THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) PATHWAY:  
A SIGNALING CONDUIT FOR PHOTIC ENTRAINMENT  
OF THE CENTRAL MAMMALIAN CIRCADIAN CLOCK  

DISSERTATION  

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ABSTRACT

The central biological clock, located in suprachiasmatic nuclei (SCN) of the hypothalamus in mammals, coordinates biochemical, physiological and behavioral processes to an approximate 24 hr (circadian) rhythm. The endogenous timing mechanism of the SCN remains synchronized to the exogenous environment through the influence of timing cues (zeitgebers), such as light, by a process known as entrainment. The studies presented in this dissertation describe a functional and mechanistic assessment of intracellular signaling via the p42/44 mitogen activated protein kinase (MAPK) cascade (Raf-MEK-ERK) and provide evidence suggesting this pathway functions as an input conduit for photic entrainment. The following hypotheses are sequentially tested using a variety of behavioral, cellular, molecular and biochemical techniques. 1) Light-induced behavioral phase shifting in mice is coupled via a MAPK dependent mechanism. 2) Light activation of the MAPK pathway (as assessed by phosphorylation of ERK1/2) is stringently regulated with respect to the time course of activation / inactivation and subcellular localization of ERK within neurons. 3) Light-induced phosphorylation of p90 ribosomal S6 kinases (RSKs) within the SCN is phase restricted to the subjective night and is dependent upon activation of the MAPK pathway. 4) Light-induced phosphorylation of mitogen- and stress-activated kinases (MSKs) within the SCN is dependent on a signaling cassette consisting of the neuromodulator
pituitary adenylate cyclase activating polypeptide (PACAP) and the MAPK pathway. 5) Light-induced phase advances, but not phase delays, are attenuated in mice lacking MSKs. Conversely, light-induced phase delays, but not phase advances will be attenuated in mice lacking RSKs.

The data discussed herein demonstrate functional coupling between photic stimuli and behavioral phase shifting using intraventricular infusion of a specific MEK inhibitor, which blocks activation of ERK1/2. Confocal fluorescent microscopy was then used to determine the temporal and spatial dynamics of light-induced ERK activation and subcellular localization following brief or continual light treatment. Finally, two substrate kinases that are regulated by the MAPK pathway, RSK1 and MSK1 were examined. Immunohistochemical labeling directed against phosphorylated forms each kinase was used to determine their activation profiles following photic stimulation presented during the day, early or late subjective night. Rapid RSK1 and MSK1 phosphorylation in the SCN following light treatment occurred in a phase-specific and ERK-dependent fashion. Differential profiles of maximal light-induced activation for RSK1 and MSK1 suggest that these kinases may occupy divergent functional roles within the entrainment mechanism. Preliminary data testing this possibility indicates that RSK and MSK signalling may function in a partially compensatory and complimentary manner rather than exclusively distinct pathways.
Dedicated to my wife Anne

and our firstborn, Phineas or Lucy
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My family and friends: For always encouraging me to follow my passions, wherever they may lead.
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- Circadian Biology
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<tr>
<td>3V</td>
<td>third ventricle</td>
</tr>
<tr>
<td>A-CREB</td>
<td>dominant negative CREB</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>BMAL1</td>
<td>brain and muscle Arnt-like protein 1</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>CA</td>
<td>constitutively active or control animal</td>
</tr>
<tr>
<td>CaMK</td>
<td>calcium/calmodulin kinase</td>
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<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CK</td>
<td>casein kinase</td>
</tr>
<tr>
<td>CL</td>
<td>constant light</td>
</tr>
<tr>
<td>CLOCK</td>
<td>circadian locomotor output cycle kaput</td>
</tr>
<tr>
<td>Chr</td>
<td>chromosome</td>
</tr>
<tr>
<td>CRE</td>
<td>cyclic AMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CRY1/2</td>
<td>Cryptochrome 1/2</td>
</tr>
<tr>
<td>CT</td>
<td>circadian time</td>
</tr>
<tr>
<td>CYC</td>
<td>cyclophilin</td>
</tr>
<tr>
<td>DD of D:D</td>
<td>dark/dark</td>
</tr>
<tr>
<td>DEC1/2</td>
<td>deleted in esophageal cancer 1/2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>DM</td>
<td>dissociation media / dorsomedial</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DN</td>
<td>dominant negative</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>evening oscillator</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminotetraacetic acid</td>
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<td>EGTA</td>
<td>ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid</td>
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<td>Elk-1</td>
<td>ETS like protein 1</td>
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<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>GFP</td>
<td>green-fluorescent protein</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
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<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperizine-N’-(2-ethanesulfonic acid)</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun amino-terminal kinase</td>
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<tr>
<td>LD or L:D</td>
<td>light/dark</td>
</tr>
<tr>
<td>LP / LF</td>
<td>light pulse / light flash</td>
</tr>
<tr>
<td>M</td>
<td>morning oscillator</td>
</tr>
<tr>
<td>MAP-2</td>
<td>microtubule-associated protein-2</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MEK</td>
<td>MAPK/Erk kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MKP</td>
<td>MAP kinase phosphatase</td>
</tr>
<tr>
<td>MLP</td>
<td>multiple light pulse</td>
</tr>
<tr>
<td>mPER1/2/3</td>
<td>mouse Period 1/2/3</td>
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<tr>
<td>MSK</td>
<td>mitogen- and stress-dependent kinase</td>
</tr>
<tr>
<td>NEN</td>
<td>New England Nuclear</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization sequence</td>
</tr>
<tr>
<td>OC</td>
<td>optic chiasm</td>
</tr>
<tr>
<td>ON</td>
<td>optic nerve</td>
</tr>
<tr>
<td>OT</td>
<td>optic tract</td>
</tr>
<tr>
<td>OTS</td>
<td>oscillating tissue slicer</td>
</tr>
<tr>
<td>P</td>
<td>proline / phosphorylated</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>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate-buffered saline + triton X-100</td>
</tr>
<tr>
<td>PC</td>
<td>personal computer</td>
</tr>
<tr>
<td>PDK1</td>
<td>2-phosphoinositide-dependent protein kinase-1</td>
</tr>
<tr>
<td>pERK/P-ERK</td>
<td>phosphorylated ERK</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>pMSK</td>
<td>phosphorylated MSK</td>
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<tr>
<td>pRSK/pp90RSK</td>
<td>phosphorylated RSK</td>
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PVDF  polyvinylidene diflouride
R     arginine
RHT   retinohypothalamic tract
RORα  retinoid-related orphan receptor alpha
RORE  retinoic acid-related orphan receptor response elements
RT-PCR reverse transcriptase polymerase chain reaction
RSK/p90RSK p90 ribosomal S6 kinase
S / ser serine
SB203508 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl) phenyl]-1H-imidazol-4-yl]pyridine
SCN   suprachiasmatic nuclei
SDS   sodium dodecyl sulphate
SDS-PAGE SDS-polyacrylamide gel electrophoresis
SE    standard error
SEM   standard error of the mean
SLP   single light pulse
SRE   serum response element
SRF   serum response factor
T / Thr threonine
TPA   12-O-tetradecanoylphorbol-13-acetate
TTX   tetrodotoxin
Tyr   tyrosine
U0126 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenyl mercapto) butadiene

VL ventrolateral

WT wild type

ZT zeitgeber time
CHAPTER 1

General Introduction

Rhythmic biologic cycles with a period of approximately one day (circadian) have been found in numerous plant and animal species (for comparative reviews see Dunlap, 1998; Wilsbacher and Takahashi, 1998; Reppert and Weaver, 2000). While basic rhythmicity at the cell and tissue level is often directly controlled by peripheral timekeepers (Schibler et al., 2002; Panda and Hogenesch, 2004), historically a central oscillator or “clock” has been credited with maintaining body-wide, cellular and tissue synchronization (Moore, 1983; Hastings, 1997; but note Davidson et al., 2003). Regardless of their physical location within an organism, biological clocks are thought to serve an adaptive purpose by providing the means to optimize the allocation and utilization of physiological resources, ultimately promoting reproductive fitness (Ouyang et al., 1998; Michael et al., 2003). Over the past decade, a category of genes specifically related to biological timing have been identified for a growing number of species (Harmer et al., 2001; Panda et al., 2002; Fukada and Okano, 2002; Murphy et al., 2005; Van Oort et al., 2005). While many of the initial studies to identify genes critical to circadian rhythmicity were conducted in drosophila and neurospora (for review see Dunlap, 1990), the proliferation of custom mouse strains lacking key timing components
has facilitated the examination of the mammalian clock and is the primary focus of work described in this dissertation.

1.1 The Molecular Clock

In the majority of model systems studied to date, interactions between a relatively small category of “clock genes” manifest as a cyclic series of tightly regulated, positive and negative transcriptional / translational feedback loops. Expression of these genes and interactions between their corresponding proteins are temporally segregated, thereby giving rise to the central oscillation mechanism essential to rhythmicity. In mice, characterization of these genes began with the identification and subsequent cloning of *circadian locomotor output cycle kaput* (*clock*; Vitaterna et al., 1994; King et al., 1997) and *brain and muscle Arnt-like protein 1* (*Bmal1 / MOP3*; Ikeda & Nomura et al., 1997; Hogenesch et al., 1997). Shortly thereafter orthologs of the drosophila *period* gene, *mPer1* (Sun et al., 1997, Tei et al., 1997), *mPer2* (Shearman et al., 1997), and *mPer3* (Zylka et al., 1998) were reported. With the more recent discoveries of a light-independent role for mammalian *cryptochrome 1* and 2 (*Cry1* and *Cry2*; Griffin et al., 1999) and transcriptional regulation by two families of orphan nuclear receptors, REV-ERB and ROR (Sato et al., 2004; Guillaumond et al. 2005), the fundamental elements of the murine central clock appear to have been defined. Additional genes such as *Timeless* (compare Zylka et al., 1998 with Barnes et al., 2003), *Dec1*, and *Dec2* (Honma et al., 2002; Kawamoto et al., 2004; Sato et al., 2004) have been reported to contribute to circadian rhythmicity. However, their precise function has yet to be fully described.
The basic mechanism of the murine central clock has been extensively reported elsewhere (for reviews see King and Takahashi, 2000; Lowrey and Takahashi, 2000; Reppert and Weaver, 2002) and is only briefly described here. The transcriptional / translational feedback loop arbitrarily begins with expression of *Bmal1*. Peak transcription of this clock gene occurs during the early- to mid-night portion of the light / dark cycle (see figure 1.1; Honma et al., 1998). Following translation, BMAL1 reenters the nucleus and binds with the non-rhythmically expressed protein CLOCK (Maywood et al., 2003) to form a heterodimer complex. Regulation of BMAL1 nuclear translocation and subsequent complex formation may occur through phosphorylation of the protein, or its binding partners, by multiple kinases including members of the mitogen-activated protein kinase (MAPK; Sanada et al., 2004) and casein kinase (CK; Eide et al., 2002) families. CLOCK/BMAL1 complexes enhance transcription of *period, cryptochrome*, and *rev-erb* genes by binding to E-boxes within their promoter regions (Gekakis et al., 1998; Darlington et al., 1998; Hida et al., 2000). Maximal CLOCK/BMAL1 mediated expression of *Per1, 2* and *Cry1, 2* occurs approximately 12 hours following transcription of *Bmal1*, providing half of the temporal delay essential to maintain a circadian (~24 hr) cycle. As the concentration of translated protein increases, PERs and CRYs begin to form homo- and hetero-dimers, which then translocate to the nucleus. Properly timed nuclear translocation and the subsequent degradation of PER/CRY complexes, are essential for correct rhythmicity and several putative regulatory mechanisms explaining these events have been proposed. Among these, phosphorylation of PERs and CRYs has been reported to mediate various effects including conformational stabilization of the
dimers (Takano et al., 2004), masking of a nuclear export signal (Vielhaber et al., 2001) and tagging of complexes for ubiquitylation and eventual degradation of the constituent proteins (Yagita et al., 2000; Takano et al., 2004; Miyazaki et al., 2004). As PERs and CRYs contain putative phosphorylation sites for several kinases (see figure 1.2), it is likely that multiple second messenger signaling events contribute to the regulation of these proteins. Indeed phosphorylation of these proteins by CK (Takano et al., 2004), MAPK (Sanada et al., 2004), and possibly glycogen synthase kinase (Iitaka et al., 2005) has been reported.

**FIGURE 1.1 Fundamental components of the mammalian circadian clock.** Activation of a light responsive element or the binding of the BMAL1 / CLOCK dimer to an E-box within the 5’ regulatory region of Cry, Per and Rev-erb genes, promotes their transcription. Upon translation, CRY and PER form homo- and hetero-dimers, which reenter the nucleus and inhibit BMAL1 dependent transcription. REV-ERB also functions as a negative regulator of its own transcription by inhibiting the expression of Bmal1, thereby reducing additional gene expression. Phosphorylation of clock proteins (not shown) may serve to temporally segregate these elements through regulation of their stability, activity, and intracellular localization.
FIGURE 1.2 Predicted MAPK and known CKIε phosphorylation sites within mPER1. NetPhos 2.0 software (Blom et al., 1999) was used to determine putative phosphorylation sites within the mPER1 protein. Threonines and Serines with a high statistical likelihood of being phosphorylated are indicated by the open and closed arrowheads. Threonine 851 and Serine 970 (green text indicated by arrows), fully match the ERK phosphorylation motif (PXT/SP) and represent the most likely sites for direct regulation by ERK. The CKIε binding domain, nuclear localization sequence (NLS), and masking site are indicated as described in Vielhaber et al. (2000) and Akashi et al. (2002).

Once in the nucleus, PER/CRY complexes bind with CLOCK/BMAL1 and repress Per/Cry transcription, consequently inhibiting their own expression. PER/CRY dimers are gradually degraded, thereby removing inhibition of CLOCK/BMAL1 and completing the autoregulatory cycle. Bmal1 expression also appears to be regulated in a similar fashion as its transcription is influenced through interaction of REV-ERBα with Rev-Erb/RORα response elements within its upstream promoter region (Sato et al., 2004). The basic helix-loop-helix transcription factors DEC1 and DEC2, which competitively bind to the same E-box as the CLOCK/BMAL1 complex, may act to attenuate CLOCK/BMAL1-dependent Per1/2 expression (not shown; Honma et al., 2002). Precise, temporally-regulated interactions between molecular components of the described transcriptional/translational feedback loops result in an inherently stable timekeeper. However, to realize the full array of evolutionary advantages a clock confers, an
oscillator must also retain a degree of flexibility such that an organism maintains synchronization with seasonal changes in the 24 hour light / dark cycle.

### 1.2 Entrainment

Maintaining synchrony with seasonal changes in day length is a fundamental property of an endogenous clock (Miller et al., 1996). This process, known as entrainment, allows the transcriptional / translational loops of the clock to reset when presented with specific stimuli, such as light, during the appropriate phase of the cycle (Pittendrigh and Daan, 1976b). In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus contain neurons that make up the central oscillator and are essential for photic entrainment (Johnson et al., 1988). These small bilateral nuclei consist of approximately 20,000 neurons, which receive photic information from a subset of melanospin containing retinal ganglion cells via a branch of the optic nerve known as the retinohypothalamic tract (RHT; Provencio et al., 2000; Hannibal et al., 2004). These retinal ganglion cells maintain an endogenous rhythm independent of SCN signaling (Lee et al., 2003), which may function as a gating mechanism limiting photic responsiveness to physiologically relevant phases of the circadian cycle (discussed in Chapter 4 below). In nocturnal animals, photic stimulation during the night results in release of glutamate, pituitary adenylate-cyclase activated polypeptide (PACAP) and other neuromodulatory peptides from RHT terminals that synapse on neurons within the SCN (Ebling et al., 1996; Hannibal et al., 1997; Hannibal et al., 2000). Light-induced release of glutamate and PACAP has been implicated as events critical to photic entrainment. Furthermore, both neurotransmitter and neuromodulator induce expression of clock genes, a hallmark of
entrainment, when administered independent from light (Nielsen et al., 2001; Minami et al., 2002).

Photic stimulation during the subjective night results in the activation of multiple intracellular signaling pathways (reviewed by Golombek and Ralph, 1996; Gillette, 1997; Meijer and Schwartz, 2003). Photic stimulation during this period also induces immediate early and clock gene expression (Rusak et al., 1990; Ginty et al., 1993; Albrecht et al., 1997; Shearman et al., 1997; Caputto and Guido, 2000; Dziema et al., 2003). Specifically, light-induced transcription of the clock genes *Per1* and *Per2* has been extensively reported (Shigeyoshi et al., 1997; Akiyama et al., 1999; Yan & Silver 2002, 2004) and is thought to be a central mechanism for photic entrainment.

Interestingly recruitment and activation of transcription factors including the cyclic AMP response element binding protein (CREB), CREB binding protein (CBP), and Elk-1 have also been observed (Obrietan et al., 1999; Fiore and Gannon, 2003; Tischkau et al., 2003; Meijer and Schwartz, 2003; Coogan and Piggins, 2003). As several light-inducible immediate early genes including Jun-B, EGR-1, and c-Fos (reviewed by Kornhauser et al., 1996), as well as *Per1* and *Per2* contain CRE sites within their promoter regions (see figure 1.3; Travnickova-Bendova et al., 2002), CRE-dependent gene expression has been suggested to play a central role in entrainment (Ginty et al., 1993; Tamai et al., 1997; Kako et al., 1998; Travnickova-Bendova et al., 2002; Tischkau et al., 2003). Thus, identifying and characterizing signaling pathways that converge upon and activate CREB, has been an area of significant interest in recent years.
FIGURE 1.3 The upstream 5’ regulatory region of mPer1. Sequence analysis using Genomatix Suite software (http://www.genomatix.de/cgi-bin/eldorado/main.pl) revealed multiple putative transcription factor binding sites within the 2kb regulatory region upstream of exon1 for mPer1. Shown are sites through which MAPK activation of transcription factors could directly or indirectly regulate mPer1 expression. Asterisks (*) indicate the location identified as the BMAL1/CLOCK binding site; involved in rhythmic expression of this gene (Gekakis et al., 1998).

1.3 ERK and the Clock

One such signaling cascade, the p42/44 MAPK pathway has garnered attention as a potential regulator of the clock (Obrietan et al., 1998; Butcher et al., 2002; Coogan and Piggins 2003; Ho et al., 2003; Yadav et al., 2003; Butcher et al., 2003). The MAPK pathway consists of three protein kinases; Raf, MEK and ERK (for review see Cobb, 1999). Catalytic activity and cellular localization of ERK is regulated via phospho-activation by MEK. In its inactive form ERK is bound to cytosolic MEK. Upon activation ERK disengages from MEK, which may then phosphorylate a number of cytosolic substrates, dimerize and depending upon the strength of the stimulus, translocate to the nucleus (reviewed by Cobb, 1999; Pearson et al., 2001) where it may
facilitate transcriptional activation. For example, following nuclear entry ERK phosphorylates Elk-1, an ETS domain transcription factor, resulting in gene expression (reviewed by Treisman, 1996). Furthermore phosphorylated ERK may stimulate the enzymatic activity of the p90 ribosomal S6 kinase (RSK) and mitogen- and stress-dependent kinase (MSK) families, thereby indirectly regulating transcriptional activation (Frodin & Gammeltoft, 1999; Thomson et al., 1999).

Obrietan et al. (1998) provided the first evidence to suggest a role for MAPK signaling within the mammalian circadian clock. Maximal endogenous ERK phosphorylation in the SCN occurs during the mid- to late-subjective day (Obrietan et al., 1998). During the subjective night, a period of limited endogenous ERK activity, brief (5 min) photic stimulation elicits rapid and robust phosphorylation of the kinase. Interestingly, while ERK phosphorylation occurs within 5 min of photic stimulation (Obrietan et al., 1998), inactivation of the kinase is completed by 45 min following the onset of either brief (15 min) or continuous photic stimulation (Butcher et al., 2003). These data suggests light-induced MAPK signaling may function more as a binary switch indicating the presence but not the duration of a photic stimulus. MAPK signaling was further implicated in the process of photic entrainment with the observations that pharmacological disruption of MAPK signaling blocked light-induced phase delays in mice (discussed in Chapter 2 below; Butcher et al., 2002) and phase advances in hamsters (Coogan & Piggins, 2003). Light-induced MAPK activation during the early subjective night is localized to the central “core” region of the SCN for both species (described in Chapter 3 below; Butcher et al., 2003; Coogan & Piggins 2003), and in the case of mice,
colocalizes in neurons expressing phosphorylated CREB (Obrietan et al., 1998).
However, unlike other signaling kinases such as CaMK IV, ERK does not directly
phosphorylate CREB. Rather intermediate effector kinases are necessary to mediate
ERK-dependent activation of CREB (reviewed by De Cesare et al., 1999).

1.4 p90 Ribosomal S6 Kinases (RSKs)

Members of the 90 kDa ribosomal S6 kinase (RSK) family constitute one such
substrate. Four members of this family of serine kinases have been described (RSK1-4
respectively). RSKs are members of the larger growth factor-activated AGC protein
kinase family and consist of two kinase domains connected by an internal linker region.
They function as central mediators for a variety of complex physiological events
(reviewed by Frodin & Gammeltoft, 1999). The first three isoforms to be described,
RSK1-3, are tightly co-regulated by the p42/44 ERK / MAPK pathway and 2-
phosphoinositide-dependent protein kinase-1 (PDK1; Scimeca et al., 1992; Leighton et
al., 1995; Bjørbaek et al., 1995; Zhao et al., 1996; Fisher & Blennis, 1996; Frodin et al.,
2000). Activation of RSK1-3 involves a complex series of sequential phosphorylation
events (figure 1.4). Initially ERK binds to a docking site located within the C-terminal
region of RSK and phosphorylates two sites; a serine in the internal linker region and a
threonine in the C-terminal kinase. Dual phosphorylation by ERK results in a
conformational change in the protein, thereby allowing the RSK C-terminal kinase to
auto-phosphorylate a second serine located within a hydrophobic motif of the internal
linker region. This results in the formation of a binding site allowing PDK1 to dock and
phosphorylate a serine in the N-terminal kinase (Jensen et al., 1999). The N-terminal kinase then phosphorylates a final C-terminal site, thereby reducing the binding affinity of the kinase for ERK in RSK1 and 2 but not RSK3 (Roux et al., 2003). The N-terminal kinase also serves as the effector region of the protein, phosphorylating a number of cytosolic and nuclear substrates (reviewed by Frodin and Gammeltoft 1999). While RSK4 is structurally similar to the isoforms described above, its function appears to be markedly different from that of RSK1-3. In vivo expression of Rsk4 during late embryonic development appears restrict specific receptor tyrosine kinase signaling (Myers et al., 2004), while in vitro RSK4 has been reported to mediate p53 signaling in a variety of mammalian tissues (Dömmler et al., 2005).

Once activated, RSKs may translocate to the nucleus (Chen et al., 1992; Zhao et al., 1995; Zhao et al., 1996; Cavet et al., 2003) where they regulate the activation state of transcription factors and facilitate chromatin remodeling via phosphorylation of the histone H3 (Xing et al., 1996; Chen et al., 1992; Sassone-Corsi et al., 1999). Previous reports have suggested phosphorylation of CREB at Ser-133 occurs primarily via a RSK2-dependent mechanism (Ginty et al., 1994; Xing et al., 1998). Additional support for this position was provided by the identification of RSK2 mutation in patients with Coffin-Lowry syndrome. This mutation results in an inactive form of RSK2, which was purported to disrupt phosphorylation of CREB and histone H3 (Trivier et al., 1996; Sassone-Corsi et al., 1999; Zeniou et al., 2002). More recent evidence has also suggested that RSK2 may modulate AMPA receptor transmission via an as yet unidentified mechanism (Thomas et al., 2005).
FIGURE 1.4 Phospho-activation of RSK2 and MSK1. RSKs and MSKs are characterized by serial phosphorylation at multiple sites located within both the C- and N-terminal kinases and the internal linker region. Following docking, ERK dually phosphorylates RSK2 at T574 and S364, indicated by red circles. The C-terminal kinase then phosphorylates S381 (blue circle), opening a binding site that permits PDK1 to bind and subsequently phosphorylate S222 (green circle) located within the N-terminal kinase. This kinase then functions as an effector, activating various substrates. MSK1 activation occurs in a similar fashion with two significant exceptions. First, a second docking site allows either ERK or p38 to bind and phosphorylate T581 and S360 (purple circles). Secondly, the C-terminal kinase phosphorylates two internal sites S376 and S381, and one N-terminal site S212 (yellow circles) and does not involve activation by PDK1 in vivo.

1.5 Mitogen- and Stress-Activated Kinases (MSKs)

A second family of ERK substrates is represented by the mitogen- and stress-activated kinases (MSKs). Two isoforms, MSK1 and MSK2, have been identified
sharing approximately 75% sequence homology with each other and 43% to that of RSK2 (Smith et al., 2004). MSKs consist of the same general structure as RSKs with N- and C-terminal kinases separated by an internal linker region (figure 1.4). Likewise, members of both families are partially activated following phosphorylation by ERK. However, despite these similarities several key functional differences between RSKs and MSKs have been identified. While RSKs are activated within the cytosol then translocate to the nucleus (Cavet et al., 2003), MSKs are exclusively nuclear (Soloaga et al., 2003). A further divergence between RSKs and MSKs relates to their activation. RSKs require sequential phosphorylation by ERK and PDK1 to obtain full activation (Jensen et al., 1999). In contrast, in vivo PDK1 does not contribute to the activation of MSKs, although under appropriate in vitro conditions, PDK1 can bind to and phosphorylate MSKs (Jensen et al., 1999). In addition to ERK, MSKs are also phosphorylated by the p38 MAPK pathway (Deak et al., 1998). This convergence of multiple signaling pathways, (p42/44 and p38), has drawn considerable attention to MSKs. Indeed MSKs have been suggested to contribute to mitogen-mediated CREB phosphorylation (Arthur et al., 2000), chromatin remodeling (Soloaga et al., 2003), neurotrophin-mediated neuronal survival (Arthur et al., 2004), enhanced sensitivity to cocaine induced conditioned place preference (Brami-Cherrier et al., 2005), the pathogenesis of Alzheimer’s disease (Webber et al., 2005), and regulation the mammalian circadian clock (Butcher et al., 2005). The latter observation is discussed in Chapter 5 below.
1.6 Hypotheses

The work described in subsequent chapters of this dissertation will detail our systematic characterization of the MAPK pathway, and its substrates, as central regulators of photic entrainment in the mammalian circadian clock. Testing of the following hypotheses will be discussed below:

Chapter 2 Light-induced behavioral phase shifting in mice is coupled via a MAPK dependent mechanism.

Chapter 3 Light activation of the MAPK pathway (as assessed by phosphorylation of ERK1/2) is stringently regulated with respect to the time course of activation / inactivation and subcellular localization of ERK within neurons.

Chapter 4 Light-induced phosphorylation of RSKs within the SCN is phase restricted to the subjective night and is dependent upon activation of the MAPK pathway.

Chapter 5 Light-induced phosphorylation of MSKs within the SCN is dependent upon a signaling cassette consisting of the neuromodulator PACAP and the MAPK pathway.

Chapter 6 Light-induced phase advances, but not phase delays, will be attenuated in mice lacking MSK. Conversely, light-induced phase delays, but not phase advances will be attenuated in mice lacking RSK.
CHAPTER 2

The p42/44 Mitogen-activated Protein Kinase Pathway Couples
Photic Input to Circadian Clock Entrainment

2.1 Abstract

In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus function as the major biological clock. SCN-dependent rhythms of physiology and behavior are regulated by changes in the environmental light cycle. Currently, the second messenger signaling events that couple photic input to clock entrainment have yet to be well characterized. Recent work has revealed that photic stimulation during the night triggers rapid activation of the p42/44 mitogen activated protein kinase (MAPK) pathway in the SCN. The MAPK signal transduction pathway is a potent regulator of numerous classes of transcription factors and has been shown to play a role in certain forms of neuronal plasticity. These observations led us to examine the role of the MAPK pathway in clock entrainment. Here we report that pharmacological disruption of light-induced MAPK pathway activation in the SCN uncouples photic input from clock entrainment, as assessed by locomotor activity phase. In the absence of photic stimulation, transient

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disruption of MAPK signaling in the SCN did not alter clock-timing properties. We also report that signaling via the Ca$^{2+}$/calmodulin kinase pathway functions upstream of the MAPK pathway, coupling light to activation of the MAPK pathway. Together these results delineate key intracellular signaling events that underlie light-induced clock entrainment.

2.2 Introduction

Within the suprachiasmatic nuclei (SCN) resides an endogenous oscillator that functions as the master biological clock. The biological rhythm generated by the SCN regulates, with near 24-h periodicity, a wide array of cellular, physiological, and behavioral processes (Miller et al., 1996; Turek, 1985). Importantly, the SCN rhythm generator can be entrained by a number of external stimuli, of which light is the most potent. This ability to entrain the clock to photic cues allows animals to adjust their biological rhythms to changes in the external environment.

Recent work has revealed that photic stimulation affects clock timing in part by triggering rapid transcriptional activation in the SCN (Dunlap, 1999). In turn, these newly transcribed gene products are postulated to regulate the clock by resetting a transcription/translation feedback loop that generates the rhythm. Although many postsynaptic events including glutamate receptor activation and increased intracellular calcium have been shown to couple light to entrainment of the circadian clock (Colwell et al., 1991; Mintz et al., 1999; Ding et al., 1998), there is still significant debate regarding the second messenger signaling event that trigger rapid transcriptional activation in the
SCN. Along these lines, several reports have shown that the infusion of broad spectrum calcium/calmodulin kinase (CaMK) inhibitors into the SCN attenuates light-induced phase shifting of the circadian clock (Yodota et al., 2001; Golombeck et al., 1995; Golombeck et al., 1994; Fukushima et al., 1997). Likewise, a role for nitric oxide and protein kinase G has been implicated in light-induced resetting of the clock (Mathur et al., 1996; Weber et al., 1997; Ding et al., 1994; Ding et al., 1997; Ferreyra et al., 1998). More recently, attention has turned to the p42/44 mitogen protein kinase (MAPK) pathway as a potential signaling intermediate coupling light to clock entrainment.

Interest in this pathway results, in part, from recent studies showing that brief exposure to light during the subjective night, but not during the subjective day, triggers rapid and robust activation of the MAPK pathway in the SCN (Obrietan et al., 1998). Furthermore, light-induced activation of ERK colocalizes with and regulates the activation state of the cAMP response element-binding protein (Obrietan et al., 1998), a transcription factor proposed to be a key intermediate coupling photic stimulation to the rapid expression of clock genes (Hida et al., 2000). Additionally, in NIH-3T3 fibroblasts disruption of TPA (12-O-tetradecanoylphorbol-13-acetate)-induced activation of the MAPK pathway blocks circadian gene oscillations (Akashi et al., 2000). Thus, the MAPK pathway fulfills several criteria likely to be essential for light entrainment of the clock: it is rapidly activated by light, its activation is restricted to the subjective night, and the pathway couples to transcription factor activation. These observations raise the possibility that the MAPK pathway plays a central role in the set of signaling events that couple photic input to clock entrainment. Here we report that pharmacological disruption
of light-induced MAPK pathway activation in the SCN uncouples photic input from clock entrainment. The observations presented here reveal that the MAPK cascade functions as an SCN clock input pathway.

2.2 Material and Methods

*Cannulation and Infusion* - Adult (12–16-week-old) male C57/BL6 mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. Fur was then removed from the scalp, and protective eye drops were administered. Next, mice were placed in the stereotaxic apparatus (Cartesian Research), and the coordinates (posterior, 0.3 mm from bregma; lateral, 1.3 mm from the midline; and dorsoventral, -2.2 mm from dura with head level) were used to place the tip of a 24-gauge guide cannula in the lateral ventricle. Although the infusate is administered several millimeters from the SCN, the natural flow of cerebral spinal fluid carries the infusate to the third ventricle, the location of the SCN. In a second group of animals the coordinates (posterior, 0.3 mm from bregma; lateral, 0.0 mm from the midline; and dorsoventral, -4.75 mm from dura) were used to place cannula tips in the third ventricle. Cannulae were held in place with dental cement. A 30-gauge stylus was secured in the cannula to ensure patentcy. After surgery animals were housed individually and allowed to recover for two weeks. A stainless steel injector needle (30 gauge) extending 500 µm from the tip of the guide cannulae was used to infuse either vehicle (Me₂SO) or U0126 (10 nm/µl, Cell Signaling Research) at a rate of 0.40 µl/min. A total volume of 3 µl was delivered. Because of its photolability, care was taken to ensure minimal exposure of U0126 to light. The efficacy of another MEK
inhibitor, SL-327 (50 µg/µl), was also examined. SL-327 was dissolved in Me2SO and infused as described above. SL-327 was kindly provided by Dr. James Trzaskos (DuPont Merck Pharmaceutical Co., Wilmington, DE). Mice were restrained by hand during insertion of the injector needle and allowed to roam freely during infusion. The infusion needle was maintained in the guide cannula for an additional 30 sec after the infusion stopped. Infusions were performed under dim light (15 watt, <1 lux at cage level) using a red safelight (Kodak filter, series 2). As the results obtained using lateral and third ventricle infusions were not statistically different, data from the two groups were combined. Initially another MEK inhibitor, PD 98059, was to be used in this study. However, because of its exceedingly limited solubility, we were not able to utilize a ventricular infusion technique to effectively deliver it to the SCN. KN 62 (10 nM /µl, Biomol) was diluted in Me2SO and infused using the techniques described above.

Circadian Activity Protocol - Cannulated mice were individually housed and entrained to a 12-h: 12-h light/dark (LD) cycle for 14 days before being transferred to dark/dark (DD). Luminescence was provided by fluorescent white light (~100 lux at mid-cage level). During the DD period animals were under conditions of total darkness, except for a weekly food and water replenishment and a change of bedding. Cage maintenance occurred at varying times during the subjective night. During cage maintenance, mice were exposed to a dim red light. Circadian physiology was monitored by recording locomotor activity via a 15-cm diameter running wheel. Closures of a microswitch attached to the wheel were recorded automatically to a PC running Vital View.
(Minimiter Corp., Bend, OR) data acquisition software. Mice were organized into four groups: U0126-infused, no light (control); vehicle-infused, no light (control); U0126-infused, light-treated; and vehicle-infused, light-treated. For the two light-treated groups, 45 min after infusions animals were exposed to white light (50 lux) for 10 min and then returned to darkness. Mice were exposed to light during early subjective night, circadian time 15 (CT15). Control groups not exposed to light were handled in a manner similar to the light-treated mice. Each group was rotated through at least two of the four stimulus paradigms. Mice were maintained under DD throughout the assay period; a minimum of 9 days separated each infusion. The Ohio State University Animal Welfare Committee approved all animal handling and experimental procedures.

*Tissue Collection* - To examine MAPK pathway activation, mice were sacrificed via cervical dislocation, and their brains were removed rapidly under red light. Brains were immersed in chilled, oxygenated physiological saline and then cut into 500 µm coronal sections with a vibratome. Tissue utilized for immunohistochemical examination was fixed in a 5% formaldehyde/phosphate-buffered saline (PBS) solution for 4–6 h at room temperature, cyroprotected with 30% sucrose for 12 h, and finally cut into thin (40 µm) sections with a freezing microtome. Tissue sections destined for Western analysis were frozen immediately on dry ice, and the brain regions of interest were isolated and stored at -80° C.

*Immunohistochemistry* - To determine the ERK activation level, free floating sections were initially blocked with 10% bovine serum albu-min/1% goat serum in PBS
containing 0.1% Triton X-100 (PBST) and 1 mM NaF. Brain sections were immunolabeled (overnight at 4° C) with an affinity-purified rabbit polyclonal antibody (1:500 final dilution, Cell Signaling Research) that detects the dually phosphorylated form of ERK. The tissue was washed six times (5 min/wash) with PBST and then was incubated with an Alexa-488-conjugated secondary antibody directed against rabbit IgG (4 µg/ml final concentration, Molecular Probes). ERK activation levels were also examined using the HRP-ABC technique (Vector Laboratories), following the instructions of the manufacturer. Nickel-intensified diaminobenzidine was used to visualize the signal. Images were captured using a 16-bit digital camera, and data were quantified using Metamorph image analysis software (Universal Imaging). To quantitate fluorescent signal intensity, a coronal-central SCN image was captured with a 10X objective, and a 140 (x-axis) X 180 (y-axis)-pixel oval was placed over the digitized SCN, and intensity was measured. For each section, the SCN signal intensity was normalized by subtracting the immunofluorescent signal from the hypothalamic area immediately lateral to the SCN. Data from three consecutive SCN sections were averaged for each animal analyzed.

Western Blotting - Isolated brain regions were resuspended and sonicated (15 sec) in 50 µl of protease inhibitor buffer (50 mM β-glycerophos-phate, 1.5 mM EGTA, 0.1 mM Na3VO4, 1mM dithiothreitol, 10 µg/ml aprotinin, 2 µg/ml pepstatin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). 50 µl of 6X sample buffer then was added, and the samples were heated to 90 °C for 10 min. 30 µl of extract was
electrophoresed through a 10% SDS-PAGE gel. Following transfer to polyvinylidene difluoride membranes (Immobilon-P, Millipore) and blocking with 10% powdered milk, samples were incubated (4 °C, overnight) in PBST with primary mouse monoclonal antibody against pERK (1:10,000 final dilution; Sigma) followed by goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (1:2,000, PerkinElmer Life Sciences). Immunoreactivity was developed using the Western-star alkaline phosphatase detection system (Tropix). Membranes then were probed for total ERK expression using a goat anti-ERK polyclonal antibody (1:1,000 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 six times (5 min/wash) in PBST with 5% milk. Scion Image analysis software was used to quantitate band intensity. Band intensity for phosphorylated erk-2 was normalized to total erk-2 levels for the corresponding lane. Each experiment was repeated a minimum of three times.

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, in which a line calculating the activity onset for a period of at least 6 days preceding light treatment was determined. This line was extended to project when activity onset should occur during the period after light exposure. A second
regression line was generated to determine activity onset after light administration. Days 3–8 following light treatment were used to generate this line. The difference in the projected versus the actual activity onset after light treatment was the phase shift. Group data are expressed as mean phase shift ± standard error of the mean (SEM). Significance was assessed using the two-tailed Student’s $t$ test.

2.3 Results

To confirm that photic stimulation elicits MAPK pathway activation in the SCN, animals were exposed to light (10 min, 50 lux) during the early night (3 h after lights off; zeitgeber time 15, ZT 15) and then immediately sacrificed. SCN tissue was processed using immunofluorescent staining techniques for the activated (i.e. dually phosphorylated, Thr202/Tyr204) form of erk-1 and erk-2 (here, collectively referred to as PERK). This dual phosphorylation event is essential for ERK enzymatic activity and is a marker for MAPK pathway activation. Relative to control animals not exposed to light, photic stimulation triggered robust activation of the MAPK cascade in the SCN (Fig. 2.1A). P-ERK was observed from the ventral to dorsal regions of the SCN. Western blotting was also used to examine the activation of ERK (Fig. 2.1, B and C). Light triggered the expression of the catalytically active form of ERK in the SCN; modest levels of activation were observed in tissue isolated from the lateral hypothalamus and piriform cortex. Collectively, these data reveal that photic stimulation triggers robust SCN-specific activation of the MAPK pathway.
FIGURE 2.1 Light induces MAPK pathway activation. A, cannulated mice were infused with the MEK inhibitor U0126 (10 nmol/µl, A3) or with drug vehicle (A1, A2). Relative to control mice (no light, A1), light (50 lux, 10 min; ZT 15) triggered robust ERK activation (A2). Infusion of U0126 45 min before photic stimulation (A3) depressed light-induced ERK activation. B, similar results were observed by Western analysis of excised SCN. Light-induced ERK activation (pERK) was blocked by infusion of U0126. The blot was then stripped and probed for total ERK expression. To assess ERK activation, the erk-2 phosphorylation level was divided by the total erk-2 level for each lane. These data are expressed as fold stimulation relative to the erk-2 activation level under control conditions (no light, no U0126), which was set equal to one. C, robust light-induced ERK activation is specific to the SCN; modest induction was observed in tissue isolated from the lateral hypothalamus (lateral hypo) and piriform cortex (CTX). Membranes were also probed for total ERK expression. To quantify ERK activation levels, the erk-2 phosphorylation level was divided by the erk-2 level for each lane and expressed as fold stimulation relative to the control (no light) ERK activation level for the corresponding brain region, which was set equal to one. Each experiment was repeated a minimum of three times. 3v, third ventricle; oc, optic chiasm.
FIGURE 2.1 Light induces MAPK pathway activation.
To disrupt MAPK signaling in the SCN we employed a ventricular infusion technique. Mice were infused with the specific MEK inhibitor U0126 (10 nmol/μl, 20) 45 in prior to photic stimulation. To determine whether U0126 administration disrupted MAPK pathway activation infused animals were light flashed and sacrificed, and SCN-containing brain sections were processed for expression of activated ERK (Fig. 2.1, A and B). Infusion of the MEK inhibitor significantly attenuated (typically ~90%) light-induced ERK activation in the SCN. Similar results were obtained with a second MEK inhibitor, SL-327 (data not shown). For the behavioral studies described below, it was imperative to determine the diffusion pattern of U0126 in the brain. Thus, to track its penetration into periventricular brain regions, mice were infused with the MEK inhibitor and then, 45 min later, intraperitoneally injected with kainate (to trigger MAPK pathway activation throughout the brain) and exposed to light (15 min, 50 lux; ZT 16).

A region lacking ERK activation centered around the infusion needle delineates the area of U0126 diffusion and inhibition. Using this technique, we found that U0126 did not broadly diffuse (Fig. 2.2B) and that inhibition was limited to within ~400 μm of the ventricle. Importantly, relative to the control animals (Fig. 2.2A), U0126 infusion blocked ERK activation within the SCN. U0126-mediated inhibition of MAPK pathway activation was transient, lasting for ~90 min (data not shown). These data reveal that U0126 infusion is localized to the periventricular brain regions. Thus, the area of inhibition is not so great as to raise considerable doubt about the site of action.

Wheel-running activity was used as a circadian output to address the contribution of the MAPK pathway to light entrainment of the clock. Animals were initially entrained to
an LD cycle for 14 days and then released into total darkness (DD). In the absence of external timing cues, overt circadian rhythms are governed by the SCN clock. After 9–12 days in DD animals were infused with either drug vehicle or U0126 at CT 14.25 and exposed to light 45 min later (50 lux, 10 min). A marked phase delay in the onset of wheel-running activity was elicited by light exposure after vehicle infusion (Fig. 2.3, A, B, and D).

**FIGURE 2.2 MEK inhibition in vivo.** To track the diffusion pattern of U0126 from the ventricle into the brain, mice were infused with the MEK inhibitor (10 nmol/µl) and then, 45 min later, intraperitoneally injected with kainate (30 mg/kg). Mice also were exposed to light (50 lux, 10 min; ZT 16). By infusing the MEK inhibitor and then stimulating MAPK pathway activation, we were able to track U0126 diffusion. Using immunohistochemical analysis of ERK activation, we found that U0126 did not diffuse broadly from the infusion site (B, dashed line denotes the approximate area of MAPK pathway inhibition; the arrow identifies the location of guide cannula/infusion needle tip). The vehicle-infused control animal (A, intraperitoneally injection with kainate, light exposure) exhibited high levels of ERK activation in periventricular regions including the SCN. Bar, 500 µm.
FIGURE 2.3 Disruption of light-induced MAPK pathway activation uncouples light from circadian clock entrainment. A, representative double-plotted actogram from a mouse initially entrained to a standard LD cycle was transferred to DD and then infused with drug vehicle (Me$_2$SO$_4$) 45 min before light (50 lux, 10 min) exposure at CT 15 (asterisk). 11 days later the same animal was infused with U0126 (10 nmol/µl) and exposed to light (asterisk). B, in a second representative trace the order of drug/vehicle infusions was reversed, but the effect was the same as in A. C, acute disruption of the MAPK pathway during the subjective night (asterisk) did not phase shift the clock. D, mean ± S.E. of the phase-delaying effects of light at CT 15. Numbers above bars denote sample sizes for each condition. **, p < 0.0001; two-tailed Student’s t test.

In striking contrast, U0126 infusion blunted this light-induced phase shift, indicating that disruption of signaling via the MAPK pathway uncouples light from clock entrainment. U0126 infusion reduced the light-induced phase shift by ~90% in 60% of
mice (n = 20). In the absence of photic stimulation, the acute disruption of MAPK signaling did not influence clock timing (Fig. 2.3, C and D). In a preliminary round of experimentation (data not shown) we found that the infusion of lower concentrations (<2 nmol/µl) of U0126 that partially blocked (40–60%) light-induced ERK activation did not dramatically alter the phase shifting effects of light.

To corroborate the effects of U0126, we tested the efficacy of another MEK inhibitor, SL-327. Similar to the experiments described above, infusion of SL-327 (50 µg/µl) significantly attenuated light-induced phase shifting of the clock (Figs. 2.3D and 4). As with U0126, the infusion of SL-327 in the absence of photic stimulation did not significantly alter clock phase. Collectively these data identify a role for the MAPK pathway as a signaling intermediate coupling light to clock entrainment. Several studies have reported that CaMK signaling couples light to clock entrainment (Yokota et al., 2001; Golombek et al., 1995; Golombek et al., 1994; Fukushima et al., 1997), raising the possibility that the MAPK and CaMK pathways work in parallel to affect downstream targets. Another possibility is that these pathways are also in series, with the CaMK pathway triggering MAPK cascade activation (Wu et al., 2001; Watt et al., 2000; Soderling et al., 1999). To assess whether CaMKs trigger MAPK activation in the SCN, mice were infused with the broad acting CaMK inhibitor KN 62 at ZT 14.25. 45 min after infusion, mice were exposed to light and sacrificed, and SCN-containing sections were processed for ERK activation. Relative to vehicle-infused animals, the infusion of the CaMK inhibitor significantly attenuated light-induced ERK activation (Fig. 2.5). In the absence of light, the low basal expression of activated ERK was not significantly affected.
by CaMK inhibition. Together, these data suggest that CaMK signaling functions as an upstream regulator of the MAPK pathway in the SCN.

FIGURE 2.4 Infusion of the MEK inhibitor SL-327 depresses light-induced phase shifting of the circadian clock. A representative double-plotted actogram from a dark-adapted mouse initially infused with drug vehicle (Me₂SO₄) 45 min before light (50 lux, 10 min) exposure at CT 15 (asterisk). Thirteen days later animal was infused with SL-327 (50 µg/µl) and exposed to light (asterisk). In the absence of photic stimulation, the infusion of SL327 (asterisk) did not dramatically alter clock phase. Please refer to the legend to Fig. 3D for quantification of the effects of SL-327.
FIGURE 2.5 Disruption of CaMK signaling in the SCN attenuates light-induced activation of the MAPK pathway. A, mice were initially infused with either drug vehicle or KN 62 (10 nmol/µl) and then, 45 min later (ZT 15), exposed to light (50 lux, 10 min) and immediately sacrificed. Control animals (no light) were sacrificed 60 min after infusions. Infusion of KN 62 did not significantly alter basal ERK activation levels. B, quantitation of pERK fluorescent intensity (0–255 scale). Numbers above bars denote sample sizes for each condition. Asterisk, p < 0.005; two-tailed Student’s t test. Error bars, S.E.
2.4 Discussion

Signaling via the MAPK pathway in the central nervous system has recently become a topic of intense interests. For example, a role for the MAPK pathway has been identified in such diverse central nervous system processes as addiction, programmed cell death, and learning and memory (Valjent et al., 2000; Hetman et al., 2000; Impey et al., 1999). Along these lines, stimulus protocols that trigger hippocampal long-term potentiation also activate the MAPK pathway (English et al., 1996). More importantly, disruption of signaling via the MAPK cascade uncouples stimulation from transcriptionally dependent forms of long-term potentiation formation, indicating a causal link between the MAPK pathway and stimulus-dependent strengthening of synaptic efficacy (English et al., 1997; Impey et al., 1998). Perhaps the most dramatic example of MAPK pathway activation in the central nervous system is observed in the SCN following photic stimulation (Obrietan et al., 1998). Interest in this pathway is derived in part from its rapid activation following photic stimulation, its phase-dependent activation, and its ability to potently regulate the activation state of numerous classes of transcription factors. These observations led us to examine the contribution of signaling via the MAPK pathway to light-induced clock entrainment. Here we show that disruption of light-induced MAPK pathway activation in the SCN blocks photic input from clock entrainment. In a large percentage of animals, infusion of U0126 led to a dramatic (~90%) reduction in light-induced phase shifting, suggesting that the MAPK pathway plays a central role in the light-entrainment process.
However, a second group of animals exhibited varying amounts of inhibition (20–90%) of light-induced phase shifting of the circadian clock. One interpretation of this result is that infusion did not lead to effective MAPK pathway inhibition. Indeed, we have observed that partial inhibition (40–60%) of light-induced ERK activation did not dramatically affect the ability of light to entrain the clock. Given that the MAPK pathway signal is amplified both spatially and temporally downstream of MEK (the target of U0126 and SL327), partial MEK inhibition may have only marginal effects on cellular targets of the MAPK pathway. This difference in the efficacy of inhibition may explain the discrepancy between our work and that of Yokota et al. (Yokota et al., 2001).

It should be noted that U0126 is a recently developed inhibitor that specifically antagonizes the activity of both MEK 1 and MEK 2 without detectable effects on other kinases such as PKA, PKC, Raf, ERK, MSK I, and JNK (Favata et al., 1998; Davies et al., 2001). Likewise, the U0126 analog SL-327 is specific to MEK 1 and MEK 2 and does not have measurable effects on PKA, PKC, and CaMK II (Favata et al., 1998; Selcher et al., 1999). Furthermore, based on a number of studies that have utilized an infusion protocol to disrupt MAPK signaling in the central nervous system, it appears that the MAPK pathway specifically affects transcriptionally dependent forms of neuronal plasticity and does not alter such fundamental neurophysiological processes as sensory, motivational, or motor abilities (Berman et al., 1998; Blum et al., 1999). Even the intraperitoneal injection of SL327 did not affect sensory perception or arousal (Atkins et al., 1998). Importantly, no long-term residual effects of MAPK pathway disruption were observed in any of these studies.
The activation state of the MAPK pathway varies as a function of circadian time in the SCN, showing maximal stimulation during the subjective day and low levels of activity during the early to mid-subjective night (Obrietan et al., 1998). This observation raised the possibility that the infusion of U0126 may alter clock phase and therefore preclude us from determining the sight of drug action. However, we found that the acute disruption of MAPK activation did not alter circadian clock timing, indicating that the effects on light-induced phase shifts resulted from inhibition of the photic input signal. The absence of an effect of U0126 infusion on clock phase might result from the transient nature of U0126 inhibition and the relatively low basal level of MAPK pathway activation during the time of the infusion (CT 14.25).

A number of studies have implicated a variety of kinase pathways in the light entrainment process. For example, disruption of nitric oxide synthetase activity blocks phase shifting (Ding et al., 1997). Likewise, a role for the CaMKs has also been identified (Yokota et al., 2001; Golombek et al., 1995; Golombek et al., 1994; Fukushima et al., 1997). Interestingly, in several model systems, signaling via nitric oxide and CaMKs have been shown to function as upstream regulators of the MAPK pathway (Watt et al., 2000; Yun et al., 1998). With respect to CaMK signaling, our data support this model. We observed that infusion of the CaMK inhibitor KN 62 attenuated light-induced ERK activation. Similar results have been found in hippocampal neurons, where depolarization-induced ERK activation is attenuated by disruption of CaMK activation (Wu et al., 2001). One possible mechanism by which CaMK signaling leads to MAPK pathway activation involves CaMK II inhibition of the GTPase-activating protein p135...
SynGAP (Chen et al., 1998). It is important to note that we are not ruling out other mechanisms by which CaMK signaling affects the clock. Indeed, it is probable that CaMK IV plays a significant role in coupling light to transcription factor activation in the SCN. Thus, there are likely to be a number of essential signaling pathways that function in an orchestrated manner to couple light to clock entrainment.

Signaling via the MAPK pathway has become an area of investigation for circadian biologists working in both invertebrate and vertebrate model systems (Ko et al., 2001; Williams et al., 2001; Hayashi et al., 2001). In particular, MAPK signaling has been shown to function as an output pathway from the clock (Ko et al., 2001) and may play a role in the core clock timing mechanism (Sanada et al., 2002). The observations described here are unique in that they reveal a role for the MAPK cascade as a signaling intermediate coupling photic input to circadian clock entrainment.
CHAPTER 3

Temporal Regulation of Light-Induced Extracellular Signal-Regulated Kinase Activation in the Suprachiasmatic Nucleus

3.1 Abstract

Signaling via the p42/p44 mitogen activated protein kinase (MAPK) pathway has been implicated as an intermediate event coupling light to entrainment of the mammalian circadian clock located in the suprachiasmatic nucleus (SCN). To examine how photic input dynamically regulates the activation state of the MAPK pathway, we monitored extracellular signal-regulated kinase (ERK) activation using different light stimulus paradigms. Compared with control animals not exposed to light, a 15 min light exposure during the early night triggered a marked increase in ERK activation and the translocation of ERK from the cytosol to the nucleus. ERK activation peaked 15 min after light onset, then returned to near basal levels within ~45 min. The MAPK pathway could be reactivated multiple times by light pulses spaced 45 min apart, indicating that the MAPK cascade rapidly resets and resolves individual light pulses into discrete signaling events. Under conditions of constant light (120 min), the time course for ERK activation, nuclear translocation, and inactivation was similar to the time course observed after a 15-min light treatment. The parallels between the ERK inactivation profiles

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elicited by a 15 and a 120 min light exposure suggest that SCN cells contain a MAPK pathway signal-termination mechanism that limits the duration of pathway activation. This concept was supported by the observation that the small G protein Ras, a regulator of the MAPK pathway, remained in the active, GTP-bound, state under conditions of constant light (120-min duration), indicating that photic information was relayed to the SCN and that SCN cells maintained their responsiveness for the duration of the light treatment. The SCN expressed both nuclear MAPK phosphatases (MKP-1 and MKP-2) and the cytosolic MAPK phosphatase Mkp-3, thus providing mechanisms by which light-induced ERK activation is terminated. Collectively, these observations provide important new information regarding the regulation of the MAPK cascade, a signaling intermediate that couples light to resetting of the SCN clock.

3.2 Introduction

The endogenous pacemaker located in the suprachiasmatic nucleus (SCN) regulates numerous biochemical, physiological, and behavioral processes with ~24 h periodicity (Allada et al. 2001; Miller et al. 1996; Reppert and Weaver 2001). The inherent timing mechanism of the SCN clock is under the control of external zeitgebers (time cues). Of all the zeitgebers, light is the most effective and certainly the best characterized (Foster and Helfrich-Forster 2001; Lowrey and Takahashi 2000; Rea 1998). Photic information is relayed from the retina to the SCN via the retinohypothalamic tract (RHT). In response to photic stimulation, glutamate is secreted from RHT nerve terminals (Colwell and Menaker 1992; Liou et al. 1986), thereby triggering a cascade of intracellular signaling
events that ultimately impinge on and reset the core clock timing mechanism.

Light-induced resetting of the circadian clock is dependent on transcription activation (Cermakian and Sassone-Corsi 2002; Lowrey and Takahashi 2000). Indeed, work over the past several years has shown that exposure to light during the night triggers the expression of immediate early gene transcription factors such as Fos, EGR-1, and JunB (Aronin et al. 1990; Kornhauser et al. 1990, 1992; Rusak et al. 1990, 1992), and the core clock genes Period 1 and 2 (Albrecht et al. 1997; Zylka et al. 1998).

Both the large number of light-inducible genes and the rate at which they are transcribed indicates that SCN neurons contain second-messenger signaling pathways that are poised to couple photic stimulation to transcriptional activation. One SCN signaling intermediate that has garnered recent attention is the p42/44 mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway is a signaling cassette formed by three kinases: RAF, MEK, and ERK. In neurons, the MAPK pathway is activated by a large array of stimuli, including trophic factors, neurotransmitters, and modulatory peptides (Cavanaugh et al. 2001; Chang and Berg 2001; Dziema and Obrietan 2002; Kurino et al. 1995; Yan et al. 1999). This diverse group of signaling molecules couples to the MAPK pathway via Ras-or Rap-1-dependent mechanisms. Once in the activated, GTP-bound, form, Ras stimulates RAF, which in turn activates MEK. MEK then stimulates ERK, thus allowing ERK to disassociate from MEK, dimerize, and translocate to the nucleus (Adachi et al. 1999; Fukuda et al. 1997; Khokhlatchev et al. 1998). The nuclear translocation of ERK is a key intermediate event that couples the MAPK pathway to transcriptional activation (Cobb 1999; Grewal et al. 1999; Treisman 1996).
Interest in this pathway stems from work showing that the MAPK cascade is activated by light in a phase-dependent manner in the SCN (Obrietan et al. 1998) and that the in vivo disruption of the MAPK cascade attenuates both the phase shifting effects of light (Butcher et al. 2002; Coogan and Piggins 2003) and immediate early gene expression (Butcher et al. 2002; Dziema et al. 2003). Additional evidence supporting a role for the MAPK pathway as a signaling intermediate comes from a zebra fish cell culture system, where the expression of Period 2 is blocked by disruption of the MAPK cascade (Cermakian and Sassone-Corsi 2002). Likewise, in cultured fibroblasts, the pharmacological disruption of MAPK pathway signaling blocks 12-O-tetradecanoyl phorbol 13-acetate (TPA)-mediated clock gene expression (Akashi and Nishida 2000).

Taken together, these reports identify potential mechanisms by which the MAPK cascade couples extracellular stimuli to the clock timing mechanism as well as illustrate the need for further investigations into the processes regulating activation of this pathway. Here, we provide data on the subcellular and temporal regulation of light-induced ERK activation in the SCN. These data provide new mechanistic insights into how light and the SCN regulate the activation state of the MAPK cascade.

3.3 Materials & Methods

*Animals* - Adult C57BL6 mice (8–12 wk of age) were used for all experiments. All animal procedures were in accordance with Ohio State University animal welfare guidelines.
Light Exposure Paradigms -

SINGLE LIGHT PULSE - Initially animals were entrained to a 12:12 h light-dark (LD) cycle for >3 wk and then divided into three groups: 15-min single light pulse (SLP), constant light (CL), and no light, control animals. After photic stimulation (white light, 100 lux at cage level), animals in the SLP group were returned to darkness for 0, 15, 30, 45, 75, and 105 min, then killed. Animals in the CL group were exposed to light for 15–120 min, then killed. Control animals were handled in a similar fashion but were not exposed to light.

MULTIPLE LIGHT PULSES - Animals were presented with one, two, or three light pulses (15 min, 100 lux each). They were then returned to darkness for 45 min between each light pulse and killed immediately after the final light pulse. Another group of animals received two light pulses and were returned to darkness for an additional 60 min prior to death.

Tissue Processing - Mice were killed via cervical dislocation under dim red (Kodak Series 2 red filter, <1 lux at cage level) illumination. In preliminary experiments, we found that exposure to the red light source (10 min at circadian time 15: CT 15) did not alter the phase of activity onset, as assessed by wheel running activity. To block postmortem photic stimulation, the eyes were covered with opaque black tape until optic nerves had been severed. Brains were then excised under normal room lighting, placed in chilled oxygenated physiological saline, and cut into 500 µm coronal or horizontal sections using a vibratome. Tissues used for immunohistochemical analysis were placed
in formaldehyde/phosphate-buffered saline (PBS, 5% wt/vol) for 4 >hrs followed by
cryoprotection in 30% sucrose (wt/vol) containing 3 mM NaF for >12 hrs. Tissue
sections were then thin cut (40 µm) using a freezing microtome.

*Circadian Activity Protocol* - Animals were housed individually and entrained to a 12:12
LD cycle for >14 days before being transferred to dark/dark (DD). Wheel-running
activity was used to monitor circadian time. Thus each cage was equipped with a 15-cm-
diam running wheel. Closures of magnetic microswitches attached to the running wheels
were automatically recorded by a personal computer running Vital View data-acquisition
software (Minimitter, Bend, OR). After 9 days in DD, half of the animals received a 15
min, 500 lux light pulse 3 hrs after the beginning of the subjective night (CT 15). The
other half of the mice received a 120 min pulse of the same intensity at CT 15. After the
stimuli, all animals were returned to DD, permitted to free-run for 14 days, and then
presented with the alternate light pulse paradigm.

*Light-Induced Phase Shift Analysis* - The least-squares linear regression approach
described by Daan and Pittendrigh (1976) was used to determine the phase-shifting
effects of light. To this end, a regression line was used to determine the periodicity of
activity onset for a period of >6 days preceding light treatment. This line was extended
through the period after light exposure to predict when activity onset should occur. A
second regression line was fitted through the actual activity onset after light
administration. Days 3–10 after light treatment were used to generate this line. The
difference between the projected and the actual activity onset was the light-induced phase shift. Significance was assessed using the two-tailed Student’s $t$-test and data are expressed as mean phase shift ± SE.

**Immunohistochemistry** - Thin (40 µm), free-floating sections containing central SCN were blocked for 1 h with 10% goat serum in PBS containing 1% Triton X-100, 1 mM NaF, and 0.02% Na azide (PBST). After five rounds of washing (5 min/wash in PBST), tissue was double labeled by incubation with an affinity-purified rabbit polyclonal antibody that detects the dually phosphorylated form of ERK (pERK: 1:500 dilution, Cell Signaling) and with a mouse monoclonal antibody against NeuN (1:500, Chemicon International) overnight at 4°C. After primary antibody treatment, the tissue was washed five times in PBST, then incubated (4 h, room temperature) with an AlexaFluor-594 conjugated goat anti-rabbit IgG antibody (1:500, Molecular Probes) and an AlexaFluor-488 conjugated goat anti-mouse IgG antibody (1:500, Molecular Probes). After a final wash cycle (5 times, 5 min/wash) sections were mounted and coverslipped.

**Image Analysis** - Immunofluorescence photomicrographs were captured using a 16bit digital (Micromax YHS 1300: Princeton Instruments) camera mounted on an inverted epi-fluorescence microscope (Leica DM IRB), and quantified using Metamorph software (Universal Imaging). To quantitate fluorescent signal intensity, coronal SCN-containing images were captured with a 10X objective, and a 150 ($x$ axis) X 200 ($y$ axis)-pixel oval or crescent was placed over the digitized regions of interest (see Fig. 3.1B). The average
signal intensity was measured for each SCN and normalized by subtracting the mean fluorescence level recorded in the lateral hypothalamus immediately adjacent to the SCN. Light-induced pERK data are presented as either the fold increase relative to control animals not exposed to light or absolute pERK intensity values using a 0–255 intensity scale. Data were collected from four animals per time point.

Confocal images were obtained using a Biorad MRC 1024 scanning laser confocal microscope. For each animal, a 10 µm (rostrocaudal) Z series was captured through the ventral part of the central SCN. Five optical sections (2 µm thickness) were scanned for pERK and NeuN expression at 40X magnification. Metamorph software was used to digitally overlay the pERK and NeuN fluorescence signals and to quantify pERK intensity. NeuN labeling was used to identify cellular nuclei. Thus digital ovals outlining the NeuN staining were transferred to the pERK image and used to quantify nuclear pERK intensity. The nuclear pERK signal was then digitally subtracted from the pERK image and the remaining immunolabeling was defined as the “non-nuclear” signal. Data were collected from three animals per condition. Statistical significance was determined for all experiments using the two-tailed Student’s t-test.

Western Analysis - Samples containing the SCN and minimal surrounding hypothalamic tissue were dissected by hand from 500 µm coronal sections and sonicated in 50 µl of HEPES buffer (15 mM HEPES, 0.25 M sucrose, 60 mM KCl, 10 mM NaCl, 2 mM NaF, 2 mM Na pyrophosphate, and a protease inhibitor cocktail: complete mini tablet, Roche Diagnostics). Additional tissue was collected from the piriform cortex and processed in a
similar manner. Fifty µl of 6X sample buffer was added, and lysates were heated to 90°C for 10 min. A 25-µl volume of extract from each sample was electrophoresed through a 10% SDS-PAGE gel, transferred to PVDF membranes (Immobilon P: Millipore), and blocked with 10% (wt/vol) powdered milk in PBST for 1 hr. Membranes were probed for phosphatase expression using rabbit anti-MKP-1 and -2 polyclonal antibodies (1:1000, Santa Cruz) followed by a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP, 1:2,000, New England Nuclear, NEN). HRP was detected using Renaissance chemiluminescent HRP substrate (NEN). The membranes were then stripped and probed for ERK expression using a rabbit anti-ERK 1/2 polyclonal antibody (1:1,000, Santa Cruz). The signal was detected as described in the preceding text. Membranes were washed four times (10 min/wash) in 5% milk/PBST between each antibody treatment.

*Ras Activation Assay* - Animals were killed at zeitgeber time (ZT) 15.25–17 after exposure to light for either 15 or 120 min (100 lux). Control animals not exposed to light were killed at ZT 16. As described in the preceding text, brains were rapidly isolated, then cut into 500 µm coronal sections, and the SCN was manually dissected and pooled from three animals for each condition. Next, tissue was sonicated and digested in lysis buffer, then centrifuged. After protein determination, the samples were divided into 50 and 15 µg aliquots. Raf-1 RBD agarose beads (Upstate Biochem., Lake Placid, NY) were incubated with the 50-µg samples for 45 min then pelleted and washed three times in lysis buffer. Fifteen µl of 3X SDS loading buffer was added, and samples were boiled for
5 min, run on a 10% SDS-PAGE gel, and transferred to PVDF membranes (Immobilon P: Millipore). Blots were blocked with 5% (wt/vol) powdered milk in PBST for >1 hr then probed with an anti-Ras mouse monoclonal antibody (Upstate Biochem.). The 15-µg aliquot was run on a 10% SDS-PAGE gel, and membranes were probed for total Ras levels. Membranes were incubated with an HRP-conjugated goat anti-mouse IgG secondary antibody and the signal was visualized as described in the preceding text. Each experiment was repeated a minimum of three times.

**RT-PCR** - Animals were killed at ZT 15, and the SCN and piriform cortex were excised from 500-µm-thick coronal brain sections. Total RNA was isolated with TRIzol Reagent (Invitrogen Life Technologies) following the manufacture’s guidelines. RNA was reverse transcribed using the Superscript First Strand cDNA Synthesis System (Invitrogen), and Mkp-3 was amplified using the primer set described by Wellbrock et al. (2002): 5’-ATCCCGGGCATCATGCTGC-3’ and 5’-TGGGACAGGTTTTGGCTCC-3’.

3.4 Results

**Light-Induced MAPK Pathway Activation**

Initially, we verified that the light treatment paradigm used here elicits activation of the MAPK pathway. Thus animals were exposed to light (100 lux, 15 min) 3 hrs after lights off (ZT 15). After light exposure, animals were immediately killed, and brains were cut in either coronal or horizontal planes through the central SCN. Sections were then immunolabeled for the activated (i.e., dually phosphorylated) form of erk-1 and erk-2
(pERK), a marker of MAPK pathway activation. In agreement with previous work (Butcher et al. 2002; Obrietan et al. 1998), light produced a marked increase in the activated form of ERK relative to control animals not exposed to light (Fig. 3.1). Induction was observed throughout both the rostrocaudal and dorsoventral extent of the SCN.

To gain an understanding of the cellular mechanisms that influence light-induced MAPK cascade activity in the SCN, we tested the effects that a short light pulse and a long light treatment have on the duration of ERK activation in the ventral SCN. The ventral SCN region examined (Fig. 3.1 B: black dashed oval) falls within the retinoreceptive “core” sub-region of the SCN.

**FIGURE 3.1** Light-induced mitogen-activated protein kinase (MAPK) pathway activation in the suprachiasmatic nucleus (SCN). Coronal and horizontal SCN-containing sections were collected from control mice not exposed to light (no light: control) and from animals exposed to light (light pulse: 15 min, 100 lux) 3 hrs into the night (ZT 15). The activation state of the MAPK pathway was detected by fluorescence immunolabeling for the phospho-activated form of extracellular signal-regulated kinase (ERK). Relative to control animals (A), light triggered a robust increase in MAPK pathway activation within the SCN (B). Areas within the white-boxed regions appear at higher power to the right. Areas outlined in black represent the dorsal (crescent shape) and ventral (dashed oval) SCN regions used for quantitation. OC, optic chiasm; ON, optic nerve; OT, optic tract; 3V, 3rd ventricle. Scale bars = 100 µm for low-magnification images and 50 µm for high-magnification images.
FIGURE 3.1 Light-induced mitogen-activated protein kinase (MAPK) pathway activation in the suprachiasmatic nucleus (SCN).
In the first experiment, animals were exposed to constant light (CL) (100 lux) for 15, 30, 45, 60, 90, or 120 min, then killed. In the second experiment, mice were exposed to a single 15 min (100 lx) light pulse (SLP), returned to darkness for 0, 15, 30, 45, 75, and 105 min, then killed. As expected, both CL (Fig. 3.2 A) and a SLP (Fig. 3.2 B) triggered an initial period of ERK activation that was significantly above basal levels (*, \( P < 0.0001 \): Fig. 3.2C). In the SLP animals, activated ERK levels decayed rapidly after cessation of the light pulse, dropping to \( \sim 50\% \) of the peak value 15 min after the light pulse was terminated (Fig. 3.2, B and C). By 60 min post light onset, pERK levels in SLP animals returned to near baseline values and remained at this level throughout the remaining observations. Interestingly, after the initial burst of MAPK pathway activation, pERK levels in CL animals began to decay, exhibiting an inactivation pattern similar to the one initiated by a SLP, although pERK levels in CL animals did remain higher than both control and the SLP animals (\( P > 0.05 \)) from 60 min post light onset until the end of the experiment (Fig. 3.2 C). Integrating the pERK signal over the 2 hr period revealed that CL elicited a 43% greater level of ERK activation than the SLP (Fig. 3.4 C). A similar inactivation profile was observed using a more intense light stimulus (500 lux: data not shown). Together, these data reveal that the duration of light-induced MAPK pathway activation is determined by both the length of stimulation and by an inherent signal termination process that may be located in the SCN. The SCN signal-termination process appears to dominate, regulating the duration of maximal ERK activation even in the presence of CL.
As noted in the preceding text, light-induced ERK activation was also observed in the dorsal SCN. Quantitative analysis of the dorsal SCN (Fig. 3.1 B, region within the solid black outline) revealed that light triggered a significant ($P < 0.0001$) increase in ERK activation relative to control animals (data collected at the LP +0 time point, Fig. 3.2 B). Although light triggered ERK activation in the dorsal SCN, the mean intensity of induction was only 18% of the level observed in the ventral SCN. The ERK inactivation profile in the dorsal region of the SCN was similar to the ERK inactivation profile in the ventral SCN (data not shown).

In an attempt to clarify where within the series of intracellular events termination of MAPK signaling occurs, we monitored the activation state of Ras. Ras is a small membrane-associated GTPase that couples extracellular stimulation to the MAPK cascade (Cullen and Lockyer 2002). A 15 min light treatment triggered an increase in the GTP-bound form of Ras, indicating a light-induced increase in Ras activity (Fig. 3.2 D). Interestingly, even after 120 min of CL, the activated form of Ras was detected. Given that GTP hydrolysis occurs rapidly after termination of Ras stimulation, these findings indicate that photic information is still being propagated to the SCN and that SCN cells are still responsive to the stimulus after a 120 min exposure to light. Thus given that Ras is activated after 120 min of continuous light but that ERK activation level is markedly attenuated, these data suggest that SCN cells contain a signal termination mechanism that limits the duration of MAPK pathway activation.

In addition to its regulation by light, the MAPK pathway is also regulated by circadian timing mechanisms. For example, a subset of cells within the central SCN
exhibit high levels of activated ERK during the night (Lee et al. 2003; Obrietan et al. 1998; Fig. 3.2 E). Recently, Nakaya et al. (2003) reported that this endogenous pERK signal was suppressed 60 min after light exposure at circadian time 14 (CT 14). To estimate how long the endogenous pERK signal remained suppressed, we examined the central SCN for pERK expression at the 90 and 120 min (Fig. 3.2 E) post-light exposure time points. At both times, pERK was observed in the central SCN, indicating that rhythmic control of pERK expression is transiently repressed after light exposure.

**FIGURE 3.2 Time course for light-induced ERK activation.** Animals were exposed to either constant light (CL: from 15–120 min) or a single 15 min light pulse (100 lux, ZT 15) followed by a return to darkness for +105 min. A: representative pERK-labeled SCN sections from animals exposed to light for 15, 30, and 120 min. Control animals (no light) were not exposed to light. B: representative pERK-labeled SCN sections from animals exposed to a SLP and killed 15 min (LP +15’), 105 min (LP +105’), or immediately after (LP+0’) termination of the light pulse. Robust ERK activation is observed at the LP +0’ and LP +15’ time points. pERK returned to near basal levels by 105 min after light exposure. Note the similarity in the time course of ERK inactivation under the light pulse and constant light conditions. Boxed regions are shown at higher resolution in E. C: immunolabeling data expressed as the relative increase in pERK levels in the ventral SCN compared with control animals not exposed to light (0 –255 intensity scale). Error bars denote SE. Data were collected from 4 animals per condition. Both SLP and CL animals had a significant increase in pERK after light onset (*, P<0.001). At the 60-, 90-, and 120-min time points, the CL group had significantly greater levels of pERK than either the control or SLP animals (P > 0.05). D: animals were initially exposed to light (100 lux) for 15 or 120 min, then immediately killed, and SCN tissue was probed for the GTP-loaded (activated) form of Ras. Relative to control animals not exposed to light, a marked increase in the activated form of Ras was detected after both a 15 and 120 min exposure to light. Data are representative of triplicate determinations. E: circadian-regulated pERK expression is observed in the central SCN before and 120 min after photic stimulation. Micrographs are enlargements of the boxed regions in B, cells with high pERK levels.
FIGURE 3.2 Time course for light-induced ERK activation.
Subcellular ERK Localization After a Light Stimulation

Sustained MAPK pathway activation results in the translocation of activated ERK from the cytosol to the nucleus (Lenormand et al. 1993; Traverse et al. 1992). To address the subcellular distribution of pERK after photic stimulation, tissue was double labeled for pERK and for the neuronal specific nuclear marker NeuN, and confocal sections were captured through the central SCN. The same time points used in the SLP experiment and the CL experiments were used in this experiment to determine the percentage of SCN neurons with activated ERK, the subcellular localization of the kinase and the duration of activation.

We found a significant increase in pERK expression over control (no light) mice in both nuclear and non-nuclear regions immediately after light stimulation (Fig. 3.3 A, LP +0'). At this time pERK levels were ~30% higher in the nuclear than the non-nuclear region (Fig. 3B: * = P < 0.005), indicating a rapid nuclear translocation and accumulation of the kinase. Nuclear pERK levels reached a maximum value 15 min after termination of the light pulse, and then began to decline toward basal levels. By 45 min after light stimulation, pERK values returned to near the control level in both regions.

We also examined the subcellular distribution of activated ERK using our CL stimulus paradigm (Fig. 3.3 C). The data collected under conditions of CL were similar to the results collected with the short pulse paradigm: rapid ERK accumulation in nucleus followed by a decrease in ERK activation to near baseline levels. One major difference was the duration of activated ERK expression in the nucleus. Under the CL treatment condition, elevated levels of pERK were observed at the 60 min time point (Fig. 3.3 C),
whereas in the SLP animals, activated ERK had returned to control (no light) levels by 60 min after light onset (Fig. 3.3 B:LP +45). These data suggest that after 60 min of photic stimulation, light was still stimulating MAPK pathway activation and ERK nuclear translocation.

**FIGURE 3.3 Subcellular localization of pERK after light stimulation.** Animals were presented with a SLP (15 min, 100 lux), then returned to darkness for 0, 15, 30, 45, and 105 min prior to being killed. Coronal SCN sections were immunolabeled for pERK (red) and for the neuronal nucleus-specific marker NeuN (green) and analyzed using a confocal microscope. A: representative images from a control animal (no light) and from animals killed at multiple time points after light exposure. Regions within dashed boxes are enlarged and presented at right; scale bars correspond to 75 µm (low magnification) and 15 µm (high magnification). Immediately after light stimulation (LP +0’ to LP +30’), activated ERK was observed in both nuclear and non-nuclear regions, as indicated by colocalization of the two fluorescent labels (yellow hue). Over time, both nuclear and non-nuclear regions underwent a reduction in light-induced pERK levels, returning to control levels within ~1h. B: quantitative data show the light-induced increase in pERK values (0–255 scale). Significant differences in the intensity of pERK expression were found between nuclear and non-nuclear regions immediately after and 15 min after termination of the light pulse (*P < 0.005). C: quantification of pERK levels from animals exposed to constant light. Animals were placed under constant light (100 lux) for 15, 30, 45, 60, and 120 min prior to sacrifice. Both nuclear and non-nuclear regions were found to have a robust increase in pERK expression after light onset. As observed in the SLP paradigm, nuclear regions from CL animals had significantly greater levels of pERK than non-nuclear areas (*P < 0.01) at the 15 min time point. Even in the presence of constant light, pERK gradually returned to control (no light) levels.
FIGURE 3.3 Subcellular localization of pERK after light stimulation.
Light-Induced Phase Shifting of the Circadian Clock

If the MAPK pathway plays a central role in coupling light to entrainment of the circadian clock, it is reasonable to hypothesize that the magnitude of light-induced ERK activation will be reflected in the magnitude of the phase shift elicited by short (15 min) and long (120 min) light pulses. Thus given that a 120 min light treatment produced a 43% greater integrated level of ERK activation than a 15 min light exposure (Fig. 3.4 C, we predict that the phase shift elicited by a 120 min light stimulus will be ~40% larger than the shift elicited by a 15 min light exposure. To examine this issue, we measured the phase shifting effects triggered by these two lighting conditions. Wheel-running activity was used as the circadian clock output. Both the 15 and 120 min light pulse elicited a marked phase shift (Fig. 3.4 A).

The 15 min light pulse produced a mean phase delay of 121.9 ± 13.13 min and the 2 h light pulse a delay of 170 ± 8.13 min, representing an ~40% larger phase shift (Fig. 3.4 B, * = P > 0.004) than the one elicited by a 15 min light pulse. These behavioral data parallel the relative magnitude of ERK activation, and thus support our hypothesis that the MAPK cascade may be a factor in determining the magnitude of the light-induced phase shift.
FIGURE 3.4 The effects of short and long light pulses on the magnitude of phase delays and ERK activation. A: a representative double-plotted actogram showing circadian wheel running activity. Initially, animals were entrained to a 12:12 light/dark (LD) schedule then transferred to total darkness (DD). After 9 days in D, animals were presented with either a 15 min (open square) or 120 min light pulse (filled square) at CT 15, then returned to darkness. Two weeks after the initial light pulse, animals were presented with the alternate light pulse paradigm and locomotor activity was monitored for an additional 2 wk. The black arrowhead indicates a computer power failure (horizontal bar), where locomotor activity was not recorded. B: data were compiled from 8 animals and presented as the mean phase delay in min \pm SE. asterisk, \( P < 0.004 \). C: integrated ERK activation values over a 2h period for animals exposed to 15 min or 2h of light. Values were generated from the data sets presented in Fig. 2C.
FIGURE 3.4 The effects of short and long light pulses on the magnitude of phase delays and ERK activation.
MAPK Pathway Resolves Multiple Light Pulses

To address the capacity of the MAPK pathway to be activated by multiple rounds of photic stimulation, we compared ERK activation in animals that received a SLP to ERK activation levels in animals that received multiple light pulses (MLP). Animals in the MLP categories were exposed to one, two, or three 15 min light pulses (100 lux) starting at ZT 15. Mice were returned to darkness for 45 min between each light pulse. Immediately after the final light exposure, animals were killed. Animals in the SLP group were either killed immediately after a single light exposure or killed at 60 or 120 min after photic stimulation.

As expected, the SLP group showed a robust increase in ERK activation immediately after photic stimulation (Fig. 3.5 A). Activated ERK levels returned to near baseline at the 60 and 120 min post-light pulse time points (Fig. 3.5, A and C). The administration of a second light pulse 60 min after the initial light pulse triggered a significant increase in ERK activation relative to the SLP group at the 60 min post-light pulse time point (**, \( P < 0.0001 \), Fig. 3.5, B and C). A third light pulse triggered an additional rise in ERK activation. In a control experiment, we found that pERK expression returned to near basal levels at the 120 min time point following the presentation of light pulses at time 0 and 60 min (data not shown).

It is of interest to note that the intensity of ERK activation diminished with the second and third light pulses (*, \( P < 0.05 \), Fig. 3.5 C), indicating desensitization of the MAPK pathway. This attenuation in ERK activation following MLPs paralleled the attenuation in ERK activation observed during a 2 hr CL treatment, thus raising the possibility that
similar mechanisms may be employed to control the gain of ERK activation under CL and MLP conditions. However, when data from the groups were compared (Fig. 3.5 D), the MLP paradigm triggered a significant increase in pERK relative to CL exposure at the 60 and 120 min CL time points (*, \( P < 0.005 \) in both cases). These data indicate that the MAPK signaling pathway is “reset” by intervening periods of darkness and thus is able to respond to multiple light pulses.

**FIGURE 3.5 The MAPK pathway resolves multiple light pulses.**
Animals were given either a single 15 min light pulse (100 lx, ZT 15) and returned to darkness for 0, 60, or 120 min or multiple light pulses separated by 45 min of darkness. *A*: representative pERK-labeled sections from animals killed immediately after a single light pulse (15’) or 60 min (60’) and 120 min (120’) after a single light pulse. *B*: representative pERK-labeled sections from animals killed after 1, 2, or 3 light pulses. A comparison on the pERK levels from mice killed at the 60 and 120 min time points reveals that multiple rounds of photic stimulation trigger multiple rounds of ERK activation. *C*: comparison of the pERK levels from animals exposed to a single light pulse to pERK levels from animals exposed to 1, 2, or 3 light pulses. Fold-stimulation relative to control pERK levels is shown on the y axis; the time course of the sequential light treatments (min) is shown on the x axis. While pERK expression in the single light pulse group rapidly returned to control levels by the 60’ and 120’ time points, light pulses presented at these time points triggered significant increases in the levels of activated ERK (**, \( P < 0.0001 \)). Significant desensitization of the light-induced pERK response was observed between each successive light pulse (*, \( P < 0.05 \)). *D*: pERK values from the multiple light pulse group were compared with pERK levels from animals presented with constant light exposure for identical periods. The pERK intensity values for the multiple light pulse group were significantly higher at the 60 and 120 min time points (*, \( P < 0.005 \)), indicating that the pathway was “reset” by the intervening dark period and reactivated by light. Error bars denote the SE.
FIGURE 3.5 The MAPK pathway resolves multiple light pulses.
MKP’s

We then sought to identify a potential mechanism by which signaling via the MAPK cascade is terminated. To this end, we probed SCN tissue for the expression of MAPK phosphatases 1, 2 and 3, (MKP-1, -2, and -3, respectively). MKP-1 and -2 are nuclear specific phosphatases that effectively inactivate ERK, and MKP-3 is an ERK phosphatase expressed specifically in the cytosol. Tissue from the SCN and piriform cortex was harvested at ZT 15. Western analysis revealed that both MKP-1 and -2 were present in SCN and cortical tissue (Fig. 3.6 A). As a protein-loading control, the tissue was also probed for the expression of total ERK. PCR analysis of cDNA samples derived from the SCN and piriform cortex confirmed the presence of Mkp-3 in both brain regions (Fig. 3.6 B). These data reveal the presence of all three phosphatases in the SCN and provide a mechanism by which ERK signaling may be terminated.
FIGURE 3.6 MAPK phosphatase (MKP) expression in the SCN. Animals were killed 3 h after lights off (ZT 15). Tissue from the SCN and piriform cortex (CTX) was isolated and probed for expression of MKP-1, -2, and -3. A: Western analysis revealed that both brain regions expressed the nuclear-specific phosphatases MKP-1 and -2. As a protein-loading control, the MKP-1 blot was also probed for total ERK 1/2 expression. B: RT-PCR was used to detect mRNA for the cytosolic phosphatase MKP-3. The expression of these dual-specificity phosphatases in the SCN provides a potential mechanism by which light-induced activation of the ERK/MAPK signaling cascade is terminated.

3.5 Discussion

The role of the MAPK pathway as a regulator of circadian physiology has recently been examined in a number of studies (Butcher et al. 2002; Hayashi et al. 2001; Ko et al. 2001; Obrietan et al. 1998; Sanada et al. 2000, 2002; Williams et al. 2001). Building on this work, we sought to examine the subcellular and temporal regulation of light-induced ERK activation in the SCN.

Effect of Light Duration on ERK Activation

As we have previously reported, exposure to light during the subjective night triggers a rapid increase in the level of activated ERK (Butcher et al. 2002; Obrietan et al. 1998).
After returning animals to darkness, the level of activated ERK returns to near basal levels within ~45 min. Interestingly, in the presence of CL, a similar time course for ERK inactivation occurred. These observations indicate that the MAPK pathway is a highly sensitive light detector, but that it is not nearly as effective at sensing light duration. If it were an effective sensor of light duration, one might expect light to elicit sustained high levels of ERK activity for the duration of the stimulus. Rather, there appears to be an inherent signal termination process that determines the duration of maximal ERK activity. To examine the upstream events that may regulate the duration of light-induced MAPK pathway activation, we monitored the activation state of Ras, a small GTPase that couples extracellular stimulation to the MAPK cascade (Cullen and Lockyer 2002). A 15 min light treatment triggered an elevation in the GTP-bound form of Ras, indicating an increase in Ras activity.

Interestingly, the activated form of Ras was detected even after 120 min of photic stimulation. Given that rapid GTP hydrolysis occurs after cessation of Ras stimulation, these data suggest that photic information is still being relayed to the SCN after 120 min of CL. Consistent with this observation, recent work has shown that light triggers a slow and sustained level of excitation (>20 min) in melanopsin-expressing retinal ganglia cells that project to the SCN (Berson et al. 2002). The finding that Ras is still activated at the end of a 2hr light exposure but that the level of activated ERK drops to near baseline, supports our hypothesis that SCN neurons determine the duration of light-induced MAPK pathway activation. This termination event appears to occur between Ras and ERK. Further work will be required to determine the exact location and mechanism of this
termination signal. Given that the MAPK pathway is a potent regulator of a large array of cellular physiological processes, including cell proliferation, differentiation, survival, and neuronal plasticity, it may not be surprising to find that its duration of activation is tightly regulated. Indeed, the length of MAPK pathway activation determines which physiological processes are affected. For example, in PC12 cells transient activation of ERK triggers cell differentiation, whereas prolonged activation results in cell proliferation (Traverse et al. 1992). Thus signaling via the MAPK pathway in the SCN is likely to be tightly regulated to ensure that the proper biochemical and physiological response is achieved.

Recently, Nakaya et al. (2003) reported that light also functions as a negative regulator of ERK activation in the SCN. Specifically, photic stimulation during the early night blocked a rhythmically regulated ERK activation pattern within the central SCN for >60 min after light exposure. Interestingly, this endogenous rhythm in ERK activation results from a retinal input signal (Lee et al. 2003). Our data show that rhythmically regulated pERK expression, returned to baseline by 90 min after termination of the photic stimulus. The mechanism underlying transient light-induced pERK inactivation is not known. Possible mechanisms may include light-induced activation of a MAPK pathway termination mechanism in SCN neurons or a light-induced alteration in the retinal input signal.

Disruption of light-induced MAPK pathway activation blunts the phase shifting effects of light (Butcher et al. 2002). Here we extend this observation and examine whether the MAPK pathway may also influence the duration of the phase shift. To this
end, we compared the relative magnitude of ERK activation elicited by short (15 min) and long (120 min) light pulses to the relative magnitude of the phase shift elicited by the same light treatments. We found that the 120 min light pulse elicited a 40% larger phase shift than the 15 min pulse. Paralleling this observation, a 120 min light pulse elicited a 43% greater integrated level of ERK activation than a 15 min light exposure. With respect to the nonlinear phase-shifting/ light duration relationship, similar observations have been reported by several groups (Daymude and Refinetti 1999; Nelson and Takahashi 1999). The similarities between the relative magnitude of ERK activation and phase shifting triggered by the two lighting paradigms raises the possibility that the MAPK cascade is not only required for light-induced phase shifting but also influences the magnitude of the phase shift. Additional work will be required to generate a definitive link between the MAPK cascade and the phase-shift magnitude.

**Rapid Resetting of the MAPK Pathway**

Given the rapid dephosphorylation of ERK after a light pulse, we were interested in examining the capacity of the pathway to “reset” and respond to a second light treatment. Here we showed that the MAPK cascade is activated by light pulses spaced 60 min apart. This ability to reset and resolve multiple light pulses presents a potential mechanism by which multiple rounds of light-induced gene transcription occur. Along these lines, Best et al. (1999) observed two discrete rounds of c-Fos transcriptional and cAMP-responsive element binding protein (CREB) phosphorylation elicited by light pulses presented 1–2 h apart.
At the behavioral level, integration of multiple light pulses has also been described, indicating that the clock is able to reset within a “temporal window” of 1–2 h after light stimulation (Best et al. 1999; Sharma and Chandrashekaran 1997). Furthermore, Daymude and Refinetti (1999) demonstrated that multiple light pulses were “perceived” by the circadian system and produced phase changes of varied direction and magnitude related to the duration and order of the pulses presented. Intriguingly, the time course of these observations correlates with that of the MAPK activation and further supports the role of the MAPK cascade as an input pathway driving transcriptionally dependent resetting of the circadian clock.

**ERK Nuclear Translocation**

In the inactive state ERK is anchored to MEK in the cytoplasm (Fukuda et al. 1997). On MEK-induced ERK activation, ERK is released, thus allowing it to dimerize and translocate to the nucleus (Adachi et al. 1999; Fukuda et al. 1997; Khokhlatchev et al. 1998). To characterize the subcellular regulation of pERK after light stimulation, animals received either a SLP or CL, and pERK expression was examined using confocal microscopy. Immediately after photic stimulation, activated ERK was found in both nuclear and non-nuclear regions of SCN neurons. Interestingly, nuclear levels of activated ERK were significantly higher than the levels in the non-nuclear regions. Given that ERK activation occurs in the cytoplasm in a MEK-dependent manner, these data reveal that light triggers the rapid disassociation of ERK from MEK, thus leading to the nuclear translocation and accumulation of activated ERK. The rate at which light elicited
the nuclear accumulation of pERK suggests that an active transport process is involved. Rapid (minutes) translocation of ERK has been shown to be dependent on an active transport mechanism, whereas slow (hours) ERK nuclear translocation results from passive diffusion of the kinase (Adachi et al. 1999).

Under the 15 min light-pulse paradigm, nuclear pERK levels reached a peak 30 min after the light pulse then rapidly returned to near basal levels. The rapid nuclear and cytoplasmic rate of ERK inactivation indicates that both subcellular regions possess ERK phosphatases. It should be noted that under CL conditions, residual ERK activation was observed at the 120 min time point using epifluorescent microscopy, but that low residual activity was observed using confocal microscopy. Possible explanations for this difference include the different criteria used to designate the regions of interest and differences in the inherent sensitivity of these two techniques.

Once in the nucleus, ERK functions as a potent regulator of transcription activation (Treisman 1996). Thus on translocation, numerous nuclear kinases and transcription factors are targeted by ERK. However, it should be noted that while ERK is still in the cytosol, it activates kinases that in turn translocate to the nucleus and affect transcriptional activation (Chen et al. 1992; Pouyssegur et al. 2002; Zhao et al. 1995). These observations suggest that the nuclear translocation of ERK and ERK-regulated kinases may be a key event in coupling light to transcriptional activation in the SCN.

**MAPK Phosphatases**

The reversible nature of protein phosphorylation is the result of a dynamic balance
between the phosphorylating activity of kinases and the dephosphorylating activity of phosphatases. Ultimately, these “stop” and “go” signals determine which signaling events are initiated and the duration of activation. As discussed in the preceding text, the physiological ramifications of MAPK pathway-dependent signaling are determined, in part, by the duration of ERK phosphorylation. Given the relatively short time frame of ERK activity after a light pulse, we were interested in identifying a possible signal termination mechanism. Within the past several years a family of dual-specificity phosphatases, termed MAPK phosphatases (MKPs) have been characterized. MKPs rapidly dephosphorylate ERK, thus inactivating the MAPK signaling pathway (Pouyssegur et al. 2002). Three members of the MKP family have been identified in the CNS: MKP-1, -2, and -3 (Boschert et al. 1998; Kwak et al. 1994; Misra-Press et al. 1995; Muda et al. 1996). MKP-1 and -2 are nuclear specific phosphatases; MKP-3 is localized to the cytoplasm. Thus depending on the subtypes and catalytic activity of the MKPs expressed, the duration of nuclear and cytosolic ERK activation can be differentially regulated. Western blot analysis revealed strong expression of MKP-1 and -2 in the SCN. These observations are in agreement with work showing high levels of Mkp-1 and -2 mRNA expression in the SCN (Kwak et al. 1994; Misra-Press et al. 1995). cDNA analysis was used to detect Mkp-3 expression in SCN tissue. Given the role of these phosphatases as potent regulators of ERK activity, these data suggest that the Mkp-1, -2, and -3 may regulate the duration of light-induced ERK activation in cytosolic and nuclear regions of SCN neurons. Additional phosphatases, such as those that are regulated by activity (Paul et al. 2003) may also play a role in determining the duration of ERK
activation.

In conclusion, the data presented here offer new insights into the mechanisms by which both light and the SCN regulate MAPK pathway activation. These results should provide important new clues about the cellular and molecular events implicated in light entrainment of the clock.
CHAPTER 4

Light-and clock-dependent regulation of ribosomal S6 kinase activity in the suprachiasmatic nucleus

4.1 Abstract

Recent work has revealed that signalling via the p42/44 mitogen-activated protein kinase (MAPK) pathway couples light to entrainment of the circadian clock located in the suprachiasmatic nucleus (SCN). Given that many effects of the MAPK pathway are mediated by intermediate kinases, it was of interest to identify kinase targets of ERK in the SCN. One potential target is the family of 90-kDa ribosomal S6 kinases (RSKs). In this study, we examined light-induced regulation of RSK-1 in the SCN. Immunohistochemical and Western analysis were used to show that photic stimulation during the early and late night triggered the phosphorylation of RSK-1 at two sites that are targeted by ERK. This increase in the phosphorylation state of RSK-1 corresponded with an approximate fourfold increase in kinase activity. Light exposure during the subjective day did not increase the phosphorylated form of RSK-1, indicating that the capacity of light to stimulate RSK-1 activation is phase-restricted. Double immunofluorescent labeling of SCN tissue revealed the colocalized expression of the activated form of ERK with the phosphorylated form of RSK-1 following a light pulse. In vivo pharmacological
inhibition of light-induced MAPK pathway activation blocked RSK-1 phosphorylation, indicating that RSK-1 activity is regulated by the MAPK pathway. PDK-1, a co-regulator of RSK-1, is also expressed in the SCN and is likely to contribute to RSK-1 activity. RSK-1 phosphorylation was also rhythmically regulated within a subset of phospho-ERK-expressing cells. Together these results identify RSK-1 as a light-and clock-regulated kinase and raise the possibility that it contributes to entrainment and timing of the circadian pacemaker.

4.2 Introduction

Light entrainment of the circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus is a well-characterized phenomenon. Photic information is communicated to the SCN by way of the retinohypothalamic tract (RHT). In response to light, RHT nerve terminals secrete glutamate onto neurons of the SCN, thereby triggering a cascade of intracellular signalling events that result in the resetting of the clock timing mechanism (Lowrey & Takahashi, 2000; Meijer & Schwartz, 2003). Light-induced resetting of the clock appears to be a transcriptionally dependent process. For example, light stimulates the rapid expression of core clock timing genes as well as a wide array of immediate early genes (Aronin et al., 1990; Kornhauser et al., 1990, 1992; Rusak et al., 1990, 1992; Albrecht et al., 1997; Zylka et al., 1998; Field et al., 2000) and the disruption of inducible gene expression attenuates light entrainment of the clock (Wollnik et al., 1995; Honrado et al., 1996; Akiyama et al., 1999). For light to stimulate rapid gene expression it must be able to activate a wide range of cellular processes that in turn both
facilitate and stabilize the formation of transcriptional activating complexes.

In a number of model systems signaling via the p42/44 mitogen-activated protein kinase (MAPK) pathway has been shown to couple extracellular stimuli to transcriptional activation (reviewed by Treisman, 1996; Cobb, 1999; Grewal et al., 1999). In turn, MAPK pathway-dependent transcription regulates a myriad of cell processes, including proliferation, differentiation, survival, and in the central nervous system, neuronal plasticity (Pearson et al., 2001; Sweatt, 2001). In the SCN, light exposure during the night leads to the rapid activation of the MAPK pathway (Obrietan et al., 1998; Coogan & Piggins, 2003), and the disruption of MAPK signaling attenuates both light-induced gene expression (Dziema et al., 2003) and entrainment of the circadian clock (Butcher et al., 2002; Coogan & Piggins, 2003). These results indicate that the MAPK pathway might be a critical component in the clock entrainment process.

The MAPK pathway consists of the kinases RAF, MEK and ERK. In large part this pathway is linear and, thus, the effector actions of the MAPK pathway are mediated by ERK. ERK directly phospho-activates transcription factors such as the SRE binding protein Elk-1. In addition, ERK regulates the activation state of intermediary kinases, which in turn regulate transcription factor activation states. One such downstream target of ERK is the family of 90-kDa ribosomal S6 kinases (RSKs). Four members of the RSK family have been identified (RSK-1, -2, -3 and -4). RSK family members are ERK substrates that have complex and, under some circumstances, redundant physiological roles (reviewed by Frodin & Gammeltoft, 1999). The MAPK pathway tightly regulates RSK activation. For example, RSKs are physically associated with ERK (Scimeca et al.,
1992; Zhao et al., 1996) and are activated specifically by the MAPK pathway (Sturgill et al., 1988; Alessi et al., 1995). In the activated state, a fraction of the RSK pool translocates to the nucleus (Chen et al., 1992; Zhao et al., 1995, 1996) where it regulates the activation state of transcription factors and alters chromatin structure via histone phosphorylation (Chen et al., 1993; Rivera et al., 1993; Xing et al., 1996; Sassone-Corsi et al., 1999). Indeed, the importance of RSKs as central effectors of the MAPK pathway has been well documented (Joel et al., 1998; Gross et al., 2000; Shimamura et al., 2000). Thus, RSKs can be considered an extension of the MAPK pathway, forming a functional signaling cassette that couples the MAPK pathway to a large number of physiological processes. In this study we examined the capacity of both light and the circadian clock to regulate RSK-1 activation in the SCN.

### 4.3 Materials & Methods

**Animals** - Adult (8-14 week-old) C57BL6 mice were entrained to a 12 hr: 12 hr light: dark (L: D) cycle for least 4 weeks before use. All procedures involving animals were in accordance with Ohio State University animal welfare guidelines.

**Light Treatment and Tissue Processing** - Light onset was defined as zeitgeber time 0 (ZT 0) and dark onset as ZT 12. Two procedures were used to examine light-induced RSK-1 phosphorylation. First, animals were either presented with a light pulse (100 lux, 15 min) during the early night (ZT 15) or late night (ZT 22). In the second approach, animals were dark-adapted for 2 days and then exposed to light during the early subjective night
(circadian time 15; CT 15), late subjective night (CT 22), or during the middle of the subjective day (CT 6). Immediately after the light treatment, mice were killed under dim red light (Kodak filter, series 2, <1 lux at cage level). Control animals for each group were handled in a similar fashion but were not exposed to light. Following decapitation, opaque black tape was placed over the eyes to prevent postmortem photic stimulation. Brains were removed and placed in chilled oxygenated physiological saline, cut into 500-µm-thick sections using an oscillating tissue slicer then placed in formaldehyde/phosphate-buffered saline (PBS, 5% w/v) for at least 4 hrs. Tissue was then cryoprotected overnight in 30% sucrose (w/v). The following day thin, 40 µm sections were cut using a freezing microtome.

*Immunofluorescent Labeling* - Free-floating sections containing the central SCN were washed 5 X (5 min per wash) in PBS containing 1% Triton X-100, 0.03% NaF and 0.02% Na azide (PBST) then blocked for 1 h with 10% goat serum in PBST. The tissue was incubated (4º C, 12 hrs) in one, or a combination, of the following antibodies: monoclonal mouse neuronal nuclear-specific marker antibody (NeuN, 1:500 dilution, Chemicon Int., Temecula CA, USA); affinity-purified rabbit polyclonal anti-phospho-p90RSK antibody (1:1000 dilution, Cell Signaling, Beverly MA, USA); monoclonal anti-phosphorylated ERK antibody (1:1000 dilution, Sigma, St. Louis MO, USA); and/or rabbit polyclonal anti-phospho-ERK antibody (1:500 dilution, Cell Signaling). The sections were then incubated (4 hr, room temperature) with an Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (1:500 dilution, Molecular Probes, Eugene OR,
USA) and/or with an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:500 dilution, Molecular Probes). Following a final wash cycle (5 X 5 min/wash in PBST) sections were mounted and coverslipped using Gelmount (Biomedia, Foster City CA, USA). Images of labeled sections were captured using a Zeiss 510 Meta confocal microscope. Sections were captured using a Z section thickness of 1 µm.

**Immunoperoxidase Labeling** - Following an initial wash (5 X 5 min in PBST) endogenous peroxidases were quenched with a 15 min treatment in 0.3% H₂O₂. Next, sections were blocked for 1 hr with 10% goat serum/PBST then incubated (overnight at 4º C) with either p90RSK antibody (1:2000 dilution; Cell Signaling) or a rabbit polyclonal anti-phospho-ERK antibody (1:2000 dilution; Cell Signaling). The next day sections were treated (2 hr, room temperature) with a biotinylated anti-rabbit IgG antibody (1:300 dilution, Vector Laboratories, Burlingame CA, USA), then placed in an avidin / biotin enzyme complex (Vector Laboratories) for 1 hr. The signal was visualized by the addition of DAB-nickel-intensified substrate (Vector Laboratories). Sections were mounted on gelatin-coated slides, dehydrated and coverslipped using Permount. Tissue was washed a minimum of 5 X (5 min per wash) in PBST after each antibody treatment.

**Western Blotting** - The SCN was dissected by hand from 500 µm coronal sections, pooled from four animals per condition and sonicated in 50 µL protease inhibitor buffer (0.25 M sucrose, 15 mM HEPES, 60 mM KCl, 10 mM NaCl, 2 mM NaF, 2 mM Na pyrophosphate and protease inhibitor cocktail; Complete Mini tablet, Roche Diagnostics). Additional
tissue was collected from the piriform cortex and processed in a similar manner. A total of 50 µL of 6X sodium dodecyl sulphate (SDS) loading buffer was added and samples were heated to 90º C for 10 min. A 25 µL aliquot of extract from each sample was electrophoresed through a 10% SDS-PAGE gel, transferred to PVDF membranes (Immobilon P; Millipore) and blocked with 10% (w/v) powdered milk in PBST for 1 hr. Membranes were probed with p90RSK polyclonal antibody (1:2000 dilution, Cell Signaling) followed by a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP, 1:2000 dilution; New England Nuclear, Boston MA, USA). The HRP was detected using Renaissance chemiluminescent HRP substrate (New England Nuclear). The membranes were then probed for pERK expression using a mouse monoclonal anti-pERK 1/2 antibody (1:2000 dilution, Sigma). Finally, the membranes were stripped and probed for total ERK expression, as described above. Membranes were washed 4 X (10 min per wash) in PBST between each antibody treatment. Additional samples were processed as described above and probed with rabbit polyclonal antibodies against RSK-1, RSK-2, RSK3 (1:1000 dilutions, Santa Cruz Biotech. Santa Cruz CA, USA), PDK1 (1:1000 dilution, Cell Signaling), or ERK (1 : 1000 dilution, Cell Signaling).

Cannulation and Infusion - Stereotaxic surgery and infusions were performed as described in Butcher et al. (2002). Briefly, animals anaesthetized with 18 µL/g of a drug cocktail containing 7 mg/µL ketamine and 0.44 mg/µL xylazine in sterile physiologic saline were mounted in a stereotaxic frame (Cartesian Research, Sandy OR, USA) and
the coordinates, posterior, 0.22 mm from bregma; lateral, 1.0 mm from midline; and dorsoventral, ± 2.5 mm with the head level were used to implant a 24-gauge guide cannula in the lateral ventricle. Guide cannulae were secured with dental cement and sealed with a 30-gauge stainless steel plug. Following surgery, animals were housed individually and allowed to recover for 2 weeks. A stainless steel 30-gauge injector needle extending 500 µm from the tip of the cannula was used to infuse 3 µL of either vehicle (DMSO) or U0126 (10 nM/µL) at a rate of 0.40 µL/min. The injector needle was maintained in the cannula for 30 sec after the completion of the infusion and animals were returned to home cages for 30 min before any experimental manipulation. All infusions were performed under dim red light (Kodak filter, series 2, <1 lux at cage level). Animals were killed by decapitation and tissue was processed as described above.

**Digital Imaging** - Bright-field photomicrographs were captured using a 16 bit digital camera (Micromax YHS 1300; Princeton Instruments) mounted on an inverted microscope (Leica DM IRB) and data were quantified with Metamorph software (Universal Imaging). Coronal images containing the central SCN were captured using a 10X objective. To quantify pERK and pRSK-1 expression, a digital oval (150 pixels, x-axis: 200 pixels, y-axis) was placed over each SCN and the hypothalamic area just lateral to the SCN and the average signal intensities were measured. The SCN intensity measurement was then normalized to the lateral hypothalamus intensity value, which were set equal to 1. Unless otherwise indicated, data are presented as mean fold SCN signal relative to the lateral hypothalamus. Student's t-tests were performed to determine
significance. In Fig. 4.3C and D, the contrast and brightness settings for the pRSK-1 photomicrographs were digitally enhanced with Photoshop to reveal potentially subtle variations in pRSK-1 expression.

Kinase Assay - Tissue from control and light-treated mice (15 min, 100 lux, ZT 15) was isolated using the approach described above for Western blotting. Tissue was pooled from 10 animals for each condition and suspended in 250 µL of lysis buffer [1% Nonidet P-40, 0.5% Triton X-100, 10% glycerol, 1 mM Na3VO4, 1 mM EDTA, protease inhibitor cocktail (Complete Mini tablet, Roche) in 50 mM Tris-HCl, pH 7.4]. The samples were then sonicated, incubated for 10 min at 4°C and centrifuged for 10 min at 14,000 g. For each sample, 150 mg of protein was incubated with p90RSK antibody (1:100 dilution; Cell Signalling) and protein A-agarose (37.5 µL; Roche Diagnostics) overnight at 4°C. Next, the samples were washed twice in lysis buffer and twice with kinase buffer (10 mM MgCl2, 0.1 mM EGTA, 1 mM DTT, and 10 mM Tris-HCl, pH 7.4). Samples were then incubated at room temperature in kinase cocktail (50 µL kinase buffer, 10 mg S6 kinase substrate peptide; Upstate Biotech, Lake Placid NY, USA), and 2 µL of 3.3 µM[^32]ATP (2 µCi; Perkin Elmer, Boston MA, USA). Samples were centrifuged and 50 µL of supernatant was blotted onto phosphocellulose P-81 paper discs (Whatman Inc, Clifton, NJ, USA). Discs were washed 10 X with 150 mM phosphoric acid and subjected to scintillation counting.
4.4 Results

RSK Expression and Activation

We, as well as several other groups, have reported that photic stimulation during the night triggers robust activation of the MAPK pathway (Obrietan et al., 1998; Coogan & Piggins, 2003; Sigworth & Rea, 2003). To further these studies we examined whether RSK-1, a potential downstream target of the MAPK pathway, is also regulated by light in the SCN. To this end, mice entrained to a 12 h light : dark cycle were exposed to light during either the subjective day or the night and the effects on the phosphorylated form of RSK-1 (pRSK-1) were examined. Figure 1A reveals that photic stimulation (100 lux; 15 min) during either the early (ZT 15) or late (ZT 22) night triggered RSK phosphorylation within the linker region, corresponding to threonine 359 and serine 363 in human RSK-1. These sites are targeted by ERK and the phosphorylation of serine 363 has been shown to be an essential step leading to the stimulation of RSK enzymatic activity (reviewed by Frodin & Gammeltoft, 1999). According to the manufacturer, this antibody detects the phosphorylated form of murine RSK-1 and has limited cross reactivity to RSK-3. For the sake of clarity, the findings described here will refer specifically to RSK-1. High magnification microscopy revealed that pRSK-1 was found primarily in the ventral SCN, with more modest expression observed in the dorsal and lateral SCN regions. RSK-1 phosphorylation was also stimulated in dark-adapted animals during either the early or late subjective night (Fig. 4.1 C). The capacity of light to trigger RSK-1 phosphorylation was phase-dependent; light exposure during the subjective day (CT 6) did not increase RSK-1 phosphorylation (Fig. 4.1 A and B). Under control conditions (no light),
densitometric analysis of the ventral SCN did not reveal significant variations in RSK-1 expression over the three time points examined (Fig. 4.1 A and B). Together these data reveal that photic stimulation triggers a phase-dependent phosphorylation of RSK-1 in the SCN, paralleling the phase-restricted capacity of light to both activate the MAPK pathway and entrain the circadian clock.

**FIGURE 4.1 Light-induced RSK-1 phosphorylation.** Mice were exposed to light (15 min, 100 lux) during mid subjective day (CT 6), early night (ZT 15), or late night (ZT 22) then immediately killed and brain sections were processed for RSK-1 phosphorylation (pRSK-1). (A) Relative to control animals (No Light), light exposure triggered a marked increase in pRSK-1 expression at the two night time points. The highest level of antigenicity was observed in the ventral SCN. Photic stimulation during the subjective day did not increase RSK phosphorylation, indicating that the capacity of light to couple to RSK-1 is phase-restricted. (B) Quantification represents the fold pRSK-1 expression in the SCN relative to the pRSK-1 expression in the lateral hypothalamus, which was set equal to 1. The number of animals used for each data point is shown above each bar. (C) Light-induced RSK-1 phosphorylation occurs under circadian timing conditions. Animals were initially dark-adapted for two days then exposed to light during either the early (CT 15) or late subjective night (CT 22). Outlined regions appear at higher magnification to the right; scale bar = 100 mm. Error bars denote the SEM. *=P < 0.002. oc, optic chiasm, 3v, 3rd ventricle.
FIGURE 4.1 Light-induced RSK-1 phosphorylation.
To examine the specificity of the pRSK-1 antibody used above, SCN tissue from control and light-treated (100 lux, ZT 15) animals was probed for pRSK expression using Western blotting techniques. Photic stimulation specifically increased the antigenicity of an approximate 90-kDa band (Fig. 4.2 A). This band corresponds to the size of RSK1 and RSK3, thus validating the results obtained using immunohistochemical detection procedures. Following detection of pRSK-1, the membrane was probed for ERK activation. RSK-1 is a downstream target of the MAPK pathway, and thus, if RSK-1 is activated one might be able to detect MAPK pathway activation. Using an antibody directed against the threonine 202 and tyrosine 204 phosphorylated forms of erk 1 and erk 2 (pERK), a marker for MAPK pathway activation, we found that photic stimulation triggered an increase in ERK phosphorylation. As a protein loading control, the blot was stripped and probed for total ERK expression. As noted above, the phospho-RSK antibody used here detects the activated form of RSK-1 and to a lesser extent RSK-3. To examine RSK isoform expression in the SCN, tissue was isolated and probed for presence of RSK-1, -2 and -3. Bands corresponding to the three isoforms were detected in the SCN (Fig. 4.2 B).

Coordinated phosphorylation by ERK and 3-phosphoinositide-dependent kinase-1 (PDK-1) stimulates maximal RSK activation (Jensen et al., 1999). Thus, a robust light-induced increase in RSK-1 enzymatic activity would require PDK-1-mediated phosphorylation. Western analysis confirmed the presence of PDK-1 in the SCN of animals killed during the early subjective night, CT 15 (Fig. 4.2C). The two bands are likely to correspond to the alpha and beta isoforms of PDK-1 (Dong et al., 1999, 2002). PDK-1
appeared to be expressed constitutively; equivalent amounts of PDK-1 were observed over the L:D cycle (data not shown).

Next, we examined whether photic stimulation triggers an increase in RSK activity. To this end, animals were exposed to light for 15 min, then killed and the SCN was isolated from coronal brain sections. Using the pRSK-1 antibody to immunoprecipitate the kinase and ribosomal S6 peptide as the substrate, we found that photic stimulation triggered an ~ fourfold increase in RSK activity relative to kinase activity from control animals not exposed to light (Fig. 4.2D). These data reveal that photic stimulation not only triggers phosphorylation, but also stimulates RSK-1 activity in the SCN.

**RSK and ERK Colocalization**

In the inactive state RSKs are largely cytoplasmic kinases. However, after activation they accumulate in the nucleus (Chen et al., 1992; Zhao et al., 1995, 1996). To address the subcellular pRSK-1 expression pattern, tissue was double labeled for pRSK-1 and for the neuronal-specific nuclear marker NeuN, and coronal sections were captured through the central SCN using confocal microscopy. Immunofluorescent labeling detected strong light-induced RSK-1 phosphorylation following a light flash (Fig. 4.3 A). Interestingly, a majority of NeuN expressing cells (~70%, n =150) within the ventral SCN were also positive for phosphorylated RSK-1.
FIGURE 4.2 RSK-1 activation and PDK-1 expression. (A) Western analysis revealed that light (LF, 15 min, 100 lux, ZT 15) increased pRSK-1 expression relative to no light (NL) control conditions. Light also increased ERK phosphorylation (pERK), whereas total ERK levels remained unchanged. (B) SCN tissue harvested from control animals was probed for RSK-1, -2 and -3 expression. All three RSK isoforms were found within the SCN. (C) The RSK kinase PDK1 is expressed in SCN as well as in piriform cortical (CTX) tissue. (D) pRSK-1 was immunoprecipitated from the SCN of control and light-treated animals and incubated with P32 ATP and a substrate peptide. P32 incorporation was determined by scintillation counting. Data are represented as the fold-increase relative to the control (no light) condition, which was set equal to 1. Results reported here are representative of at least three independent assays.
FIGURE 4.3 pRSK-1 in the SCN: nuclear localization and pERK coexpression. (A) Tissue from light-treated animals (15 min, 100 lux, ZT 15) was double labeled for the neuronal nuclear marker NeuN (green) and for pRSK-1 (red). Analysis of the merged signal reveals a high degree of colocalization between NeuN-labeled cells and pRSK-1. The outlined SCN region approximates the location of the magnified region shown below. Scale bar, 50 mm. (B) Tissue sections were double labeled for the activated form of ERK (green) and for pRSK-1 (red) at ZT 15. Confocal analysis shows low levels of pERK and pRSK-1 expression in ventral SCN from control animals not exposed to light. Light (15 min, 100 lux) stimulated an increase in both pERK and pRSK-1 expression. Merging the pERK and pRSK-1 immunofluorescent signals generated a green/yellow-hue, indicative of colocalized expression of the two kinases. Arrows identify individual cells with strong signal colocalization. (C) pRSK-1 is expressed in the subset of SCN neurons that exhibit circadian variations in ERK activation. (c1) Double immunofluorescent labeling identifies a group of central SCN neurons exhibiting high levels of pERK and elevated levels of pRSK-1. Arrows denote cells exhibiting both pERK and pRSK-1 expression. (c2) In contrast, pRSK-1 expression was relatively low in SCN regions lacking strong pERK expression. Outlined regions within the low magnification pERK photomicrographs were used to examine pERK and pRSK-1 coexpression. Animals were killed at ZT 15. (D) pRSK-1 is not coexpressed with pERK in the SCN shell. Strong pERK expression was observed within the shell region of the SCN at CT 1. Magnification of the boxed region (middle panel).
FIGURE 4.3 pRSK-1 in the SCN: nuclear localization and pERK coexpression.
If RSK-1 is a downstream target of ERK in the SCN, then one may expect to observe colocalized activation of the two kinases following a light flash. To address this issue, SCN tissue from control and light-treated mice (100 lux, ZT 15) was processed using immunofluorescent labeling techniques for the phosphorylated forms of ERK and RSK-1 and data were collected using a confocal microscope. Under control conditions (no light), low levels of activated ERK and pRSK-1 were observed (Fig. 4.3 B). However, after photic stimulation, there was a marked increase in the phosphorylated forms of both kinases (Fig. 4.3 B). Merging the two images revealed strong colocalized expression of activated ERK and RSK-1 in a subset of cells. Double labeling appeared primarily in cell bodies. Together these results reveal that light triggers a coordinated activation of ERK and RSK-1 in the SCN.

In addition to its regulation by light, the MAPK pathway is also regulated by clock timing mechanisms. For example, a subset of cells within the SCN core exhibits high levels of activated ERK during the night (Obrietan et al., 1998; Fig. 4.3 C). Double labeling experiments revealed that the phosphorylated form of RSK-1 is also elevated in this group of cells (Fig. 4.3, c1). A similar level of pRSK-1 immunoreactivity was not observed in control SCN regions that lacked high levels of activated ERK (Fig. 4.3, c2). Activated ERK was not observed within this central group of SCN cells during the subjective day (Obrietan et al., 1998). Paralleling this observation, there was an absence of pRSK-1 staining within these cells during the subjective day (data not shown). These results suggest that the circadian rhythm in ERK activity drives the rhythm of RSK-1 activation.
In addition to the SCN core subregion, cells from the SCN shell also exhibit rhythmic pERK expression (Obrietan et al., 1998). Interestingly, in contrast to the rhythmic pRSK-1 expression observed within the core, pRSK-1 expression was not found to colocalize with pERK expression in the shell at CT 1 (Fig. 4.3 D). We did note pRSK-1 immunopositive cells within the shell from time to time, but the lack of a consistent, strong, and colocalized, signal indicates that there is a disassociation of ERK and RSK activity within the shell.

**Disruption of Light-Induced MAPK Pathway Activation**

To verify the connection between light-induced MAPK activation and RSK-1 phosphorylation, we employed a ventricular infusion technique to disrupt MAPK signalling in the SCN. To this end mice were infused with the specific MEK 1/2 inhibitor U0126 (10nM / μl) 30min before photic stimulation (100 lux, 10 min, ZT 15). Figure 4.4 C and D reveal RSK-1 phosphorylation, we employed a ventricular infusion technique that infusion of U0126 disrupted light-induced MAPK pathway activation in the SCN. A region of U0126-mediated ERK inhibition was observed surrounding the 3rd ventricle (dashed outline, Fig. 4.4 C). This outline defines the approximate dorsal and lateral extent to which U0126 diffused from the 3rd ventricle and suppressed MEK activation. Importantly, the disruption of MAPK activation blocked the capacity of light to trigger RSK-1 phosphorylation (Fig. 4.4A and B; P < 0.05). In conclusion, these results reveal the presence of a light-responsive MAPK/RSK signalling cassette in the SCN.
FIGURE 4.4 U0126 infusion blocks light-induced pRSK-1 expression in the SCN. Cannulated animals were infused with either U0126 (10 nM/mL) or vehicle (DMSO) 30 min before light (15 min, 100 lux) exposure at ZT 15. Alternate tissue sections were labeled for pRSK-1 and pERK expression. (A) As expected, light stimulated pRSK-1 expression in the SCN. Pretreatment with U0126 significantly attenuated light-induced RSK-1 phosphorylation. (B) Quantitative data are presented as the fold pRSK-1 expression in the SCN relative to pRSK-1 expression in the lateral hypothalamus. P < 0.05 relative to all other conditions. No significant difference (P > 0.2) was observed between any of the other conditions. (C and D) Control experiments confirmed that U0126 infusion uncoupled light from MAPK pathway activation. Boxed region in C surrounding the 3rd ventricle demarcates the approximate region within which U0126 suppressed MEK/ERK activation. Numbers above each bar indicate the number of animals used per condition. P < 0.0001 relative to all other conditions. No significant difference (P > 0.15) was observed between any of the other conditions. Error bars denote the SEM. Scale bar, 100 mm (A); 200 mm (C).
FIGURE 4.4 U0126 infusion blocks light-induced pRSK-1 expression in the SCN.
4.5 Discussion

The results presented here identify RSK-1 as a light-responsive kinase in the SCN. RSK-1 was phosphorylated during both the early and late night, but not during the subjective day. The data also show that RSK-1 is downstream of the MAPK kinase pathway, and that pRSK-1 is concentrated in neuronal nuclei following light exposure. As an effector of MAPK signalling, RSK-1 may play a pivotal role in light-induced entrainment of the circadian clock.

Light is a potent regulator of the circadian clock in mammals. 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 & Pittendrigh, 1976). Importantly, light exposure during the mid subjective day does not alter the timing process. This phase-dependent regulation of the clock has been characterized at the molecular level. For example, the capacity of light to trigger the expression of the immediate early genes c-Fos, JunB and EGR-1 is restricted to the night (Aronin et al., 1990; Kornhauser et al., 1990, 1992; Rusak et al., 1990, 1992). Likewise, photic stimulation triggers expression of the core clock timing genes period 1-and period 2 in a phase-restricted manner (Albrecht et al., 1997; Zylka et al., 1998). The observation that light-induced transcription is phase-restricted suggests that inducible transcription factors are only activated by light during the night. Indeed, both the phospho-activation of CREB and CRE-dependent transcription are elicited by light during the night (Ginty et al., 1993; Obrietan et al., 1999; Gau et al., 2002). Phase-dependence has also been reported at the level of Elk-1 phosphorylation and histone H3 phosphorylation (Crosio et al., 2000;
Coogan & Piggins, 2003). Given that these phosphorylation-dependent events are mediated by the activation of inducible kinase pathways, we have endeavored to identify and characterize light-activated kinase cascades. One pathway, the p42/44 MAPK cascade appears to be a key intermediate in the light-entrainment process.

Several studies have shown that the MAPK pathway is activated by light in a phase-restricted manner in the SCN (Obrietan et al., 1998; Coogan & Piggins, 2003) and that MAPK signalling plays a central role in coupling light to gene expression. For example, disruption of light-induced MAPK cascade activation attenuates the expression of immediate early genes such as c-Fos, EGR-1 and JunB in the SCN (Dziema et al., 2003) and period 1 and period 2 in cell culture systems (Cermakian et al., 2002; Travnickova-Bendova et al., 2002). In addition, the MAPK pathway possesses the spatial and temporal resolution properties expected for a cell signalling pathway to affect clock timing. Interestingly, the MAPK cascade also rapidly resets following a light pulse and resolves individual light pulses into discrete signalling events (Butcher et al., 2003). Furthermore, following a light pulse, high levels of activated ERK accumulate in the nuclei of SCN neurons (Butcher et al., 2003). This nuclear translocation of ERK is likely to play an important role in regulating transcriptional activation. However, it should be noted that many of the physiological effects of the MAPK cascade are mediated by intermediate, ERK-regulated, kinases. One of these intermediates is the RSK family of kinases. These serine/threonine kinases are exclusively activated through an ERK-dependent mechanism (Reviewed by Frodin & Gammeltoft, 1999). RSKs are composed of two kinases, an N-terminal kinase that phosphorylates RSK substrates, and a C-terminal
kinase that serves an autoregulatory function. These two kinases are connected by an approximate 100-amino acid residue linker region. The activation of RSK is a multi-step process. RSK activation is initiated by ERK phosphorylation of a serine residue in the activation loop of the C-terminal kinase and the phosphorylation of a serine residue in the linker region (Sutherland et al., 1993; Fisher & Blenis, 1996). The C-terminal kinase then phosphorylates a linker region serine residue (Dalby et al., 1998), which in turn allows PDK-1 to phosphorylate an N-terminal serine (Jensen et al., 1999), thus leading to full activation of RSK.

Initially we wanted to examine RSK expression patterns in the SCN. Western blot analysis revealed the expression of RSK 1-3 in the SCN. To our knowledge this is the first study to examine RSK expression specifically in the SCN, although several studies have shown RSK family members are expressed at varying levels throughout the developing and mature nervous system (Zeniou et al., 2002; Kohn et al., 2003). As noted above, full activation of RSK requires a PDK-1-mediated phosphorylation event. PDK-1 is a constitutively active kinase, and thus, unlike ERK, its activity is not likely to be elevated by cellular stimulation (Alessi et al., 1997; Pullen et al., 1998). Western analysis was used to show PDK-1 expression in the SCN. The expression of PDK-1 along with light-induced ERK activation appears to be the minimal set of signalling events required for photic stimulation to couple to RSK activation. Indeed, using an immunoprecipitation kinase assay we found that light triggered an increase in RSK-1 activity. These data suggest strong coupling between ERK, PDK-1 and RSK-1 in the SCN.

The complex and coordinated activation of RSK requires redistribution of the kinase
from the cytosol to the cell membrane, where it is phosphorylated by PDK-1. After activation by PDK-1, RSK family members can translocate to the nucleus (Richards et al., 2001). Our data showing that photic stimulation resulted in the colocalized expression of phosphorylated RSK-1 with the neuronal-specific nuclear marker NeuN, suggest that light stimulated RSK-1 nuclear translocation. This parallels work showing that strong stimulation of the MAPK pathway leads to an accumulation of RSK in the nucleus (Chen et al., 1992; Zhao et al., 1995). In addition, we also noted an increase in the overall, non-nuclear, pRSK-1 immunostaining pattern following photic stimulation in the SCN. This apparently cytoplasmic staining likely represents the pool of activated RSK-1 that did not translocate to the nucleus.

There was a marked spatial and temporal correlation between pRSK-1 and pERK expression following photic stimulation in the SCN. Colocalization appeared to be most prominent within cellular nuclei. Colocalized nuclear expression of RSKs and ERK has been reported previously and could result from the physical association of ERK and RSKs within a multi-protein complex. This association between ERK and RSKs is observed both before and during kinase activation (Scimeca et al., 1992; Zhao et al., 1996). Also evident from our double-labeling experiments was a subset of cells that showed relatively weak colocalization of pRSK-1 and pERK expression after light exposure. This discord is the likely result of transient ERK activation leading to a sustained bout of RSK-1 activation, and/or relatively weak ERK activation leading to a robust increase in RSK-1 phosphorylation.

Expression of activated pRSK-1 was also observed within a subset of core SCN
neurons that display high night-time levels of activated ERK. This endogenous rhythm in ERK activation (Obrietan et al., 1998) was recently found to result from a retinal input signal (Lee et al., 2003). Enucleation blocked the ERK activation rhythm (Lee et al., 2003), and would therefore presumably block the pRSK-1 rhythm. The absence of a pRSK-1 signal within this subset of cells during the subjective day suggests that RSK-1 activity, like ERK activity, is regulated in a rhythmic manner in the SCN.

Given the colocalized rhythmic expression of pERK and pRSK-1 within the SCN core, it was somewhat surprising to find a dissociation of the pERK rhythm from RSK-1 phosphorylation in the shell region of the SCN. Strong pERK expression is observed in the shell during both the early and late subjective day (Obrietan et al., 1998), and yet at neither time point were we able to show consistent colocalized expression of pRSK-1. Possible explanations for a lack of coordinated activation in the shell include a differential duration of activation. Thus, ERK may be activated for an extended period, whereas RSK-1 might be activated transiently. It is also possible that our immunostaining technique does not have the sensitivity required to detect subtle oscillation in RSK-1 phosphorylation. In either case, the strong pRSK1 signal induced by light, and the absence of a signal within the shell suggests that MAPK signalling may be affecting different cellular processes within different SCN subregions. It is also plausible that the unique chemoarchitecture and efferent organization of the core and shell (Moore et al., 2002) might differentially regulate the activation state of RSK-1.

To examine the relationship between ERK and RSK-1 activation in the SCN, we utilized a ventricular infusion technique to deliver the specific MEK 1/2 inhibitor U0126
into the brain. Prior studies have shown this is an effective approach to disrupt MAPK signalling within the SCN (Butcher et al., 2002; Dziema et al., 2003). U0126 is a well-characterized MEK-specific inhibitor and its apparent lack of an effect on other kinases such as PKA, PKC, and JNK (Favata et al., 1998; Davies et al., 2000) makes it a powerful tool to examine MAPK signalling. The data presented here show that light-induced RSK-1 phosphorylation is mediated by MAPK signalling. Together these data identify RSK-1 as a downstream target of the MAPK pathway in the SCN.

What role might RSKs play as light-activated signalling intermediates in the SCN? Given the central role that the MAPK cascade plays in stimulating gene expression, one may envision RSKs coupling the MAPK pathway to light-induced transcriptional activation. Support for this concept comes from studies showing that RSKs alters the transcriptional program by regulating the functional properties of a variety of transcription factors such as CREB (Xing et al., 1996), c-Fos (Chen et al., 1993) and SRF (Rivera et al., 1993). Likewise, RSKs might alter gene expression by regulating chromosomal condensation via histone phosphorylation (Sassone-Corsi et al., 1999). In conclusion, the data presented here identifies RSK-1 as a downstream target of the MAPK cascade in the SCN. Further studies will be required to determine the potential role of RSKs in coupling light to entrainment of the circadian clock.
CHAPTER 5

Light Stimulates MSK1 Activation in the Suprachiasmatic Nucleus via a PACAP-ERK/MAP Kinase-Dependent Mechanism

5.1 Abstract

Signaling via the p42/44 mitogen-activated protein kinase (MAPK) pathway has been shown to be a key intracellular signaling event that couples light to entrainment of the mammalian circadian clock located in the suprachiasmatic nucleus (SCN). Because many of the physiological effects of the MAPK pathway are mediated by extracellular signal-regulated kinase (ERK)–regulated kinases, it was of interest to identify kinase targets of ERK in the SCN. In this study, we examined whether mitogen-and stress-activated protein kinase 1 (MSK1) is a downstream target of ERK in the SCN and whether it couples to clock gene expression. Here we show that photic stimulation during the subjective night stimulates MSK1 phosphorylation at serine 360, an event required for robust kinase activation. Activated ERK and MSK1 were colocalized in SCN cell nuclei after photic stimulation. The in vivo administration of the MAP kinase kinase 1/2 inhibitor U0126 [1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene] attenuated MSK1 phosphorylation. MSK1 phosphorylation was more responsive to late-
night than early-night photic stimulation, indicating that MSK1 may differential contribute to light-induced phase advancing and phase delaying of the clock. The potential connection between pituitary adenylate cyclase-activating polypeptide (PACAP) (a regulator of clock entrainment) and MSK1 phosphorylation was examined. PACAP infusion stimulated MSK1 phosphorylation, whereas PACAP receptor antagonist infusion attenuated light-induced MSK1 phosphorylation in the SCN. In reporter gene assays, MSK1 was shown to couple to mPeriod1 via a cAMP response element-binding protein-dependent mechanism. Together, these data identify MSK1 as both a downstream target of the MAPK cascade within the SCN and a regulator of clock gene expression.

5.2 Introduction

In mammals, the dominant circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Its inherent timekeeping ability is derived from a discrete set of genes whose protein products form positive and negative limbs of arrhythmic transcriptional/translational feedback loop (for review, see Reppert and Weaver, 2001; Albrecht, 2002). Light is thought to entrain the clock by altering the expression of clock genes and, in turn, resetting the clock feedback loop (for review, see Meijer and Schwartz, 2003). For example, photic stimulation during the night triggers the expression of the core clock genes \textit{mPer1} and \textit{mPer2} (Albrecht et al., 1997; Shigeyoshi et al., 1997), and the disruption of \textit{Per} expression blocks the phase-shifting effects of light (Akiyama et al., 1999; Albrecht et al., 2001).

Photic information is relayed from the retina to the SCN via the retino-hypothalamic
tract (RHT), a branch of the optic nerve. The melanopsin expressing retinal ganglion cells that form the RHT secrete both glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) onto SCN cells (Hannibal et al., 2000, 2002; Gooley et al., 2001). The necessary role of glutamate as a signaling intermediate coupling light to clock entrainment has been well documented. For example, ionotropic glutamate receptor antagonist infusion into the SCN blocks light-induced phase shifts (Colwell et al., 1991).

PACAP has been shown to be a potent and complex regulator of circadian clock entrainment (Hannibal et al., 1997; Harrington et al., 1999; Kawaguchi et al., 2003; Colwell et al., 2004). Interestingly, PACAP appears to function as an effector of multiple signaling pathways and augments the excitatory effects of glutamate on SCN cells (Chen et al., 1999; Harrington et al., 1999; Kopp et al., 2001; Dziema and Obrietan, 2002).

Although the precise set of cellular signaling events that couple glutamate and PACAP to clock entrainment has not been determined, both transmitters have been shown to stimulate activation of the p42/44 mitogen-activated protein kinase (MAPK) pathway (Barrie et al., 1997; Dziema and Obrietan, 2002). The MAPK pathway consists of three kinases: Raf, MAP kinase kinase (MEK), and extracellular signal-regulated kinase (ERK). During activation, ERK phosphorylates a number of cytosolic substrates and, depending on the strength of the stimulus, translocates to the nucleus and facilitates transcription activation (for review, see Cobb, 1999; Pearson et al., 2001; Roux and Blenis, 2004). In the SCN, light stimulates robust, phase-dependent, MAPK pathway activation (Obrietan et al., 1998; Butcher et al., 2003), and the disruption of light-induced MAPK activation blocks clock entrainment (Butcher et al., 2002).
The mechanism by which the MAPK cascade elicits transcription-dependent clock entrainment likely involves stimulation of a number of ERK-regulated kinases. Along these lines, we reported recently that light triggers activation of the ribosomal S6 family of kinases (RSKs) in the SCN (Butcher et al., 2004). RSKs function as central effectors of the MAPK cascade, stimulating transcription factor activation and altering chromatin structure. Additionally, another family of kinases, the mitogen-and stress-activated protein kinases (MSKs), function as intermediates that couple the MAPK pathway to transcription activation (Deak et al., 1998; Arthur and Cohen, 2000; Wiggin et al., 2002; Arthur et al., 2004). MSKs have been shown to regulate gene transactivation via both transcription factor activation and histone phosphorylation (Wiggin et al., 2002; Soloaga et al., 2003). These observations raise the possibility that MSKs may couple the MAPK pathway to resetting of the circadian clock. Here we identify for the first time MSK1 as a light-responsive kinase that stimulates expression of the clock gene mPeriod1.

5.3 Materials and Methods

Light Treatment - Initially, adult (6- to 14- week-old) C57BL/6 mice were entrained to a 12 hr light/dark (LD) cycle for at least 2 weeks and then transferred to total darkness for two 24 hr cycles. After dark adaptation, animals received a single light exposure (15 min, 100 lux) at one of three times: the middle of the subjective day [circadian time 6 (CT6)], early night (CT15), or late night (CT22). CTs were approximated, based on Zeitgeber time (ZT), with ZT0 denoting light on and ZT12 denoting light off. Immediately after light treatment, animals were killed via cervical dislocation, and brains were removed
under red light (Kodak series 2 filter approximately 10 lux at cage level; Eastman Kodak, Rochester, NY). Brains were then placed in chilled, oxygenated physiological saline, cut into 500 µm coronal sections with an oscillating tissue slicer (OTS 2000; Electron Microscopy Sciences, Fort Washington, PA), and placed in 4% paraformaldehyde (w/v in PBS) at room temperature for 4 hr. In total, it took approximately 5 min from decapitation to tissue fixation. In control experiments, we found that other, more time-consuming tissue-processing techniques, such as trans-cardial perfusion, resulted in a markedly attenuated kinase phosphorylation signal. This likely resulted from the transient nature of kinase phosphorylation. Tissue was then cyroprotected in 30% sucrose (w/v) containing 2 mM Na azide and 3 mM NaF overnight at 4°C. All procedures were in accordance with Ohio State University animal welfare guidelines.

Cannulation and Infusion – Mice were cannulated in the lateral or third ventricles as described by Butcher et al. (2002) and allowed to recover for at least 10 days after cannulation. For the infusion, animals were restrained by hand under red light, and the infusate was delivered at a rate of 0.4 µl/min. To disrupt MAPKs, 3 µl of 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenyl mercapto) butadiene (U0126; 10mM), 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl) phenyl]-1H-imidazol-4-yl]pyridine (SB203508; 10mM), or a combination of the compounds was infused 45 min before photic stimulation. Control animals were infused with an equivalent volume of vehicle (DMSO). To disrupt PAC1 receptor activation, 5 µl of PACAP 6-38 (0.5mM) diluted in physiological saline was infused 15 min before photic stimulation. This concentration of
PACAP 6-38 has been shown to significantly attenuate light-induced phase advances (Bergström et al., 2003). Two microliters of PACAP (200µM) were infused at CT22; animals were killed 45 min later.

**Immunohistochemistry** – Brain sections (500µm) were thin cut (40µm) using a freezing microtome and placed in PBST (PBS with Triton X-100) with 2mM Na azide and 3mM NaF. Endogenous peroxidases were quenched with 0.3% H₂O₂ in PBST (15min). Tissue was then blocked (1 hr) in 5% goat serum/PBST and incubated (overnight, 4°C) in rabbit anti-phospho-MSK1 (1:500 final dilution; Cell Signaling Technology, Beverly, MA), rabbit anti-phospho-ERK (1:2000; Cell Signaling Technology), or rabbit anti-phospho-p38/MAPK (1:1000; Cell Signaling Technology). Next, sections were incubated (2 hr) at room temperature in biotinylated anti-rabbit IgG (1:300; Vector Laboratories, Burlingame, CA) and then placed in an avidin/biotin HRP complex for 1 hr (prepared according to instructions of the manufacturer; Vector Laboratories). The signal was visualized by the addition of DAB–nickel-intensified substrate (Vector Laboratories) and mounted on gelatin-coated slides with Permount media (Fisher Scientific, Houston, TX).

Tissue used for fluorescent labeling was thin cut and blocked as described above and then incubated (overnight, 4°C) in one or a combination of the following antibodies: rabbit polyclonal anti-phosphorylated ERK (1:1000; Cell Signaling Technology), mouse monoclonal neuronal nuclear-specific marker (NeuN) (1:1000; Chemicon, Temecula, CA), mouse monoclonal anti-phosphorylated ERK (1:5000; Sigma, St. Louis, MO), and rabbit polyclonal anti-phospho-MSK1 (1:250; Cell Signaling Technology). The following
day, sections were incubated (3 hr, 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). Sections were mounted on slides with Gelmount media (Biomedia, Foster City, CA). Brain sections were washed (five times, 5 min per wash) between each labeling step. Bright-field photomicrographs were captured using a 16 bit digital camera (Micromax YHS1300; Princeton Instruments, Trenton, NJ) mounted on an inverted microscope (DMIRB; Leica, Nussloch, Germany). Fluorescent images were captured using a Zeiss (Oberkochen, Germany) 510 Meta confocal microscope (2 µm-thick optical sections).

*Reverse Transcriptase-PCR* – Animals were killed at CT2, CT8, CT14, and CT20, and brains were removed and cut into 500µm coronal sections as described above. Tissue was then frozen on slides with dry ice, and the SCNs were manually dissected and pooled from four animals for each time point. RNA was then isolated (RNA-Bee; Tel-Test, Friendswood, TX) and reverse transcribed using standard techniques. PCR was performed with primer sets against murine MSK1 (5’-cagttccata-tggagttgaccgt-3’ and 5’-cttcatcattgcactcctggcaac-3’) and cyclophilin [CYCα (Uz and Manev, 1998)]. Reaction products were run on a 1% agarose gel containing ethidium bromide. Digital images were captured using an Eastman Kodak Image Station, and densitometric comparisons were performed to determine the ratio of CYCα to MSK1 for each time point.
Cell Culture and Stimulation – Cortical tissue was collected from embryonic (day 18) Sprague Dawley rat pups, washed in dissociation media (DM) [90mM Na$_2$SO$_4$, 30mM K$_2$SO$_4$, 16mM MgCl$_2$, 0.25mM CaCl$_2$, 32mM HEPES, and 0.01% phenol red (Sigma), pH 7.7], and then incubated in digestion solution [100U/ml papain latex (Worthington, Freehold, NJ) and 4.5mg cysteine (Sigma) in DM] for 30 min at 37° C. Tissue was then transferred to standard culture medium (Minimal Essential Medium; Invitrogen, Gaithersburg, MD) containing 1% fetal bovine serum (Invitrogen), 2% B27 (Invitrogen), and 100U/ml penicillin/streptomycin (Invitrogen) and triturated into a single cell suspension. Cells were then plated onto poly-D-lysine-coated 9 mm$^2$ glass coverslips at a density of 50,000 cells/cm$^2$. Media were changed 1 hr after plating, and cells were maintained in a Napco (Winchester, VA) 6100 incubator (37°C, 5.5% CO$_2$) for 10 days. Media was replenished every third day.

Thirty minutes before experimental stimulation, tissue culture media were replaced with HEPES buffer (in mM:137 NaCl, 25 glucose, 10 HEPES, 5 KCl, 1 MgCl$_2$, and 3 CaCl$_2$, pH 7.4) containing tetrodotoxin (1µM). Cells were then treated (10min) with PACAP (200nM) or glutamate (10µM) and fixed (15 min) with paraformaldehyde (4% w/v). Phosphorylated MSK1 (pMSK1) immunofluorescent labeling was performed as described above. To identify neurons, cells were also labeled with a microtubule-associated protein-2 (MAP2) monoclonal antibody (1:5000; Sigma). Cells were processed with Alexa Fluor-conjugated secondary antibodies, and digital images were captured as described above.
Transfection and Luciferase Assay - Initially, human embryonic kidney 293 (HEK293) cells were seeded onto 24-well plates, grown to 90% confluency in DMEM containing 10% fetal bovine serum and 1% penicillin, and then transfected using Lipofectamine 2000 (Invitrogen). A total volume of 1.0µg of DNA was transfected per well. The following plasmids were used: constitutively active MSK1 [CA-MSK1 (Frodin et al., 2000)], dominant-negative MSK1 [DN-MSK1 (Arthur et al., 2004)], dominant-negative cAMP response element-binding protein (CREB) [A-CREB (Ahnetal., 1998)], and a murine period1 (mPer1) reporter construct. The mPer1 construct consisted of a 2.2kb fragment of the 5’ regulatory region of the second of the two mPer1 promoters [exon 1B promoter (Yamaguchi et al., 2000)] cloned into the pGL3-enhancer vector (Promega, Madison, WI). This reporter construct is analogous to the reporter used by Gekakis et al. (1998). Cells were serum starved 16 hr before stimulation (8 hr) with 12-O-tetradecanoylphorbol-13-acetate (TPA; 200ng/ml). Cells were lysed 48 hr after transfection, and luciferase reporter expression was determined using the method described by Ford and Leach (1998). To verify the efficacy of our transfection approach, HEK293 cells were grown to confluency on 9mm² coverslips and co-transfected with a cytomegalovirus–green fluorescent protein (GFP) reporter construct and FLAG-tagged DN-MSK1. Forty-eight hours later, cells were fixed and blocked as described above and labeled using a poly-clonal antibody directed against GFP (1:1000; Molecular Probes) and a monoclonal anti-FLAG antibody (1:2500; Sigma) at 4° C overnight. Alexa-conjugated secondary antibodies were used (as described above) to visualize the signal. Cells were also labeled with Hoechst 33258 (1µg/ml; Molecular Probes) and then
mounted with Gelmount.

**Data Analysis** – All data were quantified using MetaMorph software (Universal Imaging Corporation, West Chester, PA). For cell counts from DAB-labeled tissue, an intensity threshold filter was initially applied to the image. The filter eliminated non-specific background labeling from analysis. Digital circles were then overlaid on regions with a detectable signal above threshold, now defined as positive cells, and counted. Cell counts were averaged over three consecutive central SCN sections for each experimental animal. For quantization of pMSK1 expression in cultured neurons, digital ovals were placed over nuclei delineated by MAP2 labeling, and signal intensity values were collected from each of these regions. Cells were counted as pMSK1 positive if the average intensity level within a given nuclear region was >75% of the mean maximal signal found across all conditions. The fluorescent images from a minimum of four coverslips (four regions per coverslip) were captured. Significance was determined using the two-tailed Student’s *t* test and was defined as *P*< 0.05. All data are expressed as the mean ± SEM.

**Materials** – PACAP and PACAP 6-38 were obtained from Bachem (Bubendorf, Switzerland). U0126 and SB203580 were purchased from Calbiochem (LaJolla, CA). Luciferin potassium salt was acquired from Promega. All other reagents were obtained from Sigma unless otherwise indicated.
5.4 Results

Light-Induced MSK1 Activation

As an initial step in our examination of MSK1 in the SCN, C57BL/6 mice were entrained to a 12 hr LD cycle and then dark adapted for 2 days. At the end of this period, mice were exposed to light (15 min, 100 lux) during either subjective day (CT6) or subject night (CT15 and CT22) time points. Immediately after photic stimulation, animals were killed, and SCN-containing brain sections were immunohistochemically processed for the serine 360 phosphorylated form of MSK1. We chose to use an antibody that detects the phosphorylation state of serine 360 for two reasons. First, because serine 360 is flanked by prolines in the +2 and -1 positions, it is a likely target for both ERK and p38/MAPK (for review, see Roux and Blenis, 2004; Smith et al., 2004). Second, serine 360 phosphorylation is required to stimulate full MSK1 activity (McCoy et al., 2005). Thus, by monitoring phosphorylation at serine 360, it is possible to infer the activation state of the kinase.

Photic stimulation during both the early (CT15) and late (CT22) subjective night produced a significant increase in the number of cells expressing pMSK1 (Fig. 5.1 A & B). In contrast, photic stimulation during the middle of the subjective day (CT6) failed to activate MSK1. Light-induced MSK1 phosphorylation was highest in the ventral SCN, corresponding to the region that receives the greatest degree of innervation from the RHT (for review, see Moore, 1996; Moore and Silver, 1998). Interestingly, pMSK1 induction was more responsive (two fold to three fold) to late-night than to early-night light pulses. For example, light exposure at CT22 stimulated pMSK1 expression in 117.0±22.6 cells
per SCN section, whereas at CT15 light stimulated pMSK1 expression in 35.6±8.3 cells per SCN section (Fig. 5.1B). In the absence of photic stimulation, we did not observe significant variations in pMSK1 expression over the three time points examined, suggesting that pMSK1 is not regulated by the clock timing mechanism. Reverse-transcriptase (RT)-PCR was used to examine MSK1 mRNA expression as a function of circadian time (Fig. 5.1 C). SCN tissue was collected at CT2, CT8, CT14, and CT20, and the ratio of MSK1 mRNA levels to cyclophilin [a non-rhythmically expressed gene (Uz and Manev, 1998)] mRNA levels was determined (Fig. 5.1 D). Relative levels of MSK1 mRNA did not significantly vary over the four time points examined, indicating that MSK1 is not rhythmically regulated. Collectively, these data reveal that light-induced MSK1 phosphorylation is phase restricted to the night and that phase-advancing and phase-delaying light pulses differentially regulate its activation.

FIGURE 5.1 Light induces MSK1 phosphorylation. A. Mice were exposed to light during the subjective day (CT6) or subjective night (CT15 and CT22). Representative images of SCN-containing coronal brain sections immunohistochemically labeled for pMSK1 are shown. Boxed regions are enlarged below each respective image. Box =100µm , 3V=Third ventricle; OC=optic chiasm. B. Mean number of pMSK1-positive cells per SCN section. Light exposure during the subjective day (CT6) did not elicit pMSK1(P> 0.1). Photic treatment at either night time point (CT15 or CT22) produced a significant increase in the number of cells containing pMSK1 relative to control animals killed at the same time points. Light treatment at CT22 was also found to produce a significant increase in the number of pMSK1-positive cells compared with light exposure at CT15. *=P< 0.05. Numbers below each bar represent the number of animals used for each condition. C. RT-PCR analysis of MSK1 and CYCα mRNA expression in the SCN. D. Densito-metric analysis of data presented in C revealed no significant variation in total MSK1 mRNA expression as a function of circadian time. Results are presented as the ratio of the MSK1 to cyclophilin. Data were averaged from triplicate determinations for each time point.
FIGURE 5.1 Light induces MSK1 phosphorylation.
Given that MSK1 is a potential target of ERK and that light stimulates ERK activation in the SCN, we examined whether light triggers the colocalized expression of phosphorylated forms of the two kinases. To this end, coronal tissue sections from animals exposed to light (CT22, 15 min, 100 lux) and control (no light) animals were double immunolabeled for pMSK1 and the activated (Thr202/Tyr204 phosphorylated) forms of ERK-1 and ERK-2 (pERK). Compared with controls, light stimulated robust pERK and pMSK1 expression in the ventrolateral SCN (Fig. 5.2 A & B). Merging of the images revealed that the majority of pMSK1-positive cells also express activated ERK (indicated by the yellow hue). pERK is expressed in both the cytoplasm and nucleus, whereas MSK1 is exclusively nuclear (Deak et al., 1998). The magnification insets in figure 5.2 B revealed this general staining pattern for the two kinases. High-magnification microscopy of SCN tissue (light exposure, CT22) for pMSK1 and the neuronal-specific nuclear marker NeuN confirmed the nuclear localization of pMSK1 (Fig. 5.2 C).

**FIGURE 5.2** Light triggers the colocalized expression of pMSK1 and pERK in neuronal nuclei. Compared with controls (No Light, A), photic stimulation (Light, B) elicited an increase in the number of pMSK1-positive and pERK-positive cells. Merging of the pERK and pMSK1 signals revealed that the expression of the activated forms of the two kinases was colocalized. Scale bar, 100 μm. The boxed region is magnified in the inset. C. A representative image of ventral SCN tissue from a light-treated animal labeled with antibodies directed against pMSK1 (red) and NeuN (green). The colocalized expression of pMSK1 and NeuN indicates that activated MSK1 is expressed in neuronal nuclei. Scale bar, 15μm.
FIGURE 5.2 Light triggers the colocalized expression of pMSK1 and pERK in neuronal nuclei.
Kinase Coupling to MSK1

Both ERK and p38/MAPK are expressed in the SCN (Obrietan et al., 1998; Pizzio et al., 2003) and capable of phosphorylating MSK1 at serine 360. To determine which of these pathways couples light to serine 360 phosphorylation, we used a ventricular infusion approach to block MEK (and thus ERK) and p38/MAPK enzymatic activity. To this end, animals were infused with drug vehicle (DMSO), the MEK1/2-specific inhibitor U0126 (10mM), the p38/MAPK-specific inhibitor SB203508 (10mM), or a combination of the two inhibitors. Compared with vehicle-infused, light-stimulated animals, U0126 significantly reduced the number of pMSK1-expressing cells (Fig. 5.3 A & B, *P<0.001), indicating that the MAPK pathway couples light to MSK1 phosphorylation. Infusion of SB203508 also attenuated MSK1 activation; however, the level of inhibition failed to reach significance relative to the DMSO-infused light-treatment condition (P>0.06). It should be noted that DMSO elicited a modest, non-significant (P>0.25) reduction in the number of pMSK1-positive cells (compare Figs. 5.1 B & 5.3 B). The infusion of both U0126 and SB203508 reduced light-induced MSK1 phosphorylation to the level of control animals. These data indicate that the MAPK cascade is the dominant regulator of MSK1 activation in the SCN. The inhibitory effects of SB203508 also suggest a role for the p38/MAPK pathway in coupling light to MSK1 activation. However, a cautionary note was raised when we were not able to detect significant light-induced p38/MAPK activation in the SCN (data not shown).
FIGURE 5.3 The MAPK and p38/MAPK pathways couple light to MSK1 activation. Cannulated animals were infused with 3μl of DMSO (vehicle), U0126, SB203508, or a combination of U0126 and SB203508 45 min before light exposure (15 min, 100 lux) at CT 22. A. Representative coronal tissue sections immunolabeled for phosphorylated MSK1. Boxed regions are magnified below each image. B. Average number of pMSK1-positive cells per SCN. Disruption of the MAPK pathway significantly reduced the number of positive cells relative to DMSO infusion. SB203508 attenuated light-induced MSK1 activation; however, this effect failed to reach significance relative to DMSO infused, light-treated animals (P>0.06). The combined administration of U0126 and SB203508 blocked MSK1 phosphorylation. *P<0.001, relative to the DMSO-infused light-treated condition. Numbers below each bar represent the number of animals used for each condition.
FIGURE 5.3 The MAPK and p38/MAPK pathways couple light to MSK1 activation.
PACAP-Dependent MSK1 Activation

The observation that MSK1 was differentially regulated by early- and late-night light pulses suggests that different upstream regulatory events are actuated by light at these two time points. Within this context, it is of interest to note that PACAP has been shown to have complex early-night- and late-night-specific effects on clock entrainment (Chen et al., 1999; Hannibal et al., 2001; Kawaguchi et al., 2003) and can stimulate clock gene expression (Nielsen et al., 2001; Minami et al., 2002). The phase-specific effects of both PACAP and MSK1 raised the possibility that PACAP and MSK1 form a signaling cassette. To begin to address this issue, we examined whether PACAP stimulates MSK1 phosphorylation. To this end, mice were infused with PACAP (200µM) at CT22. Forty-five minutes later, animals were killed and the tissue was processed for pMSK1 expression. Relative to vehicle-infused animals, PACAP elicited a marked increase in pMSK1 expression within the SCN (Fig. 5.4 A).

Consistent with the expression pattern of PACAP receptors (Kalamatianos et al., 2004), PACAP was also found to elicit pMSK1 expression in periventricular regions dorsal to the SCN. Discernable PACAP-induced pMSK1 expression was observed in 75% of the animals (n=8). Vehicle infusion did not alter MSK1 expression (n =5) relative to control, no light conditions (Fig. 5.1 A). As expected, PACAP infusion also stimulated ERK phosphorylation in the SCN (Fig. 5.4 B). To quantitate MSK activation, rat primary neuronal cultures were stimulated with PACAP (200nM) or glutamate (10µM) and then immunolabeled for pMSK1. Relative to control conditions (mock stimulation), both PACAP and glutamate elicited a significant increase in the number of neurons expressing
pMSK1 (Fig.5.4 C & D, *P<0.05). Interestingly, PACAP was a much more effective regulator of pMSK1 than glutamate; PACAP triggered pMSK1 expression in approximately twice as many neurons as glutamate. Similar results were observed using mouse neuronal cultures (data not shown).

To assess whether PACAP receptor activation regulates light-induced pMSK1 in the SCN, mice were infused with PACAP 6-38. This truncated form of PACAP functions as a potent antagonist of the PAC1 receptor (Robberecht et al., 1992) and thus should block the physiological effects of PACAP release from the RHT. Initially, mice were infused with either vehicle (saline) or PACAP6-38 15 min before photic stimulation at CT15 or CT22. Relative to control conditions (vehicle infusion with photic stimulation), the infusion of PACAP 6-38 significantly (*P<0.001) attenuated the capacity of light to stimulate pMSK1 expression (Fig.5.5 A & B). In the absence of light, PACAP 6-38 did not alter the number of pMSK1-positive cells. These data suggest that PACAP signaling through the PAC1 receptor couples light to MSK1 activation.
FIGURE 5.4 PACAP stimulates MSK1 phosphorylation. A. Representative pMSK1 immunostaining from saline (vehicle)- and PACAP(200µM)-infused mice. Mice were infused via the lateral ventricle at CT22 and killed 45 min later. Relative to vehicle infusion, PACAP elicited a marked increase in pMSK1 expression in the periventricular regions, including the SCN. B. PACAP also elicited ERK activation. Representative sections immunolabeled for pERK are from the same animals as in A. C. Primary neuronal cultures were stimulated (10 min) with PACAP (200nM) or glutamate (10µM). Confocal images of cells double immunolabeled for phosphorylated MSK1 (red) and for the neuronal-specific structural protein MAP2 (green) are shown. Arrows indicate the locations of neuronal nuclei expressing activated MSK1. D. Quantified cells counts. Relative to mock-stimulated neurons (control), both glutamate and PACAP elicited a significant increase in the number of pMSK1-positive cells (*P<0.001). Data are expressed as fold stimulation relative to control levels of pMSK, which were normalized to a value of 1. Numbers below bars indicate the number of cells examined for each condition.
FIGURE 5.4 PACAP stimulates MSK1 phosphorylation.
FIGURE 5.5 Inhibition of the PAC1 receptor significantly attenuates light-induced MSK1 phosphorylation. Animals were infused with the PAC1-specific antagonist PACAP 6-38 or saline (vehicle) 15 min before a light treatment (15 min, 100 lux) at CT15 and CT22. A. Representative pMSK1 immunolabeled sections from each of the four treatment groups at CT22. B. Compared with saline infusion, the infusion of PACAP 6-38 significantly reduced the capacity of light to elicit pMSK1 expression (*P<0.001). Although PACAP 6-38 attenuated the number of pMSK expressing cells, a modest but significant residual pMSK1 signal was observed compared with controls (**P<0.05). Numbers below bars denote the number of animals used for that condition. C. Histological examination of cannula placement within the dorsal third ventricle (3V). Black arrow denotes the location of the cannula tip.
FIGURE 5.5 Inhibition of the PAC1 receptor significantly attenuates light-induced MSK1 phosphorylation.
MSK1 Stimulates mPer1

As noted in Introduction, much of the transcriptional potential of the MAPK cascade is exerted by ERK-regulated kinases. As a potent regulator of CREB phosphorylation and histone modification, MSK1 is well positioned to couple light to immediate early clock gene expression.

To examine the role of MSK1 as a regulator of clock gene expression, HEK293 cells were transfected with an mPer1 luciferase reporter construct and DN-MSK1 or CA-MSK1 (Frodin et al., 2000). TPA (200ng/ml) induced a marked increase in mPer1 expression that was blocked by co-transfection with DN-MSK1 (Fig. 5.6 A & B). Pretreatment with U0126 (10µM) also blocked TPA-induced mPer1 expression, indicating that TPA couples to mPer1 via a MAPK/MSK1-dependent mechanism. To further examine the role of MSK1 as a regulator of mPer1 expression, cells were co-transfected with mPer1-luciferase and CA-MSK. Compared with an mPer1-luc / empty vector (pcDNA3.1) transfection, CA-MSK1 triggered a significant six fold increase in mPer1-dependent transcription (Fig. 5.6 A). Finally, to determine whether MSK1 couples to mPer1 via a CREB-dependent mechanism, cells were also transfected with A-CREB, a dominant -negative regulator of CREB-mediated transcription (Ahn et al., 1998). A-CREB attenuated the capacity of MSK to stimulate mPer1-dependent transcription (Fig. 5.6 A). Together, these data suggest that a PACAP / MAPK / MSK1 / CREB signaling cassette couples photic information to period1 expression.
FIGURE 5.6 MSK1 stimulates mPer1-dependent transcription. A. HEK 293 cells were transfected with an mPer1-luciferase reporter construct (200ng/well) and one or a combination of the following constructs: DN-MSK1 (800ng/well), CA-MSK1 (200ng/well), or A-CREB (800ng/well). pcDNA3.1 (empty vector) was cotransfected as needed to bring the total to 1µg/well. Relative to mock-stimulated cells, TPA (200 ng/ml) elicited a marked increase in mPer1 luciferase expression. In contrast, TPA-induced mPer1 expression was attenuated by cotransfection with DN-MSK1 and by pretreatment (30 min) with U0126 (10µM). CA-MSK1 (black bars) stimulated robust mPer1-luciferase expression. Co-transfection with A-CREB significantly attenuated the effects of CA-MSK1, indicating that MSK couples to mPer1-dependent transcription via a CREB-dependent mechanism. Data are expressed as absolute intensity units. Experiments were performed four times, and representative data were averaged from quadruplicate determinations. *P<0.001. B. HEK 293 cells were immunolabeled for FLAG-tagged DN-MSK1 (red) and the cotransfection marker protein GFP (green) and stained with Hoechst 33258 (blue). Scale bar, 25µm.
5.5 Discussion

The goal of this study was to begin a systematic characterization of the ERK-regulated kinase MSK1 in the SCN. Here we report that photic stimulation triggered the phase-dependent phosphorylation of MSK1 at serine 360. Stimulation was maximal during the late subjective night, and signaling via the PAC1 receptor appeared to be central to MSK1 activation. As a regulator of *period1* expression, MSK1 may be a key intermediate in the set of signaling events that couples light to clock entrainment.

Light entrainment of the circadian clock appears to be a transcriptionally dependent process. Correlative support for this concept has been gathered from studies showing that light-induced gene expression precedes clock entrainment (Kornhauser et al., 1990; Rusak et al., 1990; Albrecht et al., 1997; Shigeyoshi et al., 1997; Best et al., 1999; Bae and Weaver, 2003). Causal evidence comes from studies showing that the disruption of clock gene expression disrupts the phase-shifting effects of light and glutamate (Akiyama et al., 1999; Albrecht et al., 2001; Wakamatsu et al., 2001; Trischkau et al., 2003).  

One cellular signaling pathway that has been shown to couple photic information to clock entrainment is the MAPK pathway. We and several other groups have demonstrated that light-induced MAPK pathway activation is phase restricted to the night time portion of the 24 hr cycle (Obrietan et al., 1998; Butcher et al., 2003; Coogan and Piggins, 2003). Furthermore, disruption of signaling via the MAPK cascade blocks both light-induced gene expression (Dziema et al., 2003) and light entrainment of the circadian clock (Butcher et al., 2002; Coogan and Piggins, 2003; Cheng et al., 2004). Interestingly, dysfunctional SCN pacemaker activity removes the phase-restricted responsiveness of the
MAPK pathway to light (Hughes et al., 2004). Together, these data indicate that the MAPK pathway is a key signaling intermediate in light entrainment of the clock and that the clock imposes phase dependency over light responsiveness of the MAPK pathway.

Within the context of gene regulation, two families of kinases, RSKs and MSKs, appear to be central effectors of the MAPK cascade. In a previous study, we found that both light and the endogenous clock timing mechanism regulate the activation state of RSK1 (Butcher et al., 2004). MSK1 is related to the RSK family of kinases (Deak et al., 1998). It is a serine/threonine kinase that is composed of two distinct domains: an N-terminal kinase that phosphorylates MSK1 substrates, and a C-terminal kinase that functions in an autoregulatory role (Deak et al., 1998; Smith et al., 2004; McCoy et al., 2005). MSK1 activation is mediated in part by a series of phosphorylation steps within an approximate 50 amino acid residue linker region that connects the C-and N-terminal kinases. Although these steps are analogous to those that drive RSK activation, there are distinct differences between the two kinases. Principal among them is that MSK1 activation is not dependent on PDK1 (phosphoinositide-dependent protein kinase-1) phosphorylation within the N terminus (Williams et al., 2000). Furthermore, unlike RSKs, MSK1 is exclusively localized to the nucleus, and its activation is regulated by both the MAPK pathway and the p38/MAPK pathway (Deak et al., 1998; Pierrat et al., 1998). Interestingly, MSK1 has been shown to be a much more effective CREB kinase than RSK2 (Deak et al., 1998; Pierrat et al., 1998; Arthur and Cohen, 2000).

Initially, we found that light exposure during the night triggered a marked increase in the number of cells expressing the serine 360 phosphorylated form of MSK1 in the SCN.
Under control (no light) conditions, there was a near total absence of pMSK1 expression in the SCN. Furthermore, pMSK1 expression was not detectable within the surrounding hypothalamic and thalamic brain regions or within the forebrain (data not shown). This absence of pMSK1 expression is surprising given that activated ERK is found in a variety of brain regions under control conditions (Obrietan et al., 1998). The lack of pMSK1 expression may reflect the limited sensitivity of the staining technique used. Conversely, the robust light-specific activation of pMSK1 in the SCN may indicate that MSK1 is primed to convey photic information. It should also be noted that the lack of basal pMSK1 expression in the SCN is in contrast to data showing that pRSK1 and pERK expression are rhythmically regulated (Obrietan et al., 1998; Butcher et al., 2004). This divergence in ERK/RSK1 and MSK1 activity would likely contribute to the distinct gene expression patterns occurring under control conditions and after photic stimulation.

In this study, we used several approaches to determine whether the MAPK pathway regulates pMSK1 expression. Initially, immunofluorescence-based double labeling was used to examine the expression pattern of the activated forms of the kinases after light exposure. In the majority of SCN cells, we observed a spatial and temporal colocalization of the two kinases within cellular nuclei. Given that the inactive form of ERK is anchored to MEK within the cytoplasm (Fukada et al., 1997), these data indicate that light triggers the translocation of ERK. Indeed, we have shown previously that photic stimulation triggers the nuclear accumulation of activated ERK (Butcher et al., 2003). To directly test whether ERK stimulates MSK1 phosphorylation at serine 360, we infused the MEK1/2 inhibitor U0126 into the third ventricle. This technique relies on the
diffusion of U0126 into the periventricular region containing the SCN. In control experiments, we have shown that U0126 diffuses into the SCN and effectively blocks ERK activation (Butcher et al., 2002, 2004; Dziema et al., 2003). The data presented here reveal that the disruption of MAPK signaling significantly attenuates light-induced MSK1 phosphorylation, thus identifying MSK1 as an ERK-regulated kinase in the SCN.

As noted above, MSK1 can also be activated by the p38/MAPK pathway. Because infusion of U0126 did not totally suppress MSK1 phosphorylation, we tested the potential contribution of the p38/MAPK pathway. Infusion of the p38/MAPK inhibitor SB203508 elicited a modest reduction in the number of pMSK1-positive cells after a light pulse. Given the relative specificity of SB203508 (Davies et al., 2000) for neuronally enriched p38/MAPK isoforms (Wang et al., 1997; Jiang et al., 1998), these results suggest that p38/MAPK may play an ancillary role in MSK1 phosphorylation. Interestingly, the combined infusion of U0126 and SB203508 totally blocked light-induced pMSK1 expression. This result lends additional support to the idea that both kinase pathways contribute to light-induced MSK1 phosphorylation in the SCN. As noted in Results, we were notable to conclusively show that p38/MAPK was activated by light in the SCN. This is in contrast to work by Pizzio et al. (2003) who reported that p38/MAPK is light responsive. This discrepancy is the likely result of different methodological approaches used by the two groups.

One striking finding of this study was that MSK1 was much more responsive to late-night than early-night photic stimulation. Light exposure during the late night triggered MSK1 activation in two to three times as many SCN cells as light exposure during the
early night. This difference does not appear to be the result of circadian variations in MSK1 expression; levels of the kinase transcript were approximately equivalent across a 24 hr period. One potential explanation for this disparate sensitivity is that different upstream cellular signaling events are stimulated by light during the early and late night. Interestingly, several studies have shown that the phase-advancing effects of light are more severely disrupted than the phase-delaying effects of light in PACAP-and PAC1-deficient mice (Hannibal et al., 2001; Kawaguchi et al., 2003). The parallels in the late-night effects of PACAP and the late-night responsiveness of MSK1, coupled with the finding that PACAP is a potent activator of the MAPK pathway in SCN neurons (Dziema and Obrietan, 2002), raised the possibility that PACAP couples photic information to MSK1.

To address whether PACAP and MSK1 form a functional signaling cassette in vivo, we infused the PAC1 receptor antagonist PACAP 6-38 into the third ventricle. Infusion of this peptide has been shown to attenuate the phase-shifting effects of light (Bergström et al., 2003). Pretreatment with PACAP 6-38 significantly reduced the number of pMSK1-positive cells after light treatment, indicating that PACAP potently regulates MSK activation in the SCN. In addition, infusion of PACAP stimulated MSK1 phosphorylation. Together, these data raise the possibility that a late-night signaling cassette formed between PACAP and MSK1 contributes to the phase-advancing effects of light.

The simplest route by which MSK1 could regulate clock phase would be through activation of clock gene expression. Interestingly, MSK has been shown to regulate
CREB phosphorylation at serine 133 (Deak et al., 1998; Pierrat et al., 1998; Arthur and Cohen, 2000; Arthur et al., 2004), an event necessary for CRE-mediated gene expression (Gonzalez and Montminy, 1989). Furthermore, the 5’ regulatory region of murine period1 has multiple CRE sites that have been proposed to play a central role in its activity-dependent expression (Yamaguchi et al., 2000; Travnickova-Bendova et al., 2002; Trischkau et al., 2003). In our reporter gene studies, we found that MSK1 coupled the MAPK cascade to mPer1 via a CREB-dependent mechanism. These data fit nicely with previous studies showing that both MAPK signaling and CREB facilitate period1 expression (Cermakian et al., 2002; Travnickova-Bendova et al., 2002). Collectively, these studies identify a light-activated signaling pathway that may directly impinge on the core clock timing mechanism.
CHAPTER 6

MAPK Signaling in the Mammalian SCN:

General Discussion, Conclusions and Future Directions

6.1 Introduction

The presence of an endogenous timing mechanism or “clock” allows an organism to maintain synchronization with the exogenous environment. Furthermore, when regular timing of biological events occurs in parallel with those of the external world, anticipating the need for specific physiological processes (enzyme secretion, enhanced blood flow, increased respiration, etc.) before they are actually required becomes possible, thus conferring an adaptive advantage (Ouyang et al., 1998; Michael et al., 2003). It is therefore not surprising that a fundamental timekeeping mechanism has been conserved across a broad range of organisms (for reviews see Dunlap 1998; Wilsbacher and Takahashi, 1998; Reppert and Weaver, 2000). Over the past ten years many of the genes and proteins, which make up the transcriptional / translational feedback loops at the heart of the circadian clock, have been identified and their functions characterized (for reviews see King and Takahashi, 2000; Lowrey and Takahashi, 2000; Reppert and Weaver, 2002). However, the molecular components that couple external timing cues,
such as light, to the expression of these “clock” genes are less clear and have been the
primary focus of the experiments described in this dissertation.

To briefly review, upon activation by light, a subset of melanopsin containing retinal
ganglion cells relay timing information via the retinohypothalamic tract (RHT) to a
bilateral cluster of neurons, known as the suprachiasmatic nuclei (SCN; Provencio et al.,
2000; Hannibal et al., 2004). In mammals, this region of the hypothalamus has
historically been designated by the auspicious title of “master” or “central clock” (Moore,
1983; Hastings 1997; Weaver, 1998). However, in light of evidence demonstrating
peripheral oscillators at the tissue and cellular levels, the role of the SCN as the dominant
biological clock has come into question (Schibler and Sassone-Corsi, 2002; Cermakian et
al., 2003; Hastings and Herzog, 2004; Panda and Hogenesch, 2004). Despite these
accounts, the essential function of maintaining body-wide synchronization between the
myriad of peripheral clocks, appears to rest securely in the domain of the SCN (Morse
and Sassone-Corsi, 2002; Aton and Herzog, 2005).

Within the central core of the SCN, terminals of RHT projections synapse upon
neurons and release a combination of the neurotransmitter glutamate and the
neuromodulator pituitary adenylate cyclase activating polypeptide (PACAP; Ebling,
1996; Hannibal et al., 1997; Nielsen et al., 2001; Minami et al., 2002). These agents
stimulate constituent receptors and initiate intracellular signaling via the activation of
multiple second messenger systems (Golombek and Ralph 1994, 1995; Gillette 1997;
Meijer and Schwartz, 2003). Among these signaling cascades, the mitogen-activated
protein kinase (MAPK) pathway has drawn much attention in the past few years. Light-
induced activation of this pathway is phase-restricted to the subjective night (Obrietan et al., 1998). Furthermore, MAPK signaling couples light to behavioral phase shifting (Chapter 2 above; Butcher et al., 2002; Coogan and Piggins, 2002), is involved in the expression of light-induced immediate early gene products (Dziema et al., 2003), and possesses a built-in mechanism for inactivation following brief or constant light stimulus (Chapter 3 above; Butcher et al., 2003); all key properties of a putative input pathway to the mammalian circadian clock.

As multiple lines of evidence have suggested that CRE-dependent clock gene expression underlies photic entrainment (Travnickova-Bendova et al., 2002; Cermakian et al., 2002), a signaling pathway that impinges upon CRE-mediated transcription, such as the MAPK pathway, is of significant interest. The MAPK pathway does not directly regulate CRE-dependent transcription. Rather, upon activation, MAPK signaling results in the recruitment and phosphorylation of multiple downstream substrate kinases, which may in turn phosphorylate the CRE-binding protein (CREB) and the CREB-binding protein (CBP; Nakajima et al., 1996; Xing et al., 1996; De Cesare et al., 1998; Ait-Ai-Ali et al., 1999). Therefore, light-induced signaling via the MAPK pathway presents a potential route for indirect modulation of CRE-dependent gene expression.

Phosphorylation of CREB at the physiologically relevant Ser 133 has been observed following phase-specific photic stimulation corresponding with that of light-induced MAPK activation (Ginty et al., 1993; Xing et al., 1996; Obrietan et al., 1999). Interestingly, two related families of MAPK substrates, the p90 ribosomal S6 kinases (RSKs) and mitogen- and stress-activated kinases (MSKs), are also activated by light at
these time points (Chapters 4 & 5 above; Butcher et al., 2004 and 2005 respectively) and have been shown to phosphorylate CREB at Ser 133 in other model systems (Xing et al., 1996; Deak et al., 1998; Frodin and Gammeltoft, 1999; Arthur and Cohen, 2000).

Previously we have demonstrated that RSK1 and MSK1 are activated by light in a phase-restricted manner and can be pharmacologically inhibited through blockade of MEK (Chapters 4 & 5 above; Butcher et al., 2004 and 2005). Interestingly, while light-induced activation for both RSK1 and MSK1 occurs during the subjective night, maximal activation of these kinases occurs at different time points. Figure 6.1 which combines data originally presented in Chapters 4 and 5 above and provides a relative comparison of the phase-specific activation patterns for these kinases. While RSK1 is maximally activated during the early subjective night, peak MSK1 phosphorylation occurs during the late subjective night. Behaviorally, light stimulation at these two points results in either phase delays (early night) or advances (late night; reviewed by Johnson, 1999). In conjunction, these observations suggest a potential relationship between RSK/MSK signaling and light-induced behavioral phase shifting. To test this supposition, an experimental approach whereby the activation and/or functional effect(s) of each kinase could be independently assessed would be of significant interest. We have recently begun such a characterization. As specific pharmacological inhibitors for these two kinases are not presently available, we have chosen to examine photic signaling and entrainment in three lines of knockout mice lacking MSK1, MSK1 and MSK2 (MSK1/2), or RSK2. Preliminary data presented below summarizes the initial test of our hypotheses that light-induced phase advances, but not phase delays, are mediated, in part
by a MSK-dependent mechanism, and that RSKs contribute to light-induced phase delays, but not phase advances.

Figure 6.1 Summary data comparing light-induced phosphorylation of MSK1 and RSK1 in the SCN of mice. Data presented in Chapters 4 and 5 above have been scaled to allow for a more direct comparison of the relative activation for each kinase. Grey bars represent relative expression of pRSK1 and original quantification is retained on the left axis. Black bars reflect average pMSK1 cell counts / SCN section and are quantified on the right axis. Note maximal activation for RSK1 occurs following a light flash (LF) administered during the early subjective night (CT15), while the maximal number of pMSK1 positive cells was observed following a late-night LF (CT22). Error bars represent SEM.

6.2 Materials and Methods

Animals - Breeding pairs of the MSK1 and MSK1/2 knockout mice were obtained from the lab of Dr. Simon Arthur, University of Dundee, UK. Generation of these animals is described elsewhere (Arthur & Cohen, 2000; Wiggin et al., 2002). RSK2 knockout mice were provided by Dr. Andre Hanauer, Institut de Genetique et de Biologie Moleculaire et
Cellulaire, C.U. de Strasbourg, France. Mice were generated as described in Yang et al. (2004). All three lines were produced on a C57BL/6J background and breeding colony founders were backcrossed for at least three generations prior to use. For all studies, adult (8-12 weeks of age) mice were used and wild type C57BL/6J mice served as normal control animals. Genotype of each animal was confirmed by RT-PCR using the primers CACTTCGCCCAATAGCAGCCAGTCCCTTCC (targeted knockout), TCCGCAGCT-CGTGCTTGACAGTAAGGAGC (wild type) and AATAGCGCTGGTGCTCAGGGCTGT (knockout and wild type) for MSK1. These primers result in 870bp fragment from the targeted knockout animals and a 350bp fragment in wild type mice. MSK2 was identified using the primers CGTTGGCTACCCGTAATATTGCTGAAGAGC (knockout), AAGATCTTCAGGCGATCTC-TTTATCCTACG (wild type), and TTGTGCTCCCCCATGTCAGCCCGGCCTTC (knockout and wild type), which produce 1030bp (wild type) and 600bp (knockout) fragments. RSK2 knockouts were identified using the primers AAGATGATTGCTTT-GCTTAGGTTA and TTGTGGTTTACTTTCTTTCGTTCTG, which produce 220bp and 300bp fragments for wild type and knockouts respectively. All experiments were conducted according to The Ohio State University animal welfare guidelines.

**Behavioral Phase Shifting** - Animals were housed individually under a standard 12:12 light dark (LD) cycle for at least 21 days prior to behavioral monitoring. Illumination was provided by a fluorescent white light (approximately 300 lux at mid-cage level). Locomotor activity was monitored via the revolution of a 15 cm running wheel and the
resulting closure of a magnetic microswitch. Data were automatically recorded for subsequent off line analysis by the Vital View data acquisition system (Minimitter Corp., Bend, OR). Following 14 days of baseline activity collection under the 12:12 LD, “dawn” and “dusk” were advanced by 8 hours. Animals were housed under this light cycle for 14 days, at which point the light cycle was delayed by 8 hrs, a return to the original lighting cycle. Food, water and bedding were refreshed weekly at random times during the day period. Onset of activity, a persistent period of wheel running lasting greater than 2 hrs in duration, was used to determine the number of days required for animals to resynchronize to the new light cycle. Animals were then transitioned into a dark / dark (DD) environment and allowed to free-run for 14 days to determine endogenous period length (tau) for each strain. To determine whether variability in gross activity might influence our observations, the total number of wheel revolutions for a 10 day period under free-running conditions was calculated for each strain.

6.3 Preliminary Results

Genotypic and Phenotypic Assessment - Prior to experimental manipulation, genotypes were confirmed in all animals as described above (data not shown). As the coding sequence for RSK2 is located within the p22 locus of the X chromosome (Jacquot et al., 2002), we chose to examine males and homozygous females (-/y or -/-) separately from heterozygous females (-/+). Behavioral analysis was conducted to determine whether variations in overall activity (wheel revolutions per hour) or period length (tau) were present in any of the strains tested (Fig. 6.2 A & B). Under free-running conditions, none
of the five strains were found to exhibit significantly different levels of activity compared with wild type (WT) mice (P>0.05). In contrast, free-running period (tau) in MSK1 -/- and MSK1/2-/- mice was found to be significantly longer than that of WT mice (23.93 ± 0.06, 24.07 ± 0.16, and 23.59 ± 0.05 hrs respectively). No significant difference between either RSK2 -/- or -/+ mice and that of WTs was found (P>0.1), although a trend towards an increasing tau was noted.

![Figure 6.2 Phenotypic assessments of MSK1, MSK1/2 and RSK2 knockout mouse strains.](image)

A) Overall locomotor activity as determined by average wheel revolutions per hour, assessed for a 10 day period under free-running conditions was determined for each strain. No significant differences were found between any of the strains examined (P>0.05). B) Endogenous period length (Tau) was calculated for each strain for the same 10 days. Both MSK1 -/- and MSK1/2--/- mice were found to have significantly longer free-running period than WT animals (*P<0.05). Although tau for homozygous and heterozygous RSK2 mice was not significantly different than WT (P>1), a trend towards an increase in tau was observed.

Wheel-running behavior in mice housed under a 12:12 LD cycle for greater than three weeks was monitored for 14 days to confirm that all animals were entrained. The light cycle was then advanced by 8 hrs and the time required to resynchronize to the new cycle
determined. After all mice had re-entrained, the photoperiod was delayed by 8 hrs (a return to the original light-cycle) and behavioral monitoring continued. Under our 8 hr advancing paradigm, wild type mice were found to resynchronize to the new photic period in an average of 2.83 ± 0.40 days (Fig. 6.3 A open circles). MSK1/-/ and MSK1/2 -/- mice were not found to be significantly different from each other (P>0.05). However, both strains required a significantly greater number of days that WT animals to resynchronize to the 8 hr advancing cycle (7.40 ± 1.57 and 7.00 ± 0.71 days respectively; * = P<0.05; Fig. 6.3 C1). Interestingly, two MSK1 -/- mice and one MSK1/2 -/- mouse were found to exhibit strikingly different patterns of entrainment and were separated for analysis. MSK1/-/ Atypical A (Fig. 6.3, A and C2) required the same number of days to re-entrain, but this animal underwent a pronounced phase delay of 16 hrs rather than the anticipated 8 hr phase advance. Over the initial 6 days of assessment, MSK1/-/ Atypical B (Fig 6.3A & C2) demonstrated a behavioral pattern similar to that of Atypical A, characterized by attenuated activity and the inability to resynchronize to the 8 hr advance. Beginning 7 days after the light advance, activity of Atypical B began to increase and within 24 hrs was synchronized with the new cycle. Neither typical (2.40 ± 0.98 days) nor atypical (A&B 3.00 ± 0.00 days) MSK1/-/ mice required a statistically different amount of time to resynchronize to the 8 hr delay, compared with WT animals (2.83 ± 0.40 days; P>0.05; Fig. 6.3 B). However, MSK1/2 -/- required significantly greater amount of time to synchronize to the delay (3.40 ± 0.68 days; Fig. 6.4 D; * = P<0.05).

Behavioral data was then collected from RSK2 -/-y and +/- mice under the same light-shifting paradigm. Interestingly, compared to WT mice, neither homozygous nor
heterozygous RSK2 animals were found to have significant deficits in their ability to resynchronize to either phase advances or delays (Fig. 6.4, A and B; P>.05), although a trend towards significance was observed in the heterozygous mice over the initial two days. Summary data from the five strains tested are presented in figure 6.4 D.
Figure 6.3 Behavioral resynchronization to an 8 hr advanced or delayed light cycle. Wild type (WT), MSK1 -/-, and MSK1/2 -/- mice were entrained to a 12:12 LD cycle then monitored for wheel running activity to verify entrainment. The light cycle was then advanced by 8 hrs and the time required for animals to resynchronize determined. Once all mice had re-entrained (14 days), the light cycle was delayed by 8 hrs (a return to the original lighting conditions) and behavioral monitoring continued. A) Daily changes in activity onset for WT, MSK1 -/-, and MSK1/2 -/- mice. Data from two atypical MSK1 -/- animals are also presented. Significant differences between WT and the knockout strains are denoted by * = P<0.05. B) Daily changes in activity onset following a delay in light onset. Average daily shifts in activity onset in MSK1/2 -/- mice was reduced compared with WT animals for the first two days following transition into the new light cycle (*=P<0.05). C1-3) Representative double plotted actograms from the indicated animal strains taken from the period before and following each lighting transition. Shaded areas reflect dark period of each cycle.
Figure 6.4 Behavioral resynchronization in homozygous and heterozygous RSK2 knockout mice. A & B) Average daily advance or delay (respectively) in behavioral onset in WT, RSK2 -/- and RSK -/+ mice. No significant difference from WT animals was observed over the period examined. C) Representative actograms from a RSK2 homozygous male and a heterozygous female. Shaded regions reflect the dark portion of each daily cycle. D) Summary data from each of the five strains examined. Average number of days required for each strain to resynchronize to the advance or delay in light onset is presented, error bars represent SEM. MSK1-/- and MSK1/2-/- required approximately twice the time to entrain to an advancing light cycle (*=P<0.05). Approximately 30% more time was also required for MSK1/2-/- mice to entrain to a delaying light cycle compared to WT animals (*=P<0.05). Neither strain of RSK2 mice were found to be statistically different from WT animals in their response to an advancing or delaying light cycle.
6.4 General Discussion

Beginning with the observations of Obrietan et al. (1998) and continuing with the data presented in this dissertation, our mechanistic evaluation of mammalian photic entrainment has indicated that light-induced activation of the MAPK pathway contributes to the phenomenon of photic entrainment and suggests the involvement of substrate kinases including MSKs and RSKs. Preliminary analysis of circadian phenotypes presented by MSK1, MSK1/2 and RSK2 knockout mice has begun to test several aspect of our current working hypothesis. Specifically that light-induced phase advances, but not phase delays, will be attenuated in mice lacking MSK. While the majority of MSK1-/- and MSK1/2 -/- mice required approximately twice as long to fully entrain to an 8 hr advancing light-cycle (Fig. 6.3), there are several interesting aspects of this data that should be noted. First while overall activity under free-running conditions was not significantly different in any of the strains examined (Fig. 6.2 A), during the 3-7 days initially following an 8 hr advance, most of the MSK1 -/- and MSK1/2 -/- mice underwent a reduction in overall activity. Furthermore, 80% of these animals demonstrated an initial bout of wheel-running, which failed to meet the pre-determined criteria (continual activity in excess of 2 hrs), but occurred in “statistical synchrony” with that of wild type animals (Fig. 6.5 A). When the absolute onset of activity was calculated using this initial bout, virtually all of the WT, MSK1-/- and MSK1/2-/- mice resynchronize within 48 hrs of the advancing phase shift, including those animals previously defined as atypical.
Figure 6.5 Revised behavioral resynchronization in WT, MSK1-/- and MSK1/2-/- mice. As the majority (80%) of MSK1-/- and MSK1/2 mice demonstrated a brief (<2 hr) bout of wheel running prior to the determined onset of activity (persistent activity <2hrs in duration), data presented in figure 6.3 was recalculated to reflect absolute activity onset. A) Daily advances from original lighting cycle following an 8 hr advance. Within 24-48 hrs of lighting shift, all mice were statistically resynchronized. B) Representative double plotted actogram from a MSK1-/- mouse. Dark period for each cycle is indicated by shaded regions, absolute activity onset is denoted by black arrowhead, activity onset used to calculate data presented in figure 6.3 & 6.4 is denoted by white arrowhead.

As dark onset (dusk) following the 8hr advance occurred during a period previously characterized as early subjective day, we cannot rule out the possibility that the initial bouts of activity were induced by the phase shift being perceived as a “dark pulse.” Previous work has indicated that dark pulses 6-9 hrs in duration, presented at this time point are able to suppress clock gene expression (Mendoza et al., 2004) and induce phase advances (Canal and Piggins, 2006). However such advances are typically accompanied by “an increase in the intensity of wheel-running on the day of the pulse” (Canal and
Piggins, 2006). In contrast, virtually all knockout mice underwent a reduction in activity beginning on the day of the supposed dark pulse, which persisted for several days.

A similar pattern of activity (a brief initial bout of wheel running followed by a prolonged rest period) was observed by Nagano et al. (2003) while assessing desynchronization between two sub-regions within the SCN of rats. In this study, an abrupt shift (6 hr advance or 10 hr delay) in the light cycle was sufficient to desynchronize clock gene expression in oscillators contained within the ventrolateral (VL) and dorsomedial (DM) regions of the SCN. Furthermore, increased rest (inactivity) following the light shift was inversely correlated with \( rPer1 \) expression in the DM SCN (Nagano et al., 2003), which the authors suggest may function as a signal for “subjective day.” In nocturnal animals light typically suppresses activity during the day through a process known as masking (Aschoff, 1999; Mrosovsky, 1999). Thus, according to the authors (Nagano et al., 2003), expression of \( rPer1 \) during the night is interpreted as a photic stimuli, thus inhibiting normal activity. Interestingly, all three of our knockout strains exhibited pronounced reduction in overall activity following light cycle shifts (Figs. 6.3, 6.4, and 6.5). As clock gene expression during the resynchronization period was not examined in our preliminary assessment, a direct test of the hypothesis put forth by Nagano et al. (2003) is not possible at this time. However, one could speculate that if RSK- or, more likely, MSK-mediated \( Per1 \) expression is necessary for resynchronization of the two SCN oscillators, animals lacking these kinases may require a longer period of time to fully re-entrain to a new light cycle. Furthermore, if RSK/MSK-mediated \( Per1 \) expression is attenuated in RSK/MSK knockout mice, then \( Per1 \)-induced suppression of
activity should also be reduced. Again, this was not observed in our preliminary assessment as knockout mice had greater periods of inactivity than WT's.

An alternative interpretation for the sub-criterion activity periods noted above could be derived from the dual oscillator hypothesis first proposed by Pittendrigh and Daan (1976b). This intriguing model attempts to explain the phase-specific effects of photic stimulation during the early and late night as functions of “morning” (M) and “evening” (E) oscillators. These oscillators are predicted to independently couple dawn and dusk photic information to behavioral advances and delays respectively. In response to recent data, Daan et al. (2001) have reframed this hypothesis such that the M and E oscillators are mechanistically attributed to oscillations in neuronal activity and phase-restricted expression corresponding with that of the clock genes Per1 / Cry1 and Per2 / Cry2 respectively. In their revised hypothesis, the M / Per1 / Cry1 oscillator is accelerated by late night / early morning light resulting in phase advances. Conversely, the E / Per2 / Cry2 oscillator is decelerated by light and mediates phase delays (Daan et al., 2001).

Additional evidence supporting portions of this hypothesis has been reported by a variety of groups. For example McArthur et al. (1997) reported a melatonin induced phase advances were mediated by gated PKC signaling occurring at dawn (CT23-0) and dusk (CT10-14). This study did not examine the specific neuronal population (shell versus core, AVP versus VIP expressing neurons, etc.) that responded to stimuli during each interval. Yet given evidence of discrete oscillators within sub-regions of the SCN (Shinohara et al., 1995; Jagota et al., 2000; Yan et al., 2005), one could speculate that the dawn / dusk gating mechanism reported by McArthur et al. (1997) may be functionally
restricted to different neuronal pools. It has also been suggested that neurons regulating
the M and E oscillators may in fact reside in different neuronal clusters (van den Pol and
Dudek, 1993; Miller 1998; Honma et al., 1998), possibly corresponding to the same
regions reported to exhibit differential clock gene expression (Yan and Silver, 2004). The
precise neuronal population in which light induces phosphorylation of RSKs and MSKs
is currently unknown. If activated RSKs and MSKs are also found to localize in different
neuronal populations, the phase-specific signaling cassettes PKC-MAPK-RSKs-
Per2/Cry2 (early night, phase delays) and PKC-MAPK-MSKs-Per1/Cry1 (late night,
phase advances) could mechanistically account for the E and M oscillators respectively
and would support our initial hypothesis. As our data indicates moderate, but significant
activation of RSK1 and MSK1 during non-peak periods (ZT 22 for RSK1, and ZT 15 for
MSK), it is also possible that each kinase may function to augment signaling of the other.

Within this context, data from the MSK knockout animals could be interpreted as a
byproduct of functionally muted M oscillator. Disruption of MSK signaling results in
normal absolute activity onset (Fig. 6.5). However the M oscillator, which under the
Daan et al. model (2001) should be responsive to the advanced light cycle, is unable to
drive normal levels of activity. The E oscillator, which responds more slowly to an
advancing light cycle (Steinlechner et al., 2002), requires significantly greater time to
“catch-up” with the rapid, but potentially weakened M oscillator. As the E oscillator in
both strains of MSK knockout mice functions properly (Fig. 6.3) behavioral
resynchronization to an 8 hr phase delay occurs normally. If MSK signaling functions as
the dominant and exclusive driving force of the M oscillator, as characterized by Daan et
al. (2001), light induced *Per1 / Cry1* expression and the resultant protein levels should be attenuated in MSK knockout mice. MSK1 expression and phosphorylation do not rhythmically cycle under endogenous conditions (Chapter 5; Butcher et al., 2005). However, the involvement of MSK1 and/or MSK2 in maintaining rhythmic clock gene expression has not been fully elucidated. While transfection of a dominant negative (inactive) MSK1 construct into HEK293 cells was found to attenuate expression of an mPer1-promoter driven luciferase reporter (Butcher et al., 2005), the precise contribution of MSKs to clock gene expression *in vivo* is not known. Analysis of endogenous versus light-induced clock gene expression in MSK1 and MSK1/2 knockout animals should clarify this issue.

Interestingly, neither the RSK2 homozygous nor heterozygous mice required a significantly greater duration of time than WTs to resynchronize to either advancing or delaying cycles (Fig. 6.4). These data indicate that, in contrast to our original hypothesis, RSK2 does not mediate re-entrainment to a delayed light cycle and implies that an alternative mechanism contributes to regulation of the putative E oscillator and light-induced phase delays. As RSK2 knockout animals retain functional RSK1 and RSK3, we cannot, at this time, discount the possibility one or both of these isoforms is up-regulated and provides partial compensation for the missing kinase. A comparison of total and activated RSK1 / RSK3 levels in WT and RSK2 knockout animals may alleviate this concern. Furthermore, our preliminary data does not fully support the hypothesized PKC-MAPK-RSKs-*Per2/Cry2* signaling cassette described above. Rather, signaling by RSK2 may serve as a gain control that enhances MSK-mediated signaling. Although
MSK knockout mice retain normal RSK2 signaling, significant behavioral differences from wild type mice were observed in these animals. Therefore, RSK2 does not appear to completely compensate for MSK signaling and is not sufficient to drive normal behavioral activity. Phase delays, mediated by the putative E oscillator, of triple MSK1 / MSK2 / RSK2 knockout mice would likely appear similar to those of WT animals. Phenotypic behavioral assessment and analysis of clock gene expression profiles in a triple knockout animal may provide verification of this hypothesis. However, lacking the driving signal of MSKs and the augmentation of RSK2, such an animal may also undergo splitting of their behavioral rhythms.

Behavioral splitting is characterized as bimodal bouts of activity centered on subjective “dawn” and “dusk” and has been well documented in hamsters (Pittendrigh and Daan, 1976) and in the CS strain of mice (Staats, 1985; Abe et al., 1999). Behaviorally, CS mice exhibit uncoupling of the M and E oscillators resulting in spontaneous rhythm splitting under certain lighting conditions (Abe et al., 1999). While the functional mechanism that produces splitting is unclear (compare Shinohara et al., 1995 and Jagota et al., 2000 with Abe et al., 2001), quantitative trait locus analysis has implicated the distal region of chromosome 19 (Chr 19), and the mid-region of Chr 12 and 19 (Suzuki et al., 2001). Interestingly basic local alignment search tool (BLAST) analysis has determined the MSK1 coding sequence is also located on Chr 12 (National Center for Biotechnology Information [NCBI] Gene ID 73086), while MSK2 resides within Chr 19 (NCBI Gene ID 56613; see also Deak et al., 1998). Clarifying whether MSK signaling, or disruption thereof, contributes functionally to the CS mouse
phenotype would be of significant interest and may allow for refinement of the M / E oscillator hypothesis.

6.5 Future Directions

The studies presented in this dissertation describe the systematic analysis of a molecular signaling cascade, the MAPK pathway, which couples light to behavioral entrainment via regulation of a circadian clock located in the SCN. The following hypotheses were tested: 1) Light-induced behavioral phase shifting is coupled via a MAPK dependent mechanism. 2) Light activation of the MAPK pathway is stringently regulated with respect to the time course of activation / inactivation and subcellular localization of ERK within neurons of the SCN. 3) Light-induced activation of RSKs within the SCN is phase-restricted to the subjective night and is dependent upon activation of the MAPK pathway. 4) Light-induced activation of MSKs within the SCN is dependent upon a signaling cassette consisting of the neuromodulator PACAP and the MAPK pathway. 5) Light-induced phase advances, but not phase delays, will be attenuated in mice lacking MSKs and, conversely, light-induced phase delays, but not phase advances will be attenuated in mice lacking RSKs.

Preliminary data (see above) was not able to sufficiently confirm or refute our fifth hypothesis in its entirety. Behavioral assessment of MSK and RSK knockout mice was able to demonstrate that MSK1 and, to a lesser extent, MSK2 contribute to re-synchronization following an 8 hr shift advance in the light cycle. Interestingly, RSK2 knockout mice were not significantly different from WT mice in their ability to re-entrain
to an advancing or delaying shift in light cycle. It should be noted that light-induced activation of RSK2, has not yet been experimentally confirmed. Data presented in Chapter 4 describe light-induced activation of RSK1 within the SCN (Butcher et al., 2004). As antibodies specific to RSK2 were not available when the study was completed, RSK2 signaling was not examined. Verification of light-induced RSK2 phosphorylation in the SCN is ongoing and should provide clarification of this issue. However, due to the significant homology with RSK1 (Frodin and Gammeltoft, 1999), it is probable that light-induced RSK2 phosphorylation will be observed and its activation is predicted to parallel that of RSK1.

![Figure 6.6 Putative MSK and RSK phosphorylation sites within regulators of clock gene expression. Serine and Threonine residues within RORα, SRF, Elk-1, CBP and CREB that contain the appropriate motif (RXXS : MSK1 and RRXS/T : RSKs) are indicated for each protein. Residues appearing in a bold face, underlined or italicized type represent sites exclusive to MSKs, RSKs or both kinases respectively. Response elements are color coded for their corresponding transcription factors.](image)

A second, yet equally intriguing, line of work concerns the regulation of clock gene expression by MSKs and RSKs. Previous work has suggested that the clock proteins mPER1 and mPER2 may contribute differentially to phase advances and phase delays
Indeed functional differences between mPER1 and mPER2 have been suggested to manifest in the form of the M and E oscillators described above. If a mechanism to segregate MSKs and RSKs mediated activation of transcription factors is present, then RSKs and MSKs may differentially regulate phase-specific expression of clock genes and corresponding levels of clock protein. Evidence in favor of such a mechanism would provide additional support for the M / E hypothesis. Figure 6.6 illustrates several putative phosphorylation sites within the retinoid-related receptor α (RORα), serum response factor (SRF), ETS like protein 1(Elk-1), CBP and CREB through which MSKs and/or RSKs could regulate the expression of clock genes. In the case of RORα regulation of Per and Cry genes has been suggested to occur through an indirect, the transcriptional regulation of BMAL1. The BMAL1/CLOCK complex also promotes expression of additional transcription factors, which bind to retinoic acid-related orphan receptor response elements (ROREs) found within the promoter region of the Bmal1 gene (Sato et al., 2004). Among these REV-ERB and RORα have been shown to enhance or attenuate (respectively) the expression of Bmal1 (Guillaumond et al., 2005; Akashi & Takumi, 2005). A reduction in the levels of Bmal1 would correspond to reduced BMAL1/CLOCK mediated transcription. Thus regulation of RORα via MSK or RSK signaling presents an indirect mechanism for Per and Cry expression.

Regulation of RORα mediated transcription by CaMK IV has also been observed (Kane and Means, 2000). Previously CaMK signaling has been implicated in glutamate- and light-induced phase shifting (Fukushima et al., 1997; Golombek and Ralph, 1995),
the expression of clock genes (Nomura et al., 2003), and as an upstream regulator of light-induced MAPK signaling in the SCN (Chapter 2; Butcher et al., 200). As signaling via the CaMK and MAPK pathways can occur in series (Soderling, 1999; Watt et al., 2001; Wu et al., 2001) or in parallel (Yokota et al., 2001), work to clarify the degree of cross-talk and/or synergistic signaling between these pathways is of interest. It should also be noted that Kane and Means (2000) attributed regulation of RORα-mediated gene expression to an indirect mechanism involving the transcriptional co-factors CBP / p300. Both CBP and p300 contain consensus motifs for MSK1 and RSK1/2 phosphorylation sites (Fig. 6.5; Sassone-Corsi et al., 1999; Meirienne et al., 2001; Janknecht, 2003). In the context of light-induced gene expression, chromatin remodeling resulting from the convergence of CaMK and MAPK signaling via RSKs and MSKs may provide future insight into the regulation of clock genes.

While clock gene expression via an SRE mechanism is more speculative, both mPer1 and mPer2 contain the appropriate motif to support transcription factor binding and regulation. Phase-restricted, light-induced phosphorylation of the transcription factor Elk-1 has been observed in the SCN of Syrian hamsters (Coogan & Piggins, 2003). As the SRF and Elk-1 contain putative phosphorylation sites specific to RSKs (SRF; Thr 155) and MSKs (SRF; Ser 99 and 372; Elk-1; Ser 77, 180, 201) the potential for transcriptional regulation by these kinases is intriguing. Further work to characterize the functional involvement of these sites may provide additional mechanistic insight into direct and indirect avenues for RSK- / MSK-mediated gene expression.


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