TEMPORAL REGULATION OF LIGHT-INDUCED EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION IN THE SUPRACHIASMATIC NUCLEUS

Number of Pages: 38
Words in Abstract: 318
Words in Introduction: 616
Words in Discussion: 1771
Number of Figures: 6
Number of Tables: 0

Corresponding Author:
Karl Obrietan
Department of Neuroscience
The Ohio State University
Graves Hall, Rm 4118
333 W. 10th Ave. Columbus, OH 43210
Phone: (614) 292-4432
Fax: (614) 688-8742
E-mail: obrietan.1@osu.edu

Authors: Greg Q. Butcher, Boyoung Lee, Karl Obrietan Department of Neuroscience. The Ohio State University. 333 W. 10th Ave. Columbus, OH 43210

Running Title: Light-induced ERK regulation in the SCN

Key words: phase shift, MAPK, circadian, transcription, Ras, mouse
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 discreet 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 elicited by a 15 min 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.
INTRODUCTION

The endogenous pacemaker located in the suprachiasmatic nucleus (SCN) regulates numerous biochemical, physiological and behavioral processes with ~ 24 hr periodicity (Miller et al., 1996; Allada et al., 2001; 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 (Rea, 1998; Lowrey and Takahashi, 2000; Foster and Helfrich-Forster, 2001). 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 (Liou et al., 1986; Colwell and Menaker, 1992), thereby triggering a cascade of intracellular signaling events that ultimately impinge upon and reset the core clock timing mechanism.

Light-induced resetting of the circadian clock is dependent on transcription activation (Lowrey and Takahashi, 2000; Cermakian and Sassone-Corsi, 2002). 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; Rusak et al., 1990; Kornhauser et al., 1992; Rusak et al., 1992), and the core clock genes Period 1 and Period 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, MAPK/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK). In neurons, the MAPK pathway is activated by a large array of stimuli, including trophic factors, neurotransmitters and modulatory peptides (Kurino et al., 1995; Yan et al.,
1999; Cavanaugh et al., 2001; Chang and Berg, 2001; Dziema and Obrietan, 2002). 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 (Fukuda et al., 1997; Khokhlatchev et al., 1998; Adachi et al., 1999). The nuclear translocation of ERK is a key intermediate event that couples the MAPK pathway to transcriptional activation (Treisman, 1996; Cobb, 1999; Grewal et al., 1999).

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 et al. 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 zebrafish 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 mechanisms regulating activation of this pathway. Here, we provide data on the subcellular and temporal regulation of light-induced ERK activation and the SCN. These data provide new mechanistic insights into how light and the SCN regulate the activation state of the MAPK cascade.
MATERIALS AND METHODS

Animals

Adult C57BL6 mice (8-12 weeks 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 hr light-dark (LD) cycle for at least three weeks, then divided into 3 groups: 15-min light pulse (SLP), constant light (CL) and no light, control animals. Following photic stimulation (white light, 100 lux at cage level), animals in the LP group were returned to darkness for 0, 15, 30, 45, 75 and 105 min, then sacrificed. Animals in the CL group were exposed to light for 15 min to 120 min, then sacrificed. 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 sacrificed immediately following the final light pulse. Another group of animals received two light pulses and were returned to darkness for an additional 60 min prior to sacrifice.

Tissue Processing. Mice were sacrificed 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 post-mortem 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% w/v) for at least 4 hours followed by cryoprotection in 30% sucrose (w/v) containing 3 mM NaF for at least 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 at least 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 diameter running wheel. Closures of a magnetic microswitches attached to the running wheels were automatically recorded by a personal computer running Vital View data acquisition software (Minimitter Corp., Bend, Oregon). After 9 days in DD, half of the animals received a 15 min, 500 lux light pulse three hours 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. Following 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 at least 6 days preceding light treatment. This line was extended through the period following 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
following 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 ± SEM.

**Immunohistochemistry**

Thin (40 µm), free-floating sections containing central SCN were blocked for one hour with 10% goat serum in PBS containing 1% Triton X-100, 1 mM NaF and 0.02% Na azide (PBST). Following five rounds of washing (5 min/wash in PBST), tissue was double labeled by incubation with an affinity-purified rabbit polyclonal antibody which detects the dually phosphorylated form of ERK (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 5X in PBST, then incubated (4 hrs, 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). Following a final wash cycle (5 X, 5 min/wash) sections were mounted and coverslipped.

**Image analysis**

Immunofluorescence photomicrographs were captured using a 16 bit digital (Micromax YHS 1300: Princeton Instruments) camera mounted on an inverted epifluorescence 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 figure 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 4 animals per time point.

Confocal images were obtained using a Biorad MRC 1024 scanning laser confocal microscope. For each animal, a 10 µm (rostro-caudal) 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 quantitate 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 quantitate 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% (w/v)
powdered milk in PBST for 1 hour. Membranes were probed for phosphatase expression using rabbit anti-MKP-1 and -MKP-2 polyclonal antibodies (1:1000, Santa Cruz) followed by a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP, 1:2000, 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 (1:1000, Santa Cruz). The signal was detected as described above. Membranes were washed 4 times (10 min/wash) in 5% milk/PBST between each antibody treatment.

**Ras activation assay**

Animals were sacrificed at zeitgeber time (ZT) 15.25-17 following exposure to light for either 15 or 120 min (100 lux). Control animals not exposed to light were sacrificed at ZT 16. As described above, 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. Following protein determination, the samples were divided into 50 µg and 15 µg aliquots. Raf-1 RBD agarose beads (Upstate Biochem., Lake Placid, New York) were incubated with the 50 µg samples for 45 min then pelleted and washed 3 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% (w/v) powdered milk in PBST for at least one hour 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 above. Each experiment was repeated a minimum of three times.
**RT-PCR**

Animals were sacrificed 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'-ATCCCGGCATCAGCTGC-3' and 5'-TGGGACAGGTCCCCGCTCC-3'.

RESULTS

Light-induced MAP Kinase 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) three hours after lights off (zeitgeber time 15: ZT 15). Following light exposure, animals were immediately sacrificed 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 (Obrietan et al., 1998; Butcher et al., 2002), light produced a marked increase in the activated form of ERK relative to control animals not exposed to light (Fig. 1). Induction was observed throughout both the rostro-caudal and dorso-ventral 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. 1B: black dashed oval) falls within the retinoreceptive “core” subregion of the SCN. In the first experiment, animals were exposed to constant light (100 lux) for 15, 30, 45, 60, 90, or 120 min then sacrificed. In the second experiment, mice were exposed to a single 15 min (100 lux) light pulse, returned to darkness for 0, 15, 30, 45, 75, and 105 min then sacrificed. As expected, both constant light (CL: Fig. 2A) and a single light pulse (SLP: Fig. 2B) triggered an initial period of ERK activation that was significantly above basal levels (* = p< 0.0001: Fig. 2C). In the SLP animals, activated ERK levels decayed rapidly after cessation of the light pulse, dropping to ~ 50% of the peak value 15 min after the light pulse was terminated (Fig. 2B and 2C). 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, following 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 single light pulse, 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. 2C). Integrating the pERK signal over the 2 hr period revealed that CL elicited a 43% greater level of ERK activation than the SLP (Fig. 4C). 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 constant light.

As noted above, light-induced ERK activation was also observed in the dorsal SCN. Quantitative analysis of the dorsal SCN (Fig. 1B, 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. 2B and 2C). 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. 2D). Interestingly, even after 120 min of constant light, the activated form of Ras was
detected. Given that GTP hydrolysis occurs rapidly following 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. 2E). 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 ERK signal remained suppressed, we examined the central SCN for pERK expression at the 90 min and 120 min (Fig. 2E) post-light exposure time points. At both times, pERK was observed in the central SCN, indicating that rhythmic control of p-ERK expression is transiently repressed after light exposure.

**Subcellular ERK Localization following a light stimulation**

Sustained MAPK pathway activation results in the translocation of activated ERK from the cytosol to the nucleus (Traverse et al., 1992; Lenormand et al., 1993). To address the subcellular distribution of pERK following 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 following light stimulation (Fig. 3A, LP + 0'). At this time pERK levels were approximately 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 towards 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 constant light stimulus paradigm (Fig. 3C). The data collected under conditions of constant light 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 constant light treatment condition, elevated levels of pERK were observed at the 60 min time point (Fig. 3C), whereas in the SLP animals, activated ERK had returned to control (no light) levels by 60 min after light onset (Fig. 3B: LP+45). These data suggest that after 60 min of photic stimulation, light was still stimulating MAPK pathway activation and ERK nuclear translocation.

**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. 4C), we predict that the phase shift elicited by a 120 min light stimulus will be ~ 40%
larger than the shift elicited by a 15 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 min and 120 min light pulse elicited a marked phase shift (Fig. 4A). The 15 min light pulse produced a mean phase delay of 121.9 min (±13.13) and the 2 hr light pulse a delay of 170 min (± 8.13), representing an ~ 40% larger phase shift (Fig. 4B, * = 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.

The 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 single light pulse (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 constant darkness for 45 min between each light pulse. Immediately following the final light exposure animals were sacrificed. Animals in the SLP group were either sacrificed immediately after a single light exposure or sacrificed at 60 or 120 min following photic stimulation

As expected, the SLP group showed a robust increase in ERK activation immediately following photic stimulation (Fig. 5A). Activated ERK levels returned to near baseline at the 60 and 120 min post-light pulse time points (Fig. 5A and 5C). 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. 5B and 5C). 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 zero 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. 5C), indicating desensitization of the MAPK pathway. This attenuation in ERK activation following multiple light pulses paralleled the attenuation in ERK activation observed during a 2 hr constant light treatment, thus raising the possibility that similar mechanisms may be employed to control the gain of ERK activation under constant light and multiple light pulse conditions. However, when data from the groups were compared (Fig. 5D), the multiple light pulse paradigm triggered a significant increase in pERK relative to constant light exposure at the 60 and 120 min constant light 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.

**MKPs**

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 MKP-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 MKP-2 were present in SCN and cortical tissue (Fig. 6A). 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. 6B). These data reveal the
presence of all three phosphatases in the SCN and provide a mechanism by which ERK signaling may be terminated.
DISCUSSION

The role of the MAPK pathway as a regulator of circadian physiology has recently been examined in a number of studies (Obrietan et al., 1998; Sanada et al., 2000; Hayashi et al., 2001; Ko et al., 2001; Williams et al., 2001; Butcher et al., 2002; Sanada et al., 2002). 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 (Obrietan et al., 1998; Butcher et al., 2002). After returning animals to darkness, the level of activated ERK returns to near basal levels within ~ 45 min. Interestingly, in the presence of constant light, 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 following cessation of Ras stimulation, these data suggest that photic information is still being relayed to the SCN after 120 min of constant light. 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 2 hr 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 up to 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 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. With respect to the non-linear 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 following 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 discreet rounds of c-Fos transcriptional and cAMP responsive element binding protein (CREB) phosphorylation elicited by light pulses presented 1-2 hrs 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 to 2 hrs following light stimulation (Sharma and Chandrashekaran, 1997; Best et al., 1999). Furthermore, Daymude & 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). Upon MEK-induced ERK activation, ERK is released, thus allowing it to dimerize and translocate to the nucleus (Fukuda et al., 1997; Khokhlatchev et al., 1998; Adachi et al., 1999). To characterize the subcellular regulation of pERK following light stimulation, animals received either a single light pulse or constant light and pERK expression was examined using confocal microscopy. Immediately following 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 (min) translocation of ERK has been shown to be dependent on an active transport mechanism, whereas slow (hrs) 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 constant light 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, upon 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; Zhao et al., 1995; Pouyssegur et al., 2002). These observations suggest that the nuclear translocations 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 above, 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 following 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, MKP-2 and MKP-3 (Kwak et al., 1994; Misra-Press et al., 1995; Muda et al., 1996; Boschert et al., 1998). MKP-1 and MKP-2 are nuclear specific phosphatases; MKP-3 is
localized to the cytoplasm. Thus, depending upon 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 MKP-2 in the SCN. These observation are in agreement with work showing high levels of Mkp-1 and Mkp-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.

ACKNOWLEDGMENTS
This work was supported by a National Science Foundation Grant (IBN-0090974) and a National Institutes of Health Grant (MH-62335) to K. Obrietan.
REFERENCES


Colwell CS, Menaker M (1992) NMDA as well as non-NMDA receptor antagonists can prevent the phase-shifting effects of light on the circadian system of the golden hamster. J Biol Rhythms 7:125-136.


FIGURE LEGENDS

Figure 1. Light-induced MAPK pathway activation in the 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) three hours into the night (ZT 15). The activation state of the MAPK pathway was detected by fluorescence immunolabeling for the phospho-activated form of 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: third ventricle. Scale bars = 100 µm for low magnification images and 50 µm for high magnification images.

Figure 2. Time course for light-induced ERK activation. Animals were exposed to either constant light (CL: from 15-120 minutes) or a single 15 min light pulse (100 lux, ZT 15) followed by a return to darkness for up to 105 minutes. A) Representative pERK-labeled SCN sections from animals exposed to light for 15, 30, and 120 minutes. Control animals (No Light) were not exposed to light. B) Representative pERK-labeled SCN sections from animals exposed to a single light pulse and sacrificed 15 minutes (LP + 15’), 105 minutes (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 to control animals not exposed to light (0-255 intensity scale). Error bars denote
SEM. Data were collected from 4 animals per condition. Both LP and CL animals had a significant increase in pERK following 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 LP animals (p < 0.05). D) Animals were initially exposed to light (100 lux) for 15 min or 120 min, then immediately sacrificed 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 following both a 15 min 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). Arrows denote cells with high pERK levels.

**Figure 3. Subcellular localization of pERK following light stimulation.** Animals were presented with a single light pulse (15 min, 100 lux) then returned to darkness for 0, 15, 30, 45, and 105 min prior to sacrifice. 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 sacrificed at multiple time points following 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 following 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 approximately 1 hr. 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)
Quantitation of pERK levels from animals exposed to constant light. Animals were placed under constant light (100 lux) for 15, 30, 45, 60, and 120 minutes prior to sacrifice. Both nuclear and non-nuclear regions were found to have a robust increase in pERK expression following 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 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 nine days in DD, 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 two weeks. The black arrowhead indicates a computer power failure (horizontal bar), where locomotor activity was not recorded. B) Data were compiled from eight animals and presented as the mean phase delay in min ± SEM. * = p < 0.004. C) Integrated ERK activation values over a 2 hr period for animals exposed to 15 min or 2 hrs of light. Values were generated from the data sets presented in figure 2C.

**Figure 5. The MAPK pathway resolves multiple light pulses.** Animals were given either a single 15 min light pulse (100 lux, ZT 15) and returned to darkness for 0, 60 or 120 minutes or multiple light pulses separated by 45 minutes of darkness. A) Representative pERK labeled sections from animals sacrificed 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 sacrificed after one, two or three light pulses. A comparison on the pERK levels from mice sacrificed at the 60 min 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 one, two or three 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 to 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 SEM.

Figure 6. MAPK phosphatase (MKP) expression in the SCN. Animals were sacrificed three hours 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 MKP-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.
Figure 1

A  
No Light: Control

Coronal

Horizontal

B  
Light Pulse

Coronal

Horizontal
Figure 2

A  Constant Light

No Light  15'  30'  120'

B  Single Light Pulse

No Light  LP + 0'  LP + 15'  LP 105'

C

Relative Intensity Above Control

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>CL</th>
<th>SLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>150</td>
<td>160</td>
</tr>
<tr>
<td>15</td>
<td>200</td>
<td>220</td>
</tr>
<tr>
<td>30</td>
<td>180</td>
<td>190</td>
</tr>
<tr>
<td>45</td>
<td>160</td>
<td>170</td>
</tr>
<tr>
<td>60</td>
<td>140</td>
<td>150</td>
</tr>
<tr>
<td>90</td>
<td>120</td>
<td>130</td>
</tr>
<tr>
<td>120</td>
<td>100</td>
<td>110</td>
</tr>
</tbody>
</table>

D

Com and 15 min 120 min
Activated Ras  22kDA
Total Ras  22kDA

E

No Light  LP 105
Figure 3

A

B

C

Relative Intensity

Time From Light Onset (min)

Nuclear pERK

Non-Nuclear pERK

Relative Intensity

Time From Light Onset (min)

Nuclear pERK

Non-Nuclear pERK
Figure 5

A  Single Light Pulse

B  Multiple Light Pulses

C  D

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Single Light Pulse</th>
<th>Multiple Light Pulse</th>
<th>Constant Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fold Increase

*  **
Figure 6

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>SCN</th>
<th>CTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>42 kD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38 kD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43 kD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MKP-1

MKP-2

ERK 1/2

B

<table>
<thead>
<tr>
<th>Protein</th>
<th>SCN</th>
<th>CTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 bp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MKP-3

Wellbrock et al., 2002

Danna and Pittendrigh, 1976