Eugene, OR), Alexa Fluor-488-conjugated goat anti-mouse IgG antibody (1:500; Molecular Probes) and/or Alexa Fluor-594-conjugated goat anti-guinea pig IgG antibody (1:500; Molecular Probes). Brain sections were washed in PBS (three times, 10 min per wash) between each labeling step. Sections were mounted on slides with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

Bright-field photomicrographs were captured using a 16 bit digital camera (Micromax YHS 1300; Princeton Instruments, Trenton, NJ) mounted on an inverted Leica microscope (DM IRB; Nussloch, Germany); images were acquired with Metamoph software (Molecular Devices, Sunnyvale CA). Confocal fluorescent images were captured using a Zeiss 510 Meta confocal microscope (Oberkochen, Germany). All confocal parameters (pinhole, contrast, brightness, etc.) were held constant for all data sets from the same experiment.

4.2.4 Materials

Unless otherwise indicated, all reagents were obtained from Sigma.
4.2.5 Data analysis

All photomicrographic data sets were statistically analyzed using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA). For the pS6 and pERK intensity analysis, SCN were digitally outlined and the mean pixel values determined. Next, a digital oval (150×200 pixels) was placed on the adjacent lateral hypothalamus and this mean value was subtracted from the adjacent SCN signal to provide a normalized SCN intensity value. 3 central SCN sections were used from each animal to generate a mean value. Mean values from different animals were pooled into treatment groups and compared by one-way ANOVA followed by SNK post-tests. The values are presented as the mean ± standard error of mean (SEM).

For the pS6 and Venus cellular correlation study, an intensity threshold filter was initially applied to eliminate nonspecific background labeling on high-magnification (40X) confocal images of pS6, and then 20 cells with identifiable profiles (based on pS6 staining) were randomly picked from each image and manually outlined. Next the red (for pS6), green (for Venus) and blue (for DRAQ5) densitometry values within an outlined cell were determined. Data from 15~20 cells were pooled and Pearson's correlation analysis was performed between pS6 and Venus data or DRAQ and Venus data. \( P < 0.05 \) was accepted as statistically significant. All statistical analysis was performed using SPSS software (SPSS Inc, Chicago, IL).

4.3 Results

4.3.1 Circadian mTOR activity in the SCN

To test whether mTOR activity is under control of the circadian clock, mice that were maintained on a standard 12 hr light/dark cycle were transferred to total darkness for 2 days, and then sacrificed at 4 hr intervals over a 28 hr period. SCN-containing tissue was then fixed and immunolabeled for the expression of the Ser240/244-phosphorylated form of S6 ribosomal protein (pS6). The rationale for monitoring the phosphorylation state of Ser240/244 is based on work showing that these post-translational modifications are mediated specifically via mTOR-dependent...
p70 S6K kinase activity. Importantly, our previous work showed that microinfusion of the mTOR inhibitor rapamycin into the SCN led to a rapid and potent suppression of S6 phosphorylation. Hence, pS6 immunolabeling serves as an excellent marker of relative mTOR activity. Examination of the labeling pattern revealed a statistically-significant circadian oscillation in pS6 expression (Figure 4.1A). Specifically, relatively little pS6 was detected at the beginning of the subjective day (i.e., circadian time 0: CT 0); however, at the CT 4 time point clear upregulation of kinase phosphorylation was detected (Figure 4.1A). Elevated labeling persisted throughout the subjective day, and then declined, reaching nadir during the late subjective night time points (CT 20-0) (Figure 4.1B). Of note, total S6 level were not altered as a function of circadian time, indicating that the change in pS6 phosphorylation reflected a circadian-regulated change in mTOR/p70 kinase activity but not in the overall expression of S6 (Figure 4.1A and B). A similar pattern of rhythmic pS6 expression (peaking during the subjective day and low during the night) was detected in both rostral and caudal regions of the SCN (Figure 4.1C). Together these data indicate that the activity of the mTOR signaling is under control of the SCN circadian clock.
Figure 4.1. Circadian rhythmic expression of phosphorylated S6 ribosomal protein in the SCN.

Entrained animals were kept in dark for 2d and killed under dim red light every 4 h over a 24 h cycle. **A**, Upper row: representative microscopic images of immunohistochemical staining for phosphorylated S6 ribosomal protein (pS6) in central SCN coronal sections at different circadian times. Lower row: representative images of immune-staining for total S6 ribosomal protein at respective time points. Of note relatively high level of pS6 labeling was observed in the SCN during subjective day at CT 4, 8 and 12, and the relatively low level of pS6 staining was observed during the subjective night (CT 16, CT 20 and CT0). As control, total S6 expression was not significantly changed. **B**, Quantitation of normalized pS6 and S6 expressions in the central SCN over a 24h cycle. Error bars denote standard error of mean. A minimum of four mice were used for each time point. **C**, Circadian variations in pS6 levels were also observed in the rostral and caudal SCN.

As shown in Figure 4.1A, pS6 labeling did not appear to be localized to a distinct neuroanatomical subregion of the SCN, such as the core or shell, thus suggesting that mTOR activation was not specific to distinct peptidergic neuronal populations (e.g., arginine vasopressine [AVP], vasoactive intestinal peptide, calbindin). To test this assertion, SCN tissue isolated at CT12 was double-labeled for pS6 and AVP, a marker of dorsal and lateral SCN neurons, which comprise the shell region of the SCN. As expected, AVP was robustly expressed in dorsal medial regions of central SCN. pS6 was expressed in both dorsal and lateral regions of the SCN, which is consistent with the AVP expression pattern; however, pS6 was also robustly expressed in the ventral SCN (Figure 4.2). Merging the immunofluorescence signals revealed that subsets of only a subset of AVP positive cells expressed pS6, and, within the ventral SCN, the vast majority of pS6 cells were not AVP-positive. Together these data support the idea that pS6 is expressed in a large and functionally diverse pools of SCN neurons.

4.3.2 mTOR oscillation and rhythmic *Per1* expression

From both a neuroanatomical and temporal perspective, the circadian oscillation in pS6 activity in the SCN parallels the rhythm in the core clock protein PERIOD1,
Figure 4.2. Colocalization of pS6 and vasopressin expressions in the SCN.
Animals were entrained, kept in dark for 2 d and killed at CT12. Coronal central SCN sections were double labeled with pS6 and vasopressin antibodies. Upper row: representative confocal microscopic immune-fluorescent images of pS6 (green, left), vasopressin (red, middle) and merged (right) in the SCN. Framed regions a,b and c are magnified to the lower row. Arrows indicate cellular colocalization of the two antigens.

thus raising the possibility that rhythmic mTOR activity is restricted to the pool of SCN neurons that exhibit robust clock gene rhythms.

To begin to address this question, we profiled period1 expression and mTOR activity as a function of circadian time. To monitor rhythms in period1 gene expression, we employed a transgenic mouse line in which the Per1 promoter- drives the expression of the GFP derivative Venus (Cheng et al., 2009). Of note, in these transgenic animals, the Venus coding sequence was modified to allow for rapid protein turnover, and for nuclear localization of the reporter protein, thus allowing for a dynamic signal that closely matches the transcriptional profile of the endogenous gene (Cheng et al., 2009). As shown in Figure 4.3A, representative confocal images of triple fluorescent labeling for pS6, Venus and nuclear marker DRAQ reveal a striking cellular-level colocalization of pS6 and Venus at CT12 and CT20. Of note, cells (based on DRAQ labeling) that do exhibit Venus also express low levels of pS6. These data reveal that there is a coordinate activation of mTOR signaling and period1 gene expression. As expected, Venus was mostly expressed within the cell nuclei while pS6 was expressed within the cytoplasm. To more fully profile the
cellular-level relationship between pS6 and Venus expression, linear correlation analysis between pS6 intensity and Venus expression was examined across the circadian cycle. Interestingly, at all time points, a significant linear correlation was detected between pS6 and Venus expressions ($p<0.05$, Figure 4.3B). As a control, there is no correlation between DRAQ5 and Venus expression. Together these data suggest that the rhythmic actuation of mTOR-regulated translational is tightly linked to expression of the core clock gene period1.

4.3.3 Light-induced Per1 expression and mTOR activation

Our previous work found that mTOR activity augments light-induced PER1 expression in the SCN (Cao et al., 2010). Here we examine whether this relationship can be detected at a cellular level. To this end, Venus and pS6 expression was examined 4h after an early night (CT 15) light pulse (100 lux). Consistent with our previous results (Cheng et al., 2009; Cao et al., 2010), relative to control animals that were not exposed to light, a marked increase in pS6 and Venus expression was detected at 4 hrs after light exposure (Figure 4.4A). Analysis of the confocal images revealed that light-evoked pS6 and Venus expression occurred in the same subpopulation of SCN cells. A more modest correlation was detected in the absence and presence of light. Quantitative cellular level analysis of the control and light-treated groups revealed a significant positive correlation between pS6 and Venus ($p<0.05$, Figure 4.4B). As control, analysis of DRAQ5 and Venus following light treatment revealed that there was no correlation between the two antigens. These data further indicate that mTOR activity is restricted to cells that exhibit a high degree of clock gene expression.
Figure 4.3. Colocalization of pS6 and mPer1-Venus expressions in the SCN over the 24 h cycle.

Entrained animals were kept in dark for 2d and killed under dim red light every 4 h over a 24 h cycle. Coronal sections of central SCN were used for immuno-labeling. A, Representative confocal microscopic images of triple fluorescent labeling for pS6(red), Venus(green) and DRAQ5(blue) at CT12 and CT20. Framed regions on Merged images are magnified to the right. B, Linear correlation of cellular Venus and pS6 expressions at CT4(a), CT8(c), CT12(d), CT16(e) and CT20(f). The Y-axis denotes normalized fluorescent intensity (0–255 scale) of Venus within cells and X-axis denotes normalized fluorescent intensity of pS6. As control the relation between Venus(Y-axis) and DRAQ5(X-axis) expressions at CT4 is shown in B,. The significance (p value) for Pearson's correlation analysis was shown in each figure.

Figure 4.4. Colocalization of light-induced pS6 and mPer1-Venus expressions in the SCN.

Entrained animals were kept in dark for 2d and exposed to a light pulse (100 lux, 15 min) at CT 15 and killed under dim red light at CT19. Coronal brain sections with central SCN were used for immuno-labeling. A, Representative confocal microscopic images of triple fluorescent labeling for pS6 (red), Venus(green) and DRAQ5(blue) of “no light” and “light” groups. Framed regions on Merged images are magnified to the right. B, Linear correlation of cellular Venus and pS6 expressions without light(a), or after light(c). The Y-axis denotes normalized fluorescent intensity (0–255 scale) of Venus within cells and X-axis denotes normalized fluorescent intensity of pS6. As control the correlation between Venus (Y-axis) and DRAQ5(X-axis) expressions after light is shown in (b). The significance (p value) for Pearson's correlation analysis was
shown in each figure.

4.3.4 P42/44 MAPK activity and mTOR oscillation

Next, we turned to an examination of potential upstream regulatory pathways with drive the circadian oscillation in mTOR activity. Given their well-recognized role as regulators of mTOR, two pathways were of particular interest: the p42/44 ERK/mitogen-activated protein kinase (ERK/MAPK) pathway and the PI3 kinase/AKT pathway. Initially, emphasis was placed on the MAPK pathway, since we previously reported that activation this pathway exhibits robust circadian oscillation with a similar temporal profile to that of mTOR activity (Obrietan et al., 1998), and that the MAPK pathway couples light to mTOR in the SCN (Cao et al., 2008). To examine this question, we first profiled the circadian regulation of ERK activation by using an antibody that detects the dual phosphorylated form of threonine 202 and tyrosine 204 (here referred to as pERK), a marker of MAPK activation in the SCN. Examination of pERK expression over a circadian cycle revealed that pERK has a similar pattern as pS6 expression: high during the day and low during the night (Figure 4.5A).
Figure 4.5. Circadian p42/44 MAPK couples clock to mTOR activation.

Entrained animals were kept in dark for 2d and killed under dim red light every 4 h over a 24 h cycle. **A**, Representative microscopic images of immunohistochemical staining indicating a circadian rhythmic pERK expression of pERK in the SCN. **B**, Quantitation of circadian pERK expression in the SCN. **C**, Representative confocal images of double fluorescent labeling for pERK(green) and pS6(red) at CT 8 and CT20. Framed regions are magnified to the bottom. **D**, Infusion of U0126 and rapamycin inhibited circadian pS6 expression in the SCN. U0126 (10 mM, 2 µl), rapamycin (100 µM, 2 µl) or DMSO (2 µl) was infused into the lateral ventricle 30 min before CT8 or CT16 and animals were killed at CT8 and CT16, respectively. Representative microscopic images of immunohistochemical labeling for pERK and pS6 are shown in D and quantitative analysis is shown in Figure 4.6B. Of note, infusion of U0126 markedly blocked pERK expression in the SCN and significantly attenuated pS6 expression at both day and night time points. Rapamycin significantly blocked pS6 expression but not pERK expression at CT8.

To examine a potential cellular colocalization of ERK and mTOR signaling, tissue from animals sacrificed at CT8 and CT20 was double labeled of pERK and pS6. At CT8 there was extensive colocalization of pERK and pS6 expression in the ventral lateral region of SCN, while at CT20 (a time point where low levels of pERK are observed) significant colocalization was not detected (Figure 4.5B). Next, we employed a pharmacological approach to acutely disrupt ERK activation and assess the effects on S6 phosphorylation. MEK inhibitor U0126 (10 mM, 2µl) was infused into the lateral ventricle 30 min before sacrificing the animals. As expected, ERK phosphorylation was suppressed by U0126 within the SCN region. Further, we found that pS6 expression was moderately, yet significantly, decreased (Figure 4.5C and D) by U0126 infusion. These data suggest that circadian mTOR signaling activity is modulated by the ERK/MAPK pathway. As a control, animals were infused with the mTOR inhibitor rapamycin (100 µM, 2µl) at CT8. As expected, rapamycin significantly inhibited pS6 expression (Figure 4.5C and D). Our previous data have shown that rapamycin significantly inhibits pS6 expression during night (Cao et al.,2008). Together these data prove that pS6 expression at both day and night time points are mTOR-dependent and mTOR activity is coupled to circadian
ERK MAPK activation.

We also looked at another signaling pathway, the PI3K/AKT pathway and its potential coupling with mTOR signaling in the SCN. First we tested expression of phosphorylated form of AKT at Ser308. AKT is activated by phospholipid binding and phosphorylation at Thr308. So this site can be used as an indicator of AKT activity. Interestingly, only sparse staining of pAKT was found in the SCN (Figure 4.6A). Also light-induced AKT phosphorylation was not detected in the SCN

![Figure 4.6. Circadian p42/44 MAPK but not PI3K/AKT couples clock to mTOR activation.](image)

Entrained animals were kept in dark for 2d. U0126 (10mM,2µl), Rapamycin(100µM,2µl ), LY294002 (30 mM, 2 µl), Akti-1/2(20 mM, 2 µl) or DMSO (2 µl) was infused into the lateral ventricle 30 min before CT8 or CT16 and animals were killed under dim red light at CT8 and CT16, respectively. Representative microscopic images of immunohistochemical labeling for pAKT and pS6 are shown in A and quantitative analysis is shown in B. Infusion of U0126 and LY294002 but not Akti-1/2 significantly inhibited circadian pS6 expression in the SCN. Of note, infusion of LY294002 markedly blocked both pAKT and pS6 expression(Continued)
(unpublished observations). These data indicate minimal level of AKT activity in the SCN. Next, we used a PI3K/mTOR dual inhibitor LY294002 infusion to the SCN. As expected, both AKT phosphorylation and S6 phosphorylation were inhibited, suggesting AKT phosphorylation is dependent on PI3K activity (Figure 4.6A). To test whether AKT signaling mediates basal mTOR activation, 20 mM AKT inhibitor AKTi-1,2 was infused to the SCN. This concentration has been used in other models to completely inhibit mTOR activity by specifically blocking AKT activity (Logie et al., 2007). Notably, neither AKT nor S6 phosphorylation was affected by AKTi-1/2, suggesting AKT signaling doesn’t mediate basal mTOR activity in the SCN (Figure 4.6A and B).

4.4 Discussion

We have previously identified a light-responsive mTOR signaling cascade in the SCN that modulates clock entrainment. As a logical extension of these studies, we were curious about whether mTOR signaling might also under the control of the core clock timing process in the SCN. Here we characterized the 24 h temporal profile of mTOR signaling expression in the SCN. Of note, this study reports that 1) mTOR activity exhibits marked circadian oscillation, 2) rhythmicity is not limited to subregions of the SCN, but rather is broadly distributed, 3) at a cellular level mTOR rhythms are highly correlated with rhythms of the circadian clock gene Per1 and 4) rhythmic mTOR activity is influenced by the p42/44 MAPK pathway. Together these data raise the prospect that inducible mRNA translation contributes to circadian clock physiology.

Circadian profiling revealed that mTOR activity peaked during the subjective day, and reached its nadir during the late subjective night. Given work in other model systems showing that mTOR signaling is a linker between cellular energy
metabolism and neuronal excitability (Potter et al., 2010), this daily activity profile parallels the rhythmic regulation of SCN cellular physiology. Along these lines, SCN neuronal activity and glucose metabolism, peak during the middle of the subjective day (Schwartz and Gainer, 1977; Shibata and Moore, 1988). Hence, the parallel profile of these data sets raises the possibility that elevated mTOR activity during the daytime is required to meet the physiological demands of the elevated level of metabolic and firing activities of SCN cells. In some senses, this may be similar to the recent work from the arcuate nucleus, which showed that mTOR activity is directly and tightly regulated by energy intake (Cota et al., 2006).

Of note, even during the middle of the subjective day (when maximal mTOR activity was detected) only a subset of SCN neurons exhibited marked S6 phosphorylation. In some respects, this contrasts with the robust rhythmic activity that is found throughout the SCN. Along these lines, Nakamura et al. (2003) showed that circadian rhythmicity, as assessed by the examination of neuronal firing properties, is detected in ~76% of SCN neurons. Hence, if mTOR activity was simply a reflection of elevated metabolic demand, a larger percentage of SCN cells should have been immunoreactive for pS6.

Another, related, possibility is that mTOR activity is restricted to cells that exhibit robust transcriptional rhythms. As described in the Introduction, the molecular underpinning of a cellular-level 24-hr oscillator is a transcription/translation feedback loop that is centered on the rhythmic expression of Per and Cry genes. Both Per1 and Per2 exhibit a marked increase in mRNA expression that is coincident with the beginning of the subjective daytime (Shearman L et al., 1997). However, at a neuroanatomical level Per1 and Per2 appears to be quite distinct. For example, during the middle of the subjective day, Per1 is detected throughout the entirety of the SCN, whereas Per2 expression is largely localized to the dorsal, medial and lateral regions of the SCN, with only limited expression in the SCN “core” (Takumi et al., 1998; Yan and Okamura, 2002; Yan and Silver, 2004; Cheng et al., 2009). These data suggested that mTOR activity may simply reflect transcriptional rhythms, but rather
may be associated with a particular subset of SCN neurons. Interestingly, over the circadian cycle, the anatomical expression pattern of pS6 appeared to closely approximate the expression pattern of *Per1*, thus raising the possibility robust *Per1* and mTOR activity oscillations are occurring in the same cell populations. We pursued this question by examining the composite profile S6 phosphorylation and *Per1* transcriptional rhythms. With the use of a *Per1*-Venus transgenic mouse strain that nicely recapitulates the temporal and spatial pattern of endogenous *Per1* expression, we showed that there is a statistically significant cellular-level correlation between pS6 and period1-mediated Venus expression, thus indicating that high-levels of clock-regulated mTOR activity occurs in SCN oscillator cell populations. An obvious extension of this line of inquiry was to perform a parallel analysis of pS6 and *Per2* expressing SCN neurons. Intriguingly, in contrast with the *per1* pS6 data, mTOR activity was not correlated with *Per2* expression. Further, an analysis of clock-regulated c-Fos expression (Guido et al., 1999) revealed that pS6 was not colocalized with expression of the immediate early gene. If mTOR were simply responding to a circadian actuated increase in cellular activity, then one would expect strong colocalization of pS6 with *per2* and Fos. Rather, the lack of a correlation indicates that the circadian clock limits mTOR activity to a subset of cells that exhibit robust transcriptional drive for the clock gene *Per1*. Interestingly, *Per1*-expressing SCN neurons appear to be a distinct subclass of oscillator cells. Work by Belle et al. (2009) showed that *Per1* neurons exhibit markedly altered electrophysiological characteristics, relative *Per1*-negative SCN oscillator neurons. On its own, the distinct 24-hr electrophysiological signature of Per-1 neurons would likely not likely drive high levels of mTOR signaling. Rather, enhanced mTOR activity may be a reflection of the unique phenotype of *per1*-expressing SCN cells. As further support for the unique relationship between mTOR and period 1, we provide data showing that photic stimulation lead to a concordant, cellular-level, upregulation of both pS6 and Venus.

Interestingly our previous work showed that abrogation of mTOR signaling via rapamycin infusion decreased light-evoked PERIOD expression (Cao et al., 2010),
but that basal (i.e., clock-regulated) PER1 expression was not affected. This reported lack of an effect should be viewed with caution. Of note, the paradigm used in the prior study was designed to specifically test the effects of light-evoked mTOR. Further, the rapamycin infusion paradigm led to a transient (~ 90 min) repression of mTOR, which may not be sufficient time to reveal an effect of mTOR on mPER1 expression. Finally, S6 maintains activity for an extended period, following transient mTOR and p70 S6K activity, thus raising the possibility that long-term suppression of mTOR (which in turn would suppress S6) would be required to detect an effect on rhythmic PER1 expression or circadian clock function. Interestingly, a recent study in Drosophila (Zheng and Sehgal, 2010) found alteration of TOR activity by genetic approach affects circadian period. Although the effect of TOR on Per expression was not examined, the results indicate a critical role of TOR signaling in regulating circadian oscillation.

To gain insights into the upstream signaling cascade that couples clock to mTOR signaling, we looked at the p42/44 MAPK signaling and the PI3K/AKT pathways. The rationale is that our previous work has shown a circadian oscillation of this signaling in the SCN (Obrietan et al., 1998) and that this signaling couples light to mTOR activation during the photic entrainment of the clock (Cao et al., 2008). Further, recent studies from other groups have shown that pharmacological inhibition of MAPK signaling significantly affected the robustness of circadian clock gene expression and SCN neuron firing (Akashi et al., 2008). These data support a role of MAPK in autonomous clock function. As a potential downstream signaling of MAPK, the temporal expression pattern of pS6 is similar to that of pERK in the SCN. Double labeling revealed colocalization of pS6 and pERK at CT8 but not CT20. The failure to see a clear colocalization at CT20 may be due to the low level of pERK expression at that time. By using MEK inhibitor U0126, we found pS6 expression in the SCN was significantly inhibited at both time points. These results suggest that ERK/MAPK couples clock to mTOR signaling. However, there was still considerable pS6 expression in the presence of U0126.
As PI3K/AKT signaling is another canonical upstream pathway of mTOR signaling, we were interested to see if this signal is also involved in mediating circadian mTOR activity in the SCN. To address this question, we first looked at AKT phosphorylation in the SCN. Notably, pAKT was expressed at a very low basal level in the SCN, compared to its surrounding brain regions. Light stimulation couldn’t induce further AKT activation. Then we used a PI3K/AKT inhibitor LY294002 to the SCN. Interestingly, LY294002 significantly decreased basal pS6 expression both at day and at night time points. As LY294002 can directly inhibit mTOR activity (independent of PI3K/AKT), we used a specific AKT inhibitor AKTi-1/2 to evaluate the role of AKT on mTOR activation. AKTi-1/2 didn’t have effects on S6 phosphorylation. Together, these data suggest PI3K-AKT pathway is not involved in basal mTOR activation in the SCN.

It still remains to be determined, what other signaling pathway mediates mTOR activation, besides ERK MAPK, since there is still considerable pS6 expression in the presence of U0126. This may be because mTOR signaling can be activated by multiple intracellular signals such as energy status, nutrient and stress, etc (Hay and Sonenberg, 2004; Wullschleger et al., 2006), and/or because S6 maintains activity for an extended period in the SCN. Under in vivo conditions it is impossible to control all the upstream signals that potentially can lead to mTOR activation. However, we were able to decrease mTOR activity to the minimal level in SCN neurons by putting neurons in ACSF for several hours (unpublished observation).
Chapter 5. Mitogen and Stress-Activated Protein Kinase Modulates Photic Entrainment of the Suprachiasmatic Circadian Clock

5.1 Introduction

The master circadian clock in mammals, localized in the suprachiasmatic nucleus (SCN), is continuously adjusted (entrained) by signals from the external environment (Reppert and Weaver, 2002). Thus, light signal received from the retina is transmitted via retinohypothalamic tract (RHT) to the SCN cells. At the RHT terminal, synaptic release of neurotransmitters leads to activation of multiple intracellular signal transduction pathways, which in turn transduce extracellular signals into the cell nucleus and regulate expression of clock genes, e.g. Period (Per) (Golombek and Rosenstein, 2010). The clock is intrinsically driven by several interlocking transcriptional/translational feedback loops. The major negative feedback loop is formed by the interactive transcription and translation of Per genes. Briefly, CLOCK and BMAL1 form heterodimers and activate transcription of the Per, Cry and Rev-Erbα genes by binding to E-box enhancers. As the levels of PERIOD (PER) proteins increase, they form complexes with CRY and CKIα/CKIβ proteins, translocate into cell nucleus and associate with CLOCK–BMAL1 heterodimers to shut down their own transcriptions (Ko and Takahashi, 2006). Light-induced rapid upregulation of Per genes leads to reconstruction of the dynamic molecular feedback loop and this process is behaviorally manifested as phase shift.

There has been significant interest in unraveling the intracellular signaling processes that couple light to clock gene expression. Per gene expression is driven by E-Box elements that respond to BMAL-CLOCK (King et al., 1997; Gekakis et al., 1998; Darlington et al., 1998; Sangoram et al., 1998; Shearman, et al., 2000), as well as CRE elements in its promoter region (Travnickova-Bendova et al., 2002; Tischkau et
Light at night induces rapid CREB phosphorylation and CRE-dependent clock gene transcription (Ginty et al., 1993; Obrietan et al., 1999). Several signaling pathways have been implicated in mediating photic induction of CREB phosphorylation in the SCN. For example, CaMKII inhibition blocks light-induced CREB phosphorylation (Golombek and Ralph, et al., 1995). Elevated cytosolic Ca$^{2+}$ has been shown to trigger CREB phosphorylation through a nitric oxide dependent mechanism (Ding et al., 1997). Our lab and other groups have identified the p42/44 MAPK pathway as a major regulator of photic entrainment of the clock (Obrietan et al., 1998; Coogan and Piggins, 2003). Along these lines, light activates ERK in the SCN in a phase-specific manner (Obrietan et al., 1998). Activated ERK mediates light-induced immediate early gene (IMG) expression and CREB phosphorylation in the SCN (Obrietan et al., 1999; Dziema et al., 2003). At the behavioral level, disruption of ERK activation uncouples light from the circadian phase shift in animals (Butcher et al., 2002; Coogan and Piggins, 2003; Cheng et al., 2006).

The ERK MAPK signaling cascade involves multiple downstream effectors which can regulate clock gene expression at different levels. At the transcriptional level, ERK activates protein kinases that may regulate CREB activity by phosphorylation, including RSK (Anjum and Blenis, 2008) and MSK (Deak et al., 1998; Arthur and Cohen, 2000; Wiggin et al., 2002; Arthur et al., 2004; Vermeulen et al., 2009). In the SCN, MSK1 is activated through PACAP/ERK pathway. Activated MSK1 can stimulate Per gene expression in vitro (Butcher et al., 2005). However, the role of MSK in circadian clock function has not been examined in animals. In this study, we utilized MSK null mouse lines to address this question. We find that the entrainment of clock by light is undermined in MSK null mice. Also in these mice phase-shifting response at night was decreased and the capacity to be entrained to new light cycle was compromised, although circadian rhythm is conserved under free running conditions. At the molecular level, light-induced IMG c-Fos expression and CREB phosphorylation were significantly attenuated in these mice. As a result, light-induced $Per1$ expression was decreased.
5.2 Materials and methods

5.2.1 Animals

The Msk1 and Msk2 knockout animals were obtained from Dr. J. Simon C. Arthur (University of Dundee, Scotland) as generous gifts. Generation of these animals has been described elsewhere (Wiggin et al., 2002). Msk1<sup>−/−</sup> mice were crossed to Msk2<sup>−/−</sup> mice to generate Msk1<sup>−/−</sup>,2<sup>−/−</sup> animals. The genotypes of pups were confirmed by PCR-based genotyping methods with tail biopsy samples. For Msk1 knockout genotyping, the following three primers were used: primers CACTTCGCCCAATAGCAGCCAGTCCCTTCC (targeted), TCCGCAGCTCGTGTGACAGTAAGGAGC (wild type), and AATAGCGCTGGTGCTACGGCTCAGGCTGT (targeted or wild type), which give a fragment of 870 bp from a targeted gene (for Msk1 knockout) and 350 bp from a wild-type gene. Genotyping for Msk2 knockout mice was carried out with primers CGTTGGCTACCCGTAATATTGCTGAAGAGC (targeted), AAGATCTCTCCAGGGCATCTCTTTATCCTACG (wild type), and TTGTGCTCCCCATGCTGCAGCCCGGCCTTC (targeted or wild type), which give a fragment of 1,030 bp for a wild-type gene and 600 bp for a targeted gene (for Msk2 knockout). The PCR program consisted of a hot start at 94°C, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min.

5.2.2 Behavioral rhythm recording

For wheel-running recording, adult (8~10-week-old) male Msk null mice or wild-type littermates were individually housed in polycarbonate cages equipped with running wheels. Wheel rotation was detected via the closure of a magnetic switch and recorded using ActiView software (MiniMitter, Bend, Oregon). For core body temperature recording, adult (8~10-week-old) male Msk1<sup>−/−</sup>,2<sup>−/−</sup> animals were anesthetized with ketamine hydrochloride (91 mg/ml) and xylazine (9 mg/ml) and implanted with transmitters (E-Mitters; MiniMitter) in the intraperitoneal cavity, as
previously described (Cao et al., 2010). After surgery, animals were housed singly in cages placed on receiving platforms (ER-4000 receiver) that communicated with the E-Mitter: data were collected every 60 s. The signal was acquired via the VitalView software program (MiniMitter) and analyzed via the ActiView software program (MiniMitter). To clearly visualize the circadian rhythm of body temperature in an actograph format, a threshold was set to 2–2.5°C below the maximum recorded temperature during the entire experimental period, and only signals above this threshold temperature were displayed.

For the light flash experiment, animals were entrained to a 12 h Light/Dark (LD) cycle (100 lx) for 14 d and transferred to continuous darkness (DD). Short white light treatment (100 lx, 15 min) was applied at CT15 and CT22. Mice were then returned to their home cages for at least 10 d to record their post-light wheel-running or core body temperature rhythms. For the phase-shifting experiment, animals were put in DD or 12h LD (7 lx) cycle for ~14 d. Then the lightening period was abruptly advanced for 8 h. 14–21 d later the light cycle was shifted back to the original one (delayed for 8 h).

5.2.3 Assessment of circadian phenotypic characteristics

The free-running circadian period (τ) and overall activity were determined with ActiView software. Variance in activity onset was defined as number of days that the actual activity onset time is largely off (>15 min) the regression line. The linear regression method described by Daan and Pittendrigh (1976) was used to assess light-induced phase shifts. Specifically, the difference in activity onset before and after the day of light exposure was determined by a least-squares method. Thus, a line calculating the activity onset for a period of at least 6 d preceding light treatment was calculated. This line was extended to project to when activity onset should occur during the period after light exposure. A second regression line was generated to determine activity onset after light administration. Wheel running or body temperature actogram 3–10 d after light treatment was used to generate this line.
The difference in the projected versus the actual activity onset after light treatment was the phase shift. Values are expressed as mean phase shift ± SEM. Significance was assessed using one-way ANOVA analysis followed by the Student–Newman–Keuls (SNK) test. A value of $p < 0.05$ was accepted as statistically significant.

5.2.4 Brain tissue processing

Mice were entrained and transferred to total darkness for two consecutive 24 h cycles. After dark adaptation, animals received a single light exposure (100 lux, 15 min) at CT 15 and were sacrificed under dim red light (Kodak series 2 filter ≈5 lux at cage level; Eastman Kodak, Rochester, NY) immediately or 4 hr later at CT19. Brains were harvested, cut into 1.5 mm coronal slices with a vibrotome (OTS 2000; Electron Microscopy Sciences, Fort Washington, PA), fixed in 4 % paraformaldehyde for 6 hr at room temperature and then transferred into 30% sucrose (w/v, with 2 mM sodium azide and 3 mM NaF) overnight at 4 °C. All procedures were in accordance with Ohio State University animal welfare guidelines and approved by the Institutional Animal Care and Use Committee.

5.2.5 Immunohistochemistry

Coronal brain slices (1.5 mm) containing the SCN were cut into thin sections (40 µm) using a freezing microtome and placed in PBS containing 2 mM sodium azide and 3 mM NaF, pH 7.4. For the immunohistochemical staining, sections were first treated with 0.3 % H$_2$O$_2$ and 20 % methanol in PBS for 10 min to deactivate endogenous peroxidases and permeabilize the tissue and then blocked for 1 h in 10% goat serum/PBS and incubated in one of the following antibodies: rabbit anti-phospho-MSK1 (Ser-360, 1:1000; Cell Signaling Technology, Danvers, MA), rabbit anti-MSK1 (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal phospho-CREB (Ser-133) (1:1000; Cell Signaling Technology), rabbit anti-phosphorylated ERK (pERK, Thr-202, Tyr-204) (1:2000; Cell Signaling
Technology); rabbit polyclonal anti-c-Fos (1:3000; Calbiochem, Gibbstown, NJ) overnight at 4°C. Next, tissue was incubated for 1.5 h in biotinylated anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) at room temperature and then placed in an avidin/biotin/HRP complex for 1 h (Vector Laboratories). Sections were washed in PBS (three times, 10 min per wash) between each labeling step. The signal was visualized using nickel-intensified DAB substrate (Vector Laboratories) and sections were mounted on slides with Permount media (Fisher Scientific, Houston, TX).

For immunofluorescent labeling, tissue was permeabilized with PBST (PBS with 1% Triton X-100) for 30 min, blocked as described above and then incubated (overnight, 4°C) in 5% goat serum/PBS with Chicken GFP antibody (staining for Venus, 1:2000; Abcam, Cambridge, MA). The following day, sections were incubated (3 h, room temperature) in Alexa Fluor-488-conjugated goat anti-chicken IgG antibody (1:500; Molecular Probes, Eugene, OR). Brain sections were washed in PBS between each labeling step. Sections were mounted on slides with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

Bright-field photomicrographs were captured using a 16 bit digital camera (Micromax YHS 1300; Princeton Instruments, Trenton, NJ) mounted on an inverted Leica microscope (DM IRB; Nussloch, Germany). Images were acquired with Metamoph software (Molecular Devices, Sunnyvale, CA). Confocal fluorescent images were captured using a Zeiss 510 Meta confocal microscope (Oberkochen, Germany). All confocal parameters (pinhole, contrast, brightness, etc.) were held constant for all data sets from the same experiment.

5.2.6 Materials

Unless otherwise indicated, all reagents were obtained from Sigma.

5.2.7 Densitometry data analysis

All photomicrographic data sets were statistically analyzed using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA). For the
c-Fos, pERK, pCREB and Venus intensity analysis, ventrallateral SCN were digitally outlined and the mean pixel values were determined. Next, a digital oval (150×200 pixels) was placed on the adjacent lateral hypothalamus and this mean value was subtracted from the adjacent SCN signal to provide a normalized SCN intensity value. Three central SCN sections were used from each animal to generate a mean value. Mean data from different animals were pooled into treatment groups and compared by one-way ANOVA followed by SNK post-tests. The values are presented as the mean ± standard error of mean (SEM). $P < 0.05$ was accepted as statistically significant. All statistical analysis was performed using SPSS software (SPSS Inc, Chicago, IL).

5.3 Results

5.3.1 Genotyping for Msk knockout animals

The genotypes of mice were confirmed by PCR-based genotyping methods with tail biopsy samples. PCR were done separately for detecting $Msk1$ knockout and $Msk2$ knockout. As shown in Figure 5.1 A, for $Msk1$ PCR reaction, DNA samples from homozygous knockout animals generate one band of 870 bp (lane 2 and 5), heterozygous generate two band of 870bp and 350bp (lane 1 and 7), while wild-type generate one band of 350bp (lane 6). For Msk2 PCR, DNA samples from homozygous knockout animals generate one band of 1030 bp (lane 4) while heterozygous generate two bands of 1030bp and 600bp (lane 3). Only homozygous $Msk1$ or $Msk1, 2$ knockouts were used in the study. These mice were viable and fertile and had no obvious health problems. To confirm MSK1 expression was eliminated in $Msk1$ knockout mice, immunohistochemical staining was done to detect MSK1 expression in the SCN and piriform cortex of these animals. As shown in Figure 5.1B, compared to wild-type litter mates, MSK1 expression was completely abrogated in these brain regions. Further, light-induced phospho-MSK1 (Ser360) expression in the SCN was also abolished in $Msk1$ knockout animals (Figure 5.1C).
Figure 5.1. Genotyping and MSK1 expression in the Msk knockout animals

A. Genotypes of Msk knockout animals. Images show agarose gel electrophoresis results of PCR products from DNA samples of Msk1 or Msk2 knockout animals. For Msk1 PCR, a fragment of 870 bp indicates inclusion of the targeted gene (for gene knockout) and 350 bp indicates a wild-type gene. For Msk2 PCR, a fragment of 1,030 bp indicates inclusion of a wild-type gene and 600 bp indicates a targeted gene (for gene knockout). One band indicates homozygous genotype while two bands indicate heterozygous genotype. Only homozygous Msk knockout or wild-type animals were used in the study. B. MSK1 expression was completely eliminated in Msk1-/- animals. Representative microscopic images of immunohistochemical staining show MSK1 expression in suprachiasmatic nuclei (SCN) and piriform cortex (CTX) of wild-type (WT, top) and Msk1-/- animals (bottom). C. Light-induced phospho-MSK1 (Ser360) expression was abolished in Msk1-/- animals. Animals were entrained, put in dark for 48 hr and given a light flash (100 lx, 15 min) at circadian time (CT)15. Microscopic images of immunohistochemical staining show light-induced phospho-MSK1(Ser360) expression in the suprachiasmatic nuclei(SCN) of wild-type(WT) animals (top). In the Msk1-/- animals, light didn’t induce phospho-MSK1 expression.

5.3.2 Circadian phenotype of Msk knockout animals

To characterize potential circadian phenotype of Msk1-/- animals, we first tested light-induced phase delaying response in these mice. For this purpose animals were entrained in 12h LD cycle for 10 d and put in DD for 8 d. A light flash (100 lx, 15 min) was applied at CT15. As expected, both Msk1-/- and wild-type animal were entrained to the 12LD cycle and free-ran in DD. There was no difference in overall wheel-running activity in LD (Table1, Parameter 4) between Msk1-/- and their
wild-type littermates. However, *Msk1*<sup>−/−</sup> showed significant decrease of wheel-running activity in DD (Table 1, Parameter 5). The period length “tau” value of the *Msk1*<sup>−/−</sup> mice was slightly larger than that of the wild-type littermates, but didn’t reach a statistical significance (Table 1, Parameter 1).

Notably, both in LD and DD, *Msk1*<sup>−/−</sup> mice showed larger variance in activity onset, as indicated by larger number of days when activity onset was “off” the regression line (Table 1, Parameter 2 and 3). After a short light exposure at CT15, animals showed a marked phase delay. Compared to the wild-type litter mates, the light-induced phase delay was significantly decreased in *Msk1*<sup>−/−</sup> mice (Figure 2A and Table 1, Parameter 9). However, we did not find significant differences in light-induced phase advances at CT22 (Table 1, Parameter 10). When animals were re-entrained to LD cycle from free-running DD condition, the *Msk1*<sup>−/−</sup> animals required significantly longer time to adapt to the LD cycle (Figure 2B and Table 1, Parameter 6). Next, after animals are entrained to the LD cycle for 10 d, the LD cycle was abruptly advanced for 8 h. As expected, animals were entrained to the new LD cycle. Remarkably, the entraining processes to a new LD cycle were drastically different in *Msk1*<sup>−/−</sup> and wild-type mice. *Msk1*<sup>−/−</sup> mice exhibited more gradual shift of wheel-running phase to the new dark period while wild-type animals adapted to the new cycle more rapidly (Figure 5.2C). The number of days required to be entrained to the advancing new cycle was significantly larger in *Msk1*<sup>−/−</sup> mice than in wild-type littermates (Table 1, Parameter 7). After ~14 d in the new cycle, the LD cycle was shifted back to the original by delaying 8 h. Again, *Msk1*<sup>−/−</sup> mice showed slow entrainment and required significantly larger number of days to adapt to the delayed cycle (Figure 2C and Table 1 Parameter 8). Together, these data indicate compromised capacity to be entrained by light in *Msk1*<sup>−/−</sup> mice.
Table 1. Phenotypic Characteristics of Msk \(^{-/-}\) Mice

<table>
<thead>
<tr>
<th>Parameter (mean±standard error)</th>
<th>Wild-Type</th>
<th>Msk(^{-/-})</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Circadian period(\tau) in DD(h)</td>
<td>23.55±0.12</td>
<td>23.78±0.13</td>
<td>0.245</td>
</tr>
<tr>
<td>2. Variance in activity onset in LD(d)</td>
<td>0.40±0.24</td>
<td>3.00±0.58</td>
<td>0.005**</td>
</tr>
<tr>
<td>3. Variance in activity onset in DD(d)</td>
<td>1.40±0.51</td>
<td>3.43±0.61</td>
<td>0.038*</td>
</tr>
<tr>
<td>4. Overall activity in LD(rounds/5 min)</td>
<td>64.9±6.01</td>
<td>66.26±10.56</td>
<td>0.923</td>
</tr>
<tr>
<td>5. Overall activity in DD(rounds/5 min)</td>
<td>76.58±4.25</td>
<td>50.50±6.69</td>
<td>0.014*</td>
</tr>
<tr>
<td>6. DD to LD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days to retrain(d)</td>
<td>1.00±0.32</td>
<td>5.29±0.87</td>
<td>0.003**</td>
</tr>
<tr>
<td>Day 1 lag in onset(hr)</td>
<td>1.13±0.53</td>
<td>2.29±0.69</td>
<td>0.242</td>
</tr>
<tr>
<td>7. 8h phase-advancing light</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days to retrain(d)</td>
<td>3.00±0.32</td>
<td>11.29±0.92</td>
<td>0.000**</td>
</tr>
<tr>
<td>Day 2 lag in onset(hr)</td>
<td>1.12±0.40</td>
<td>7.81±0.20</td>
<td>0.000**</td>
</tr>
<tr>
<td>8. 8h phase-delaying light</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days to retrain(d)</td>
<td>3.0±0.32</td>
<td>5.71±0.64</td>
<td>0.008**</td>
</tr>
<tr>
<td>Day 1 lag in onset(hr)</td>
<td>4.90±0.84</td>
<td>3.37±1.16</td>
<td>0.351</td>
</tr>
<tr>
<td>9. Photic phase delay at CT15(h)</td>
<td>1.85±0.24</td>
<td>0.86±0.13</td>
<td>0.003**</td>
</tr>
<tr>
<td>10. Photic phase advance at CT22(h)</td>
<td>0.66±0.13</td>
<td>0.51±0.14</td>
<td>0.465</td>
</tr>
</tbody>
</table>

\*\(P<0.05\), \**\(P<0.01\)

---

**A**

![Wild type Circadian activity](image1)

![Msk\(^{-/-}\) Circadian activity](image2)

**B**

![Wild type Activity onset](image3)

![Msk\(^{-/-}\) Activity onset](image4)

**C**

![Wild type Activity delayed](image5)

![Msk\(^{-/-}\) Activity delayed](image6)
**Figure 5.2.** *MskI*<sup>−/−</sup> mice show compromised capability to be entrained by light in wheel-running activity.

A. Light-induced phase delay is decreased in *MskI*<sup>−/−</sup> mice. Representative double-plotted actographs of wheel-running activity. Initially, mice were entrained on a 12 h LD cycle and then transferred to total darkness. After ~10 d under DD, mice were exposed to light (100 lux, 15 min) at CT15 (red asterisks). Regression lines and arrows approximate the phase-delaying effects of light. Grey area indicates the period when light was off. B. *MskI*<sup>−/−</sup> mice require longer time to be entrained to LD from DD. Representative double-plotted actographs of wheel-running activity. Initially, mice free-ran in DD for 10 d and then transferred to an 8 h phase-delaysing LD cycle. Grey area indicates the period when light was off. C. *MskI*<sup>−/−</sup> mice require longer time to be entrained to a new LD cycle from LD. Representative double-plotted actographs of wheel-running activity. Initially, mice were entrained on a 12 h LD cycle for 10 d and then transferred to an 8 h phase-advancing LD cycle. Mice were kept in the new LD cycle for 14 d and transferred back to the original LD cycle.

To complement the wheel-running data of *MskI*<sup>−/−</sup> mice, we conducted a parallel set of experiments with *MskI*<sup>−/−</sup>,<sup>2−/−</sup> mice, in which both isoforms of *Msk* were deleted. Using core body temperature rhythm as an indicator of the clock function, we first observed their response to light flash at night. As in *MskI*<sup>−/−</sup> mice, light exposure at CT15 induced smaller phase-delay in *MskI*<sup>−/−</sup>,<sup>2−/−</sup> mice, compared to their wild-type littermates (Figure 5.3A). In the “jet lag” experiment, *MskI*<sup>−/−</sup>,<sup>2−/−</sup> mice exhibited a dramatically different way to adapt to the new LD cycle: instead of phase advancing to the new cycle, they phase-delayed to the 8 h phase-advancing cycle (Figure 5.3B). The rhythmicity analysis by FFT graph during the transition period is shown at the bottom of Figure 5.3B. Compared to the wild-type control, *MskI*<sup>−/−</sup>,<sup>2−/−</sup> exhibited larger degree of arrhythmicity during this period (Figure 5.3C). Also, they required significantly longer time to be entrained to the new cycle, both in advancing phase-shift and delaying phase-shift (Figure 5.3B).

5.3.3 Light-induced immediate early gene (IEG) but not pERK expression is decreased in *MskI*<sup>−/−</sup> mice

To gain insight into the molecular mechanisms of the circadian phenotype of *Msk* null mice, we first looked at light-induced IEG expression in the SCN. Thus, animals
were entrained in LD for 14 d and put in DD for at least 2 d. At CT15, a short light pulse (7 or 100 lx, 15 min) was applied to animals and animals were sacrificed immediately after the light. The weak light intensity (7 lx) was the same as the one

A. Light-induced phase delay is decreased in Msk1<sup>1<sub>−/−</sub></sup> mice. Representative double-plotted actographs of core body temperature recording. Initially, mice were entrained on a 12 h LD cycle for 10 d and then transferred to total darkness. After 14 d under DD, mice were exposed to light (100 lux, 15 min) at CT15 (red asterisks). Regression lines and arrows approximate the phase-delaying effects of light. Grey area indicates the period when light was off. B. Msk1<sup>1<sub>−/−</sub></sup> mice require longer time to be entrained to a new LD cycle from LD. Representative double-plotted actographs of core body temperature recording. Initially, mice were entrained on a 12 h LD cycle for 10 d and then transferred to an 8 h phase-advancing LD cycle. Mice were kept in the new LD cycle for 21 d and transferred back to the original LD cycle. FFT(fast fourier Transformation) analysis of the periods of various cycles during phase-advancing entrainment is shown at the bottom.

Figure 5.3. Msk1<sup>1<sub>−/−</sub></sup> mice show compromised capability to be entrained by light in core body temperature rhythm.

A. Light-induced phase delay is decreased in Msk1<sup>1<sub>−/−</sub></sup> mice. Representative double-plotted actographs of core body temperature recording. Initially, mice were entrained on a 12 h LD cycle for 10 d and then transferred to total darkness. After 14 d under DD, mice were exposed to light (100 lux, 15 min) at CT15 (red asterisks). Regression lines and arrows approximate the phase-delaying effects of light. Grey area indicates the period when light was off. B. Msk1<sup>1<sub>−/−</sub></sup> mice require longer time to be entrained to a new LD cycle from LD. Representative double-plotted actographs of core body temperature recording. Initially, mice were entrained on a 12 h LD cycle for 10 d and then transferred to an 8 h phase-advancing LD cycle. Mice were kept in the new LD cycle for 21 d and transferred back to the original LD cycle. FFT(fast fourier Transformation) analysis of the periods of various cycles during phase-advancing entrainment is shown at the bottom.
used in the “jet lag” phase-shifting experiments while the strong light (100 lx) was same as the one used in behavioral analysis for light-induced phase shifting. C-Fos expression in the SCN was examined by immuno-staining. Interestingly, compared to that in wild-type littermates, 100lx light-induced c-Fos expression in \( Msk^{+/−} \) mice was significantly decreased by \( \sim \) 30%, although light still induced significant expression of c-Fos in the SCN (Figure 5.4A and B). 7lx light-induced c-Fos wasn’t significantly changed in \( Msk^{+/−} \) mice (Figure 5.4B). The basal (no light) c-Fos expression in the SCN wasn’t significantly different in \( Msk^{+/−} \) mice compared to wild-type control (Figure5. 4A and B). These data indicate the coupling mechanisms between light and gene expression in SCN was compromised in \( Msk \) null mice. To test if the ability of these animals to sense light was undermined by \( Msk \) deletion, we detected light-induced phospho-ERK expression in the SCN. Interestingly, light induction of ERK in these animals wasn’t significantly different compared to the wild-type control (Figure 5.4C). ERK phosphorylation is an upstream event of light-induced Msk phosphorylation. Light-induced phospho-ERK expression was intact means that the coupling pathway wasn’t affected upstream of \( Msk \) signaling.

5.3.4 Light-induced CREB phosphorylation is decreased in \( Msk^{+/−} \) mice

Light-induced CREB phosphorylation at Ser133 is a key event that couples light to CRE-mediated gene expression in the SCN. MSK has been shown in other model systems to be the kinase that can phosphorylate CREB at Ser133. We are therefore interested to see if light-induced CREB phosphorylation is affected in \( Msk \) null mice. To pursue this line of inquiry we entrained animals in LD for two weeks and put them
Figure 5.4. Light-induced c-Fos expression was decreased in Msk knockout animals.

A. Representative microscopic images of immunohistochemical staining for c-Fos expression in the SCN. Animals were entrained, put in dark for 48 hr and given a light flash (100 lx, 15 min) at CT15. Light induced robust c-Fos expression in the SCN of wild-type (WT) animals (top). In the Msk1\(^{-/-}\) animals (KO, bottom), light-induced c-Fos expression was significantly decreased. B and C. 7 lx and 100 lx light (15 min) induced c-Fos (B) and pERK (C) expressions are plotted. 100 lx light-induced c-Fos expression was significantly decreased in Msk1\(^{-/-}\) mice while pERK expression was not changed, comparing with that in wild-type animals.

in DD for at least two days. At CT15, we applied short light pulse (100 lx, 15 min) to animals and sacrificed them immediately after the light. Phospho-CREB expression was analyzed by immune-staining with phospho-specific CREB antibody at Ser133. As expected, we found considerable amount of basal level phospho-CREB expression in the SCN (Figure 5.5 A). And short light exposure induced moderate but significant amount of phospho-CREB expression in the ventral lateral region of SCN in the wild-type animals (Figure 5.5 A and B). Interestingly, we still saw high-level of p-CREB expression in Msk1\(^{-/-}\) mice at the basal level (no light) (Figure 5.5 A). Light also induced moderate increase of CREB
phosphorylation in these mice but the induction wasn’t significantly increased compared to the “no light” control (Figure 5.5 B) and it was significantly decreased compared to the wild-type mice.

**Figure 5.5. Light-induced phospho-CREB expression was decreased in Msk1-/- animals.**

A. Representative microscopic images of immunohistochemical staining for phospho-CREB (at Ser133) expression in the SCN. Animals were entrained, put in dark for 48 hr and given a light flash (100 lx, 15 min) at CT15. Light induced moderate increase of phospho-CREB expression in the SCN of wild-type (WT) animals (top). In the Msk1-/- animals (KO, bottom), light-induced phospho-CREB expression was decreased. B. Histograms show a statistical analysis of light-induced phospho-CREB expression in wild-type and Msk1-/- animals. The phospho-CREB expression in the SCN under “no light” condition were set to be 1 in wild-type animals. Light induced significant increase of phospho-CREB expression in the SCN. In Msk1-/- animals, the basal level of phospho-CREB expression was moderately decreased comparing with that in wild-type control. Light induced moderate but insignificant increase of phospho-CREB expression in the SCN of Msk1-/- animals.
5.3.5 Light-induced Per1 expression is decreased in Msk1^{-/-} mice

Light-induced Per gene expression is considered to be one of the major mechanisms of SCN clock entrainment. To examine if light-induced Per expression is affected by Msk deletion, we crossed Msk1^{-/-} mice with Per1-Venus mice. The Per1-Venus mouse is a Per1 gene expression reporter strain by using Per1 promoter-driven GFP-derivative fluorescent protein Venus expression. To look at light-induced Per1 expression (as indicated by Venus expression), we entrained animals in LD for 14 d and put them in DD for at least 2 d. At CT15, we applied short light (100 lx, 15 min) to animals and sacrificed them 4 h after light. Immunofluorescent labeling was used to analyze light-induced Venus expression. As expected, in wild-type animals significant-induction was found in the ventral lateral SCN (Figure 5.6 A and B), the pattern of which was similar as reported by us and other groups. In the Msk1^{-/-} mice, light-induced Venus expression was also detected, in a similar pattern but the intensity of expression was significantly decreased compared to that in wild-type animals(Figure 5.6 A and B). These data indicate that light-induced Per1 expression was decreased in Msk1^{-/-} mice.

5.4 Discussion

As an adaptive response to the ever changing eternal environment, the circadian clock is continuously adjusted according to the environment cues (Zeigebers) (Reppert and Weaver, 2002; Golombek and Rosenstein, 2010). For mammals, light signal from the retina serves as the most prominent zeigeber. The SCN clock is monosynaptically connected to retina and receives photic information in the form of neurotransmitter release. There has been considerable interest unraveling the intracellular signal transduction mechanisms in the SCN that couple the light-evoked synaptic signals to the intracellular clock gene expression. Our lab and other groups
Figure 5.6. Light-induced Per1-Venus expression was decreased in $Msk1^{-/-}$ animals.

A. Representative microscopic images of immunohistochemical staining for Venus expression in the SCN. Animals were entrained, put in dark for 48 hr and given a light flash (100 lx, 15 min) at CT15. Animals were sacrificed 4 hr later at CT19. Light induced increased expression of Venus in the SCN of wild-type (WT) animals (top). In the $Msk1^{-/-}$ animals (KO, bottom), light-induced Venus expression was markedly decreased. B. Histograms show a statistical analysis of light-induced Venus expression in wild-type and $Msk1^{-/-}$ animals. The Venus expression in the SCN under “no light” condition were set to be 1 in the wild-type animals. Light induced significant Venus expression in the SCN. In $Msk1^{-/-}$ animals, the basal level of phospho-CREB expression was moderately decreased comparing with that in wild-type control. Light induced moderate but insignificant increase of Venus expression in $Msk1^{-/-}$ animals.

have identified p42/44 MAPK signaling pathway as a pivotal mediator during this process(Obrietan et al.,1998; Butcher et al.,2002; Coogan and Piggins 2003). As further investigation of the molecular mechanisms along this line, we have identified a nuclear serine/threonine protein kinase MSK1 as a downstream target of ERK that
can be regulated by light in the SCN. As a continuation of this line of work, here we utilized Msk null mice to analyze the potential function of MSK in the circadian clock entrainment process in vivo. Our data prove that the capacity to be entrained by light is significantly compromised in Msk null mice. Mechanistically these phenotypes can be explained by reduction in light-induced CREB phosphorylation and Per1 expression in these mice.

MSK is a nuclear serine/threonine protein kinase originally described in 1998 (Deak et al., 1998). It has two isoforms, MSK1 and MSK2, which is 64% identical to each other in human. MSKs can be activated by either ERK1/2 or p38 MAPK in vitro. MSKs contain an AGC kinase family related N-terminal domain and a CaMK family related C-terminal domain. MAPK phosphorylates the C-terminal domain, which in turn phosphorylates and activates the N-terminal domain. Once activated, the N-terminal domain phosphorylates substrates including CREB, NFkB, HMGN1 and histone H3 in cells. The physiological roles of of MSK1 are not completely understood. It has been indicated functional importance in immunity, neuronal activity and cell death. Our previous study has identified that MSK1 can be activated in the SCN by light stimulation at night and that ERK1/2 is the major activator of MSK1 during the light entrainment process. Thus, in the SCN circadian clock, MSK1 can be activated by PACAP release from the RHT during the night but not during the day. In reporter gene assays, MSK1 was shown to couple to mPeriod1 expression via a cAMP response element-binding protein-dependent mechanism (Butcher et al., 2005). However, a role of MSK in circadian clock in vivo has not been examined.

In this study, we first characterized the behavioral phenotype of the MSK null animals. As MSK is known to be a downstream effector of ERK MAPK, a major mediator of photic entrainment, we tested whether clock entrainment is compromised in these animals. We found that these mice were able to be entrained in 12h LD cycle. However, they showed significant larger variance in activity onset both in LD, indicating relatively unstable entrainment of the clock. Similarly, the larger variance
was also observed in free-running condition, indicating the stability of clock oscillation is also undermined in the *Msk* null animals. To further determine the entrainability of *Msk* null mice, we employed a standard Aschoff type I paradigm to observe light-induced phase-shift in these mice. As expected, we found the early-night-light induced phase delay was significantly decreased in these mice, also supporting compromised entraining mechanism in these mice. Next, we conducted "jet lag" experiments to look at the phase shifting response. Compared to the light pulse experiment, we used a low intensity light (7 lx) as entrainment cue in wheel-running experiments, because high intensity light has significant masking effect on animal wheel-running behavior, which may disguise the real rhythm of the clock. However, in core-body temperature recording experiments, we still used a very strong light (400 lx) as entraining signal. In both sets of experiments, we observed drastically different phase-shifting responses in *Msk* null mice, compared to wild-type littermates: the knockout animals required more time to be entrained to the new light cycle. These data means the ability to be entrained by light is compromised in *Msk* null mice. To further locate the affected step on the entrainment pathway, we did a control experiment to see if upstream of MSK is affected in *Msk* knockout mice by detecting light-induced ERK activation. We found ERK activation was not influenced in these mice, at both a weak and a strong light intensity. These data suggest the signaling pathway from retina to SCN is not changed upstream of MSK.

To continue to dissect the molecular mechanisms of compromised light entrainment in *Msk* null mice, we looked at the light-induced IMG c-Fos expression. C-Fos expression can be used as a non-specific neuronal activity marker in light entrainment of the clock. Our previous work indicates that MAPK couples light to IMG expression in the SCN. In this study, we found ~20% decrease in light-induced c-Fos expression in *Msk* null mice, indicating that at least part of the IMG expression is dependent on MSK signaling. Also it suggests that the coupling mechanism from light to gene expression in the SCN is deteriorated in *Msk* null mice. To look at
more specific molecular mechanism, we tested CREB phosphorylation in these mice. CREB phosphorylation and CRE-mediated gene transcription serves as a central conduit that links several signaling transduction pathways to clock gene expression and clock resetting. Interestingly, CREB has been implicated as a substrate of MSK in other systems. This information makes it intriguing to look at CREB phosphorylation in Msk null mice. We found a moderate decrease in phospho-CREB basal level in Msk−/− mice, although it didn’t reach statistical significance. Light at night also induced a moderate expression of phospho-CREB in the SCN. However, the induction was significantly ablated compared with wild type control. These data indicate that MSK is a key mediator of light-induced CREB phosphorylation in the SCN. There are other mechanisms that can phosphorylate CREB in Msk null animals so that phosphorylated CREB is still robustly expressed in the Msk null mice. For example, RSK, another downstream target kinase of ERK MAPK, is also shown to phosphorylate CREB in other systems. Interestingly, RSK activity can also be activated by light via ERK-mediated mechanism in the SCN (Butcher et al., 2003).

As an ultimate step of clock entrainment, clock protein expression level can be changed by light signal so that the dynamic balance of the transcriptional/translational feedback loop is temporarily disrupted until a new oscillatory cycle is established. A number of studies have suggested light-induced Per gene expression plays a major role in this process (Albrecht et al., 1997; Shigeyoshi et al., 1997; Akiyama et al., 1999; Albrecht et al., 2001; Wakamatsu et al., 2001). Period gene has several CRE sequence in its promoter region and subjective to CREB mediated transcriptional activation (Travnickova-Bendova et al., 2002). Since CREB phosphorylation is decreased in Msk knockout mice, we were interested to determine if Per1 transcription was affected. For this purpose, we used a Venus reporter mouse strain to indicate Per1 transcription. Interestingly, we found similar pattern of decrease in Venus expression as in phosphor-CREB expression: Venus basal level was moderately but insignificant decrease in Msk knockout mice and light-induced Venus
expression was significantly decreased in these mice. Together, these date supporting a key role of the MSK-pCREB-Per1 signaling pathway in the circadian clock entrainment.
Chapter 6. Conclusion

The MAPK cascade is a complex and context-specific regulator of a vast array of physiological processes. Thus, MAPK signaling has been shown to affect the cellular protein profile via the regulation of gene transcription, mRNA translation, and posttranslational protein stability (Adams and Sweatt, 2002; Carrière et al., 2008). Since the activity of p42/44 MAPK was first characterized in the SCN (Obrietan et al., 1998), the role of this pathway as a key mediator of light signal in clock entrainment is being increasingly appreciated. In the earliest and most parsimonious examination of MAPK signaling and clock entrainment, Butcher et al. (2002) and Coogan and Piggins (2003) reported that the pharmacological inhibition of MAPK signaling led to a marked repression of light-induced clock entrainment. Given that MAPK signaling functions via multiple downstream effectors, a good amount of effort has been directed toward understanding specific routes by which the MAPK pathway regulates clock entrainment. In my dissertation work, focus was placed on two signaling pathways that are activated by ERK activation in the SCN, the mTOR and MSK signaling.

First, I revealed the presence of mTOR signaling activity in the SCN, the locus of master circadian clock in mammals. Next, I found that light, as the most prominent entrainment signal, regulates mTOR activity in the SCN in a phase-specific manner. Also, my work indicates that ERK MAPK is the upstream pathway that couples light to mTOR activation. Further, I proved a key role of mTOR signaling in regulating light-induced phase shifting by facilitating the core clock protein PERs translation in the SCN. I also identified a circadian expression pattern of mTOR signaling in the SCN, and found a close association of circadian mTOR activity and rhythmic Per1 gene expression at a cellular level. Together, these results suggest a
significant role of mTOR signaling in mammalian circadian clock entrainment and timing process.

My work with *Msk* knockout animals strongly indicates that MSK is a key mediator of circadian clock entrainment *in vivo*. MSKs, as the kinase effectors of MAPK, facilitate light-induced *Per1* expression by phosphorylating the transcription factor CREB in the SCN. Together, my work provides further information with respect to the role of MAPK in circadian clock entrainment.

To summarize the function of MAPK in circadian clock entrainment, our lab has shown that activation of the MAPK cascade is required for light-evoked, MSK-dependent, CRE-mediated transcription in the SCN (Obrietan et al., 1999; Butcher et al., 2005). My work here further proved the role of MSK as a mediator between MAPK and CREB phosphorylation *in vivo*. Interestingly, inducible PER1 transcription is mediated by MAPK signaling via the CREB/CRE pathway, thus providing one transcriptionally dependent mechanism by which the MAPK pathway regulates the clock (Travnickova-Bendova et al., 2002; Butcher et al., 2005). In addition, our recent work has revealed a role for MAPK signaling in mRNA translation regulation within the SCN. Along these lines, the MAPK pathway was shown to couple light to miR-132, thus leading to a modulation of clock entrainment (Cheng et al., 2007). Studies have also raised the possibility that MAPK signaling affects the clock through posttranslational (i.e., phosphorylation) processes. For example, MAPK signaling has been shown to evoke the phosphorylation of CLOCK (a component of the core clock timing mechanism) and affect the transactivation potential of the CLOCK/CYCLE transcriptional complex (Weber et al., 2006). Importantly, my work regarding mTOR signaling reveals a new translation-dependent mechanism by which the MAPK couples to the molecular clock (Cao et al., 2008, 2010). A diagram outlining the potential signaling pathways by which MAPK signaling influences clock entrainment is included (Figure 6.1).
Figure 6.1. Schematic overview of MAPK-regulated processes that are thought to couple light to the SCN clock during the early night.

Photic input from the RHT drives the release of the excitatory amino acid glutamate and the neuropeptide PACAP. Postsynaptic receptors trigger actuation of the MAPK signaling cassette, ultimately leading to the phosphoactivation of the effector kinase ERK. Two principal ERK-mediated signaling events are depicted: activation of MSK and the mTORC1 signaling complex. A potential signaling pathway from ERK to CLOCK (CLK) is also depicted (Weber et al., 2006). Signaling through MSK leads to activation of transcription factors such as CREB (Vermeulen et al., 2009), which in turn drives the induction of early response genes, including c-fos and the clock gene Period1, and potentially Period2 (Travnickova-Bendova et al., 2002). CREB also stimulates the expression of the microRNA miR-132, which works through an as yet unidentified mechanism to limit the resetting effects of light (Cheng et al., 2007).

ERK-dependent activation of mTORC1 causes phosphorylation-dependent activation of a p70 S6K/S6 signaling cassette, which stimulates TOP mRNA translation, and a 4E-BP1 and eEF1A signaling cassette, which increases CAP-dependent translation. These two arms of the mTOR pathway work in conjunction to enhance the rate of mRNA processivity. With respect to clock entrainment, we posit that the ERK-dependent transcription (via MSK/CREB) and ERK-dependent translation facilitation (via the mTOR pathway) lead to a robust induction of the PERIOD protein expression. As a state variable of the clock (Reppert and Weaver, 2002), the induction of PERIOD leads to a rapid resetting of the molecular oscillator.


Cheng H, Papp JW, Varlamova O, Dziema H, Russell B, Curfman JP, Nakazawa T,


Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi


Coordinate regulation of ribosome biogenesis and function by the ribosomal protein S6 kinase, a key mediator of mTOR function. Growth Factors 25:209–226.


Sangoram A, Saez L, Antoch MP, Gekakis N, Staknis D, Whiteley A, Fruechte EM,


Staber P, Vesely P, Haq N, Ott RG, Funato K, Bambach I, Fuchs C, Schauer S,
Linkesch W, Hrzenjak A, Dirks WG, Sexl V, Bergler H, Kadin ME, Sternberg
large-cell lymphoma induces JUNB transcription via ERK1/2 and JunB
Takumi T, Matsubara C, Shigeyoshi Y, Taguchi K, Yagita K, Maebayashi Y,
mammalian period gene predominantly expressed in the suprachiasmatic
Tang H, Hornstein E, Stolovich M, Levy G, Livingstone M, Templeton D, Avruch J,
Meyuhas O. (2001) Amino acid-induced translation of TOP mRNAs is fully
dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially
inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation.
rapamycin-sensitive signaling pathway contributes to long-term synaptic
plasticity in the hippocampus. Proc Natl Acad Sci U S A 99:467-472.
Circadian oscillation of a mammalian homologue of the Drosophila period
selectively inhibits translation of mRNAs encoding elongation factors and
gating of glutamatergic signaling regulates long-term state changes in the


Young M, Bray MS (2007) Potential role for peripheral circadian clock dyssynchrony


