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

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Butcher, Greg Q., Boyoung Lee, and Karl Obrietan. Temporal regulation of light-induced extracellular signal-regulated kinase activation in the suprachiasmatic nucleus. J Neurophysiol 90: 3854–3863, 2003. First published August 20, 2003; 10.1152/jn.00524.2003. 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 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.

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/p44 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 dissociate from MEK, dimerize, and translocate to the nucleus (Adachi et al. 1999; Fukuda et al. 1997; Khokhatchev et al. 1998). The nuclear translocation of ERK is a key intermediate event that couples the MAPK pathway to transcriptional activation (Cobb 1999; Greulich 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

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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.

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 lx 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 lx 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 lx 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 formaldihyde/phosphate-buffered saline (PBS, 5% wt/vol) for ≥4 h followed by cryoprotection in 30% sucrose (wt/vol) containing 3 mM NaF for ≥12 h. 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 lx light pulse 3 h 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 dual 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 AlexaFlour-594 conjugated goat anti-rabbit IgG antibody (1:500, Molecular Probes) and an AlexaFlour-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 16-bit digital (Microcam 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 ×10 objective, and a 150 (x axis) × 200 (y axis)-pixel oval or crescent was placed over the digitized regions of interest (see Fig. 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 ×40 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 outlines of 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 microliters of 6× 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 h. 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, 1:2000, 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 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 lx). 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 microliters of 3× 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 h 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 manufacturer’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’-ATCCCCGGGATCATGCTGC-3’ and 5’-TTGGGACAGGTTCGCTC-3’.

**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 lx, 15 min) 3 h 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. 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. 1B: black dashed oval) falls within the retinoreceptive “core” subregion of the SCN. In the first experiment, animals were exposed to constant light (CL) (100 lx) 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. 2A) and a 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. 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 postlight onset until the end of the experiment (Fig. 2C). Integrating the pERK signal over the 2-h 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 lx: 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. 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). 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 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

**FIG. 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 lx, 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 lx) 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 after photic stimulation. Micrographs are enlargements of the boxed regions in (B), →, cells with high pERK levels.
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 pERK signal remained suppressed, we examined the central SCN for pERK expression at the 90 and 120 min time points (Fig. 2E) postlight 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.

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 nonnuclear regions immediately after light stimulation (Fig. 3A, LP +0’). At this time pERK levels were ~30% higher in the nuclear than the nonnuclear 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. 3C). 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. 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 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. 4A). 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. 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.

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 lx) 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. 5A). Activated ERK levels returned to near baseline at the 60 and 120 min time points, indicating that the pathway was “reset” by the intervening dark period and reactivated by light.
120 min postlight pulse time points (Fig. 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 postlight pulse time point (**, $P < 0.0001$, Fig. 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. 5C), indicating desensitization of the MAPK pathway. This attenuation in ERK activation following MLPs paralleled the attenuation in ERK activation observed during a 2 h 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. 5D), 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.

**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 -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. 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 Mk-p-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 (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 2 h 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 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 Chandrashekharan 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 nonnuclear regions of SCN neurons. Interestingly, nuclear levels of activated ERK were significantly higher than the levels in the nonnuclear 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.

DISCLOSURES

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