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

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Abstract

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

Introduction

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

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

The MAPK pathway consists of the kinases RAF, MEK and ERK. In large part this pathway is linear and, thus, the effector actions of the MAPK pathway are mediated by ERK. ERK directly phospho-activates transcription factors such as the SRE binding protein Elk-1. In addition, ERK regulates the activation state of intermediary kinases, which in turn regulate transcription factor activation states. One such downstream target of ERK is the family of 90-kDa ribosomal S6 kinases (RSKs). Four members of the RSK family have been identified (RSK-1, -2, -3 and -4). RSK family members are ERK substrates that have complex and, under some circumstances, redundant physiological roles (reviewed by Frodin & Gammeltoft, 1999). The MAPK pathway tightly regulates RSK activation. For example, RSKs are physically associated with ERK (Scimeca *et al*., 1992; Zhao *et al*., 1996) and are activated specifically by the MAPK pathway (Sturgill *et al*., 1988; Alessi *et al*., 1995). In the activated state, a fraction of the RSK pool translocates to the nucleus (Chen *et al*., 1992; Zhao *et al*., 1995, 1996) where it regulates the activation state of transcription factors and alters chromatin structure via histone phosphorylation

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(Chen *et al.*, 1993; Rivera *et al.*, 1993; Xing *et al.*, 1996; Sassone-Corsi *et al.*, 1999). Indeed, the importance of RSKs as central effectors of the MAPK pathway has been well documented (Joel *et al.*, 1998; Gross *et al.*, 2000; Shimamura *et al.*, 2000). Thus, RSKs can be considered an extension of the MAPK pathway, forming a functional signalling cassette that couples the MAPK pathway to a large number of physiological processes. In this study we examined the capacity of both light and the circadian clock to regulate RSK-1 activation in the SCN.

Methods and materials

Animals

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

Light treatment and tissue processing

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

Immunohistochemistry

Immunofluorescent labelling

Free-floating sections containing the central SCN were washed 5 × (5 min per wash) in PBS containing 1% Triton X-100, 0.03% NaF and 0.02% Na azide (PBST) then blocked for 1 h with 10% goat serum in PBST.

The tissue was incubated (4 °C, 12 h) in one, or a combination, of the following antibodies: monoclonal mouse neuronal nuclear-specific marker antibody (NeuN, 1 : 500 dilution, Chemicon Int., Temecula CA, USA); affinity-purified rabbit polyclonal anti-phospho-p90RSK antibody (1 : 1000 dilution, Cell Signalling, Beverly MA, USA); monoclonal anti-phosphorylated ERK antibody (1 : 1000 dilution, Sigma, St. Louis MO, USA); and/or rabbit polyclonal anti-phospho-ERK antibody (1 : 500 dilution, Cell Signalling). The sections were then incubated (4 h, room temperature) with an Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (1 : 500 dilution, Molecular Probes, Eugene OR, USA) and/or with an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1 : 500 dilution, Molecular Probes). Following a final wash cycle (5 × 5 min/wash in PBST) sections were mounted and coversliped using Gelmount (Biomedia, Foster City CA, USA). Images of labelled sections were captured using a Zeiss 510 Meta confocal microscope. Sections were captured using a Z section thickness of 1 μm.

Immunoperoxidase labelling

Following an initial wash (5 × 5 min in PBST) endogenous peroxidases were quenched with a 15-min treatment in 0.3% H_2O_2. Next, sections were blocked for 1 h with 10% goat serum/PBST then incubated (overnight at 4 °C) with either p90RSK antibody (1 : 2000 dilution; Cell Signalling) or a rabbit polyclonal anti-phospho-ERK antibody (1 : 2000 dilution, Cell Signalling). The next day sections were treated (2 h, room temperature) with a biotinylated anti-rabbit IgG antibody (1 : 300 dilution, Vector Laboratories, Burlingame CA, USA), then placed in an avidin/biotin enzyme complex (Vector Laboratories) for 1 h. The signal was visualized by the addition of DAB-nickel-intensified substrate (Vector Laboratories). Sections were mounted on gelatin-coated slides, dehydrated and coversliped using Permount. Tissue was washed a minimum of 5 × (5 min per wash) in PBST after each antibody treatment.

Western blotting

The SCN was dissected by hand from 500-μm coronal sections, pooled from four animals per condition and sonicated in 50 μL protease inhibitor buffer (0.25 M sucrose, 15 mM HEPES, 60 mM KCl, 10 mM NaCl, 2 mM NaF, 2 mM Na pyrophospate and protease inhibitor cocktail; Complete Mini tablet, Roche Diagnostics). Additional tissue was collected from the piriform cortex and processed in a similar manner. A total of 50 μL of 6 × sodium dodecyl sulphate (SDS) loading buffer was added and samples were heated to 90 °C for 10 min. A 25-μL aliquot of extract from each sample was electrophoresed through a 10% SDS-PAGE gel, transferred to PVDF membranes (Immobilon P; Millipore) and blocked with 10% (w/v) powdered milk in PBST for 1 h. Membranes were probed with p90RSK polyclonal antibody (1 : 2000 dilution, Cell Signalling) followed by a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP, 1 : 2000 dilution; New England Nuclear, Boston MA, USA). The HRP was detected using Renaissance chemiluminescent HRP substrate (New England Nuclear). The membranes were then probed for pERK expression using a mouse monoclonal anti-pERK 1/2 antibody (1 : 2000 dilution, Sigma). Finally, the membranes were stripped and probed for total ERK expression, as described above. Membranes were washed 4 × (10 min per wash) in PBST between each antibody treatment. Additional samples were processed as described above and probed with rabbit polyclonal antibodies against RSK-1, RSK-2, RSK-3 (1 : 1000 dilutions, Santa Cruz Biotech. Santa Cruz CA, USA), PDK-1 (1 : 1000 dilution, Cell Signalling), or ERK (1 : 1000 dilution, Cell Signalling).

Cannulation and infusion

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

level). Animals were killed by decapitation and tissue was processed as described above.

Digital imaging

Bright-field photomicrographs were captured using a 16 bit digital camera (Micromax YHS 1300; Princeton Instruments) mounted on an inverted microscope (Leica DM IRB) and data were quantified with Metamorph software (Universal Imaging). Coronal images containing the central SCN were captured using a 10 × objective. To quantify pERK and pRSK-1 expression, a digital oval (150 pixels, *x*-axis: 200 pixels, *y*-axis) was placed over each SCN and the hypothalamic area just lateral to the SCN and the average signal intensities were measured. The SCN intensity measurement was then normalized to the lateral hypothalamus intensity value, which were set equal to 1. Unless otherwise indicated, data are presented as mean fold SCN signal relative to the lateral hypothalamus. Student's *t*-tests were performed to determine significance. In Fig. 3C and D, the contrast and brightness settings for the pRSK-1 photomicrographs were digitally enhanced with Photoshop to reveal potentially subtle variations in pRSK-1 expression.

Kinase assay

Tissue from control and light-treated mice (15 min, 100 lux, ZT 15) was isolated using the approach described above for Western blotting. Tissue was pooled from 10 animals for each condition and suspended in 250 μL of lysis buffer [1% Nonidet P-40, 0.5% Triton X-100, 10% glycerol, 1 mM Na_3VO_4, 1 mM EDTA, protease inhibitor cocktail (Complete Mini tablet, Roche) in 50 mM Tris-HCl, pH 7.4]. The samples were then sonicated, incubated for 10 min at 4 °C then centrifuged for 10 min at 14 000 ***g***. For each sample, 150 μg of protein was incubated with p90RSK antibody (1 : 100 dilution; Cell Signalling) and protein A-agarose (37.5 μL; Roche Diagnostics) overnight at 4 °C. Next, the samples were washed twice in lysis buffer and twice with kinase buffer (10 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, and 10 mM Tris-HCl, pH 7.4). Samples were then incubated at room temperature in kinase cocktail (50 μL kinase buffer, 10 μg S6 kinase substrate peptide; Upstate Biotech, Lake Placid NY, USA), and 2 μL 3.3 μM [γ^{32}]ATP (2 μCi; Perkin Elmer, Boston MA, USA). Samples were centrifuged and 50 μL of supernatant was blotted onto phosphocellulose P-81 paper discs (Whatman Inc, Clifton, NJ, USA). Discs were washed 10 × with 150 mM phosphoric acid and subjected to scintillation counting.

Results

RSK expression and activation

We, as well as several other groups, have reported that photic stimulation during the night triggers robust activation of the MAPK pathway (Obrietan *et al*., 1998; Coogan & Piggins, 2003; Sigworth & Rea, 2003). To further these studies we examined whether RSK-1, a potential downstream target of the MAPK pathway, is also regulated by light in the SCN. To this end, mice entrained to a 12 h light : dark cycle were exposed to light during either the subjective day or the night and the effects on the phosphorylated form of RSK-1 (pRSK-1) were examined. Figure 1A reveals that photic stimulation (100 lux; 15 min) during either the early (ZT 15) or late (ZT 22) night triggered RSK phosphorylation within the linker region, corresponding to threonine 359 and serine 363 in human RSK-1. These sites are targeted by ERK and the phosphorylation of serine 363 has been shown to be an essential step leading to the stimulation of RSK enzymatic activity (reviewed by Frodin & Gammeltoft, 1999). According to the manufacturer, this antibody detects the phosphorylated form of murine RSK-1 and has limited cross reactivity to RSK-3. For the sake of clarity, the findings described here will refer specifically to RSK-1. High magnification microscopy revealed that pRSK-1 was found primarily in the ventral SCN, with more modest expression observed in the dorsal and lateral SCN regions. RSK-1 phosphorylation was also stimulated in dark-adapted animals during either the early or late

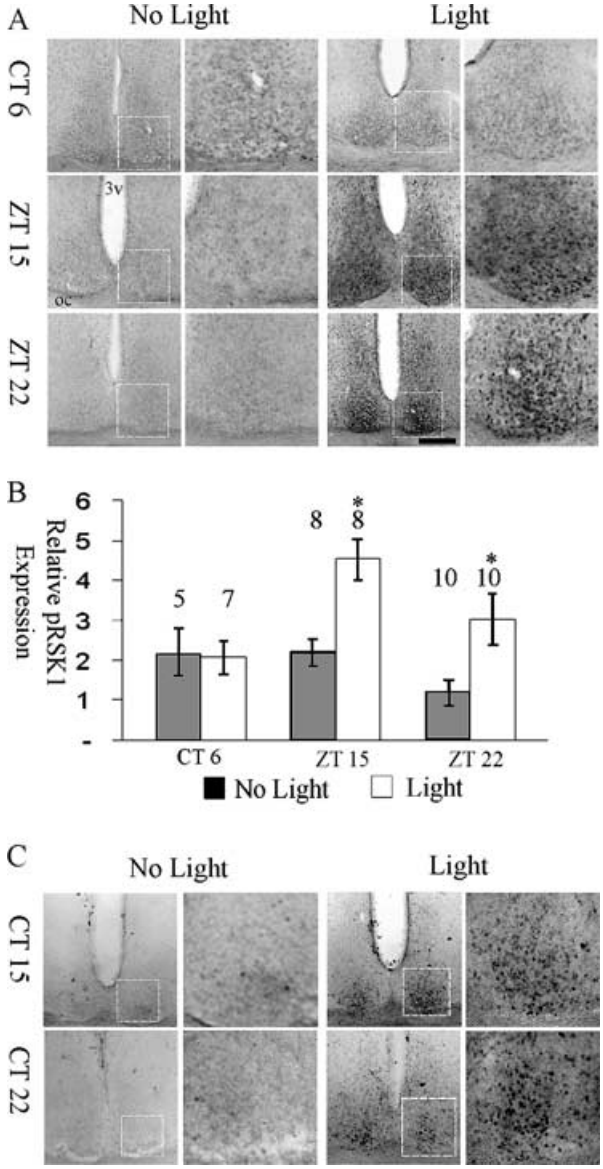

FIG. 1. Light-induced RSK-1 phosphorylation. Mice were exposed to light (15 min, 100 lux) during mid subjective day (CT 6), early night (ZT 15), or late night (ZT 22) then immediately killed and brain sections were processed for RSK-1 phosphorylation (pRSK-1). (A) Relative to control animals (No Light), light exposure triggered a marked increase in pRSK-1 expression at the two night time points. The highest level of antigenicity was observed in the ventral SCN. Photic stimulation during the subjective day did not increase RSK phosphorylation, indicating that the capacity of light to couple to RSK-1 is phase-restricted. (B) Quantification represents the fold pRSK-1 expression in the SCN relative to the pRSK-1 expression in the lateral hypothalamus, which was set equal to 1. The number of animals used for each data point is shown above each bar. (C) Light-induced RSK-1 phosphorylation occurs under circadian timing conditions. Animals were initially dark-adapted for two days then exposed to light during either the early (CT 15) or late subjective night (CT 22). Outlined regions appear at higher magnification to the right; scale bar = 100 μm. Error bars denote the SEM. $^*P < 0.002$. oc, optic chiasm, 3v, 3rd ventricle.

subjective night (Fig. 1C). The capacity of light to trigger RSK-1 phosphorylation was phase-dependent; light exposure during the subjective day (CT 6) did not increase RSK-1 phosphorylation (Fig. 1A and B). Under control conditions (no light), densitometric analysis of the ventral SCN did not reveal significant variations in RSK-1 expression over the three time points examined (Fig. 1A and B). Together these data reveal that photic stimulation triggers a phase-dependent phosphorylation of RSK-1 in the SCN, paralleling the phase-restricted capacity of light to both activate the MAPK pathway and entrain the circadian clock.

To examine the specificity of the pRSK-1 antibody used above, SCN tissue from control and light-treated (100 lux, ZT 15) animals was probed for pRSK expression using Western blotting techniques. Photic stimulation specifically increased the antigenicity of an approximate 90-kDa band (Fig. 2A). This band corresponds to the size of RSK1 and RSK3, thus validating the results obtained using immunohistochemical detection procedures. Following detection of pRSK-1, the membrane was probed for ERK activation. RSK-1 is a downstream target of the MAPK pathway, and thus, if RSK-1 is activated one might be able to detect MAPK pathway activation. Using an antibody directed against the threonine 202 and tyrosine 204 phosphorylated forms of erk 1 and erk 2 (pERK), a marker for MAPK pathway activation, we found that photic stimulation triggered an increase in ERK phosphorylation. As a protein loading control, the blot was stripped and probed for total ERK expression. As noted above, the phospho-RSK antibody used here detects the activated form of RSK-1 and to a lesser extent RSK-3. To examine RSK isoform expression in the SCN, tissue was isolated and probed for presence of RSK-1, -2 and -3. Bands corresponding to the three isoforms were detected in the SCN (Fig. 2B).

Coordinated phosphorylation by ERK and 3-phosphoinositide-dependent kinase-1 (PDK-1) stimulates maximal RSK activation (Jensen *et al.*, 1999). Thus, a robust light-induced increase in RSK-1 enzymatic activity would require PDK-1-mediated phosphorylation. Western analysis confirmed the presence of PDK-1 in the SCN of animals killed during the early subjective night, CT 15 (Fig. 2C). The two bands are likely to correspond to the alpha and beta isoforms of PDK-1 (Dong *et al.*, 1999, 2002). PDK-1 appeared to be expressed constitutively; equivalent amounts of PDK-1 were observed over the L:D cycle (data not shown).

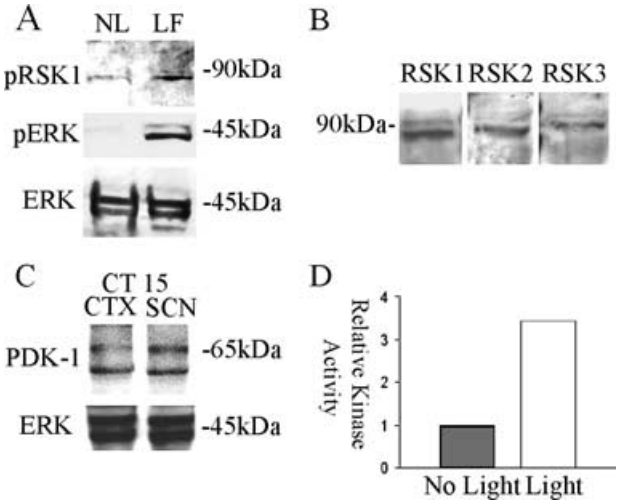

FIG. 2. RSK-1 activation and PDK-1 expression. (A) Western analysis revealed that light (LF, 15 min, 100 lux, ZT 15) increased pRSK-1 expression relative to no light (NL) control conditions. Light also increased ERK phosphorylation (pERK), whereas total ERK levels remained unchanged. (B) SCN tissue harvested from control animals was probed for RSK-1, -2 and -3 expression. All three RSK isoforms were found within the SCN. (C) The RSK kinase PDK-1 is expressed in SCN as well as in piriform cortical (CTX) tissue. (D) pRSK-1 was immunoprecipitated from the SCN of control and light-treated animals and incubated with P32 ATP and a substrate peptide. P^{32} incorporation was determined by scintillation counting. Data are represented as the fold-increase relative to the control (no light) condition, which was set equal to 1. Results reported here are representative of at least three independent assays.

Next, we examined whether photic stimulation triggers an increase in RSK activity. To this end, animals were exposed to light for 15 min, then killed and the SCN was isolated from coronal brain sections. Using the pRSK-1 antibody to immunoprecipitate the kinase and ribosomal S6 peptide as the substrate, we found that photic stimulation triggered an $\sim$ fourfold increase in RSK activity relative to kinase activity from control animals not exposed to light (Fig. 2D). These data reveal that photic stimulation not only triggers phosphorylation, but also stimulates RSK-1 activity in the SCN.

RSK and ERK colocalization

In the inactive state RSKs are largely cytoplasmic kinases. However, after activation they accumulate in the nucleus (Chen *et al.*, 1992; Zhao *et al.*, 1995, 1996). To address the subcellular pRSK-1 expression pattern, tissue was double labelled for pRSK-1 and for the neuronal-specific nuclear marker NeuN, and coronal sections were captured through the central SCN using confocal microscopy. Immunofluorescent labelling detected strong light-induced RSK-1 phosphorylation following a light flash (Fig. 3A). Interestingly, a majority of NeuN expressing cells ($\sim$70%, $n = 150$) within the ventral SCN were also positive for phosphorylated RSK-1.

If RSK-1 is a downstream target of ERK in the SCN, then one may expect to observe colocalized activation of the two kinases following a light flash. To address this issue, SCN tissue from control and light-treated mice (100 lux, ZT 15) was processed using immunofluorescent labelling techniques for the phosphorylated forms of ERK and RSK-1 and data were collected using a confocal microscope. Under control conditions (no light), low levels of activated ERK and pRSK-1 were observed (Fig. 3B). However, after photic stimulation, there was a marked increase in the phosphorylated forms of both kinases (Fig. 3B). Merging the two images revealed strong colocalized expression of activated ERK and RSK-1 in a subset of cells. Double labelling appeared primarily in cell bodies. Together these results reveal that light triggers a coordinated activation of ERK and RSK-1 in the SCN.

In addition to its regulation by light, the MAPK pathway is also regulated by clock timing mechanisms. For example, a subset of cells within the SCN core exhibits high levels of activated ERK during the night (Obrietan *et al.*, 1998; Fig. 3C). Double labelling experiments revealed that the phosphorylated form of RSK-1 is also elevated in this group of cells (Fig. 3, c1). A similar level of pRSK-1 immunoreactivity was not observed in control SCN regions that lacked high levels of activated ERK (Fig. 3, c2). Activated ERK was not observed within this central group of SCN cells during the subjective day (Obrietan *et al.*, 1998). Paralleling this observation, there was an absence of pRSK-1 staining within these cells during the subjective day (data not shown). These results suggest that the circadian rhythm in ERK activity drives the rhythm of RSK-1 activation.

In addition to the SCN core subregion, cells from the SCN shell also exhibit rhythmic pERK expression (Obrietan *et al.*, 1998). Interestingly, in contrast to the rhythmic pRSK-1 expression observed within the core, pRSK-1 expression was not found to colocalize with pERK expression in the shell at CT 1 (Fig. 3D). We did note pRSK-1-immunopositive cells within the shell from time to time, but the lack of a consistent, strong, and colocalized, signal indicates that there is a disassociation of ERK and RSK activity within the shell.

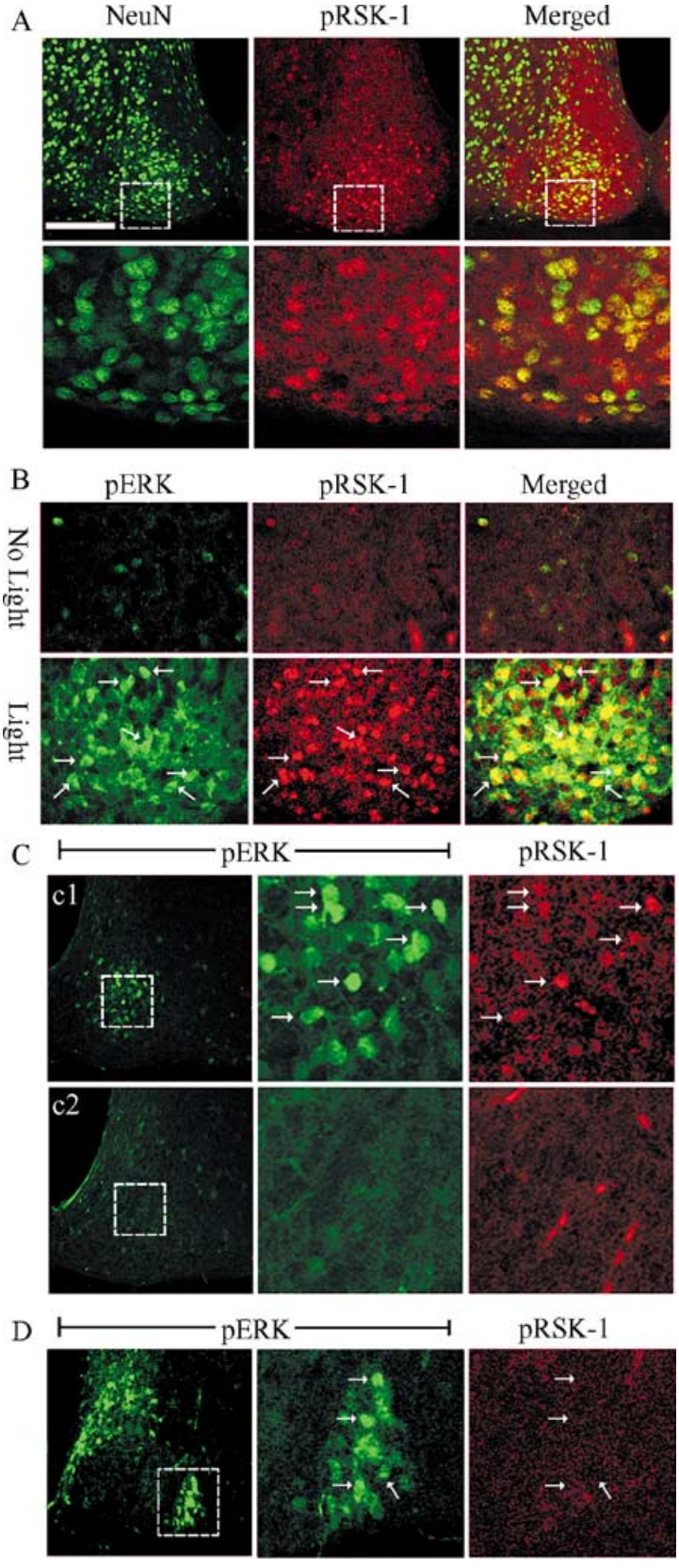

FIG. 3. pRSK-1 in the SCN: nuclear localization and pERK coexpression. (A) Tissue from light-treated animals (15 min, 100 lux, ZT 15) was double labelled for the neuronal nuclear marker NeuN (green) and for pRSK-1 (red). Analysis of the merged signal reveals a high degree of colocalization between NeuN-labelled cells and pRSK-1. The outlined SCN region approximates the location of the magnified region shown below. Scale bar, 50 μm. (B) Tissue sections were double labelled for the activated form of ERK (green) and for pRSK-1 (red) at ZT 15. Confocal analysis shows low levels of pERK and pRSK-1 expression in ventral SCN from control animals not exposed to light. Light (15 min, 100 lux) stimulated an increase in both pERK and pRSK-1 expression. Merging the pERK and pRSK-1 immunofluorescent signals generated a green/yellow-hue, indicative of colocalized expression of the two kinases. Arrows identify individual cells with strong signal colocalization. (C) pRSK-1 is expressed in the subset of SCN neurons that exhibit circadian variations in ERK activation. (c1) Double immunofluorescent labelling identifies a group of central SCN neurons exhibiting high levels of pERK and elevated levels of pRSK-1. Arrows denote cells exhibiting both pERK and pRSK-1 expression. (c2) In contrast, pRSK-1 expression was relatively low in SCN regions lacking strong pERK expression. Outlined regions within the low magnification pERK photomicrographs were used to examine pERK and pRSK-1 coexpression. Animals were killed at ZT 15. (D) pRSK-1 is not coexpressed with pERK in the SCN shell. Strong pERK expression was observed within the shell region of the SCN at CT 1. Magnification of the boxed region (middle panel) reveals a cluster of cells with strong pERK expression (arrows). Coexpression of pRSK-1 was not observed within this region. The pRSK-1 immunofluorescent labelling presented in C and D was digitally enhanced to facilitate visualization of the signal. Please refer to the Methods section for additional information.

Disruption of light-induced MAPK pathway activation

To verify the connection between light-induced MAPK activation and RSK-1 phosphorylation, we employed a ventricular infusion technique to disrupt MAPK signalling in the SCN. To this end mice were infused with the specific MEK 1/2 inhibitor U0126 (10 nM/μL) 30 min before photic stimulation (100 lux, 10 min, ZT 15). Figure 4C and D reveal that infusion of U0126 disrupted light-induced MAPK pathway

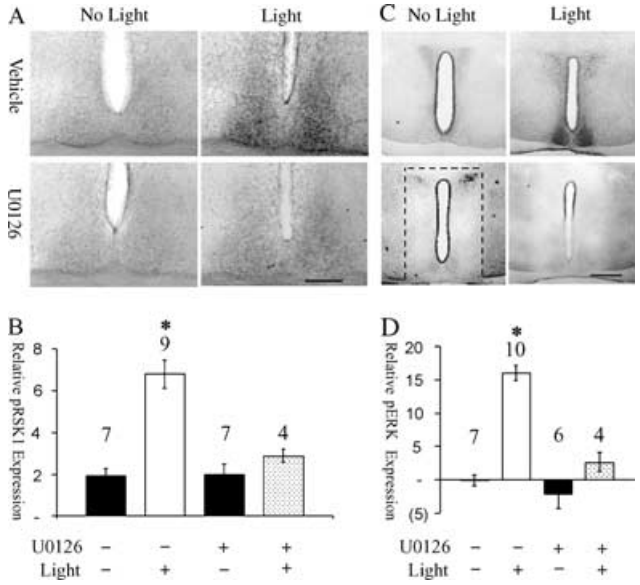

FIG. 4. U0126 infusion blocks light-induced pRSK-1 expression in the SCN. Cannulated animals were infused with either U0126 (10 nM/μL) or vehicle (DMSO) 30 min before light (15 min, 100 lux) exposure at ZT 15. Alternate tissue sections were labelled for pRSK-1 and pERK expression. (A) As expected, light stimulated pRSK-1 expression in the SCN. Pretreatment with U0126 significantly attenuated light-induced RSK-1 phosphorylation. (B) Quantitative data are presented as the fold pRSK-1 expression in the SCN relative to pRSK-1 expression in the lateral hypothalamus. $^{*}P < 0.05$ relative to all other conditions. No significant difference ($P > 0.2$) was observed between any of the other conditions. (C and D) Control experiments confirmed that U0126 infusion uncoupled light from MAPK pathway activation. Boxed region in C surrounding the 3rd ventricle demarcates the approximate region within which U0126 suppressed MEK/ERK activation. Numbers above each bar indicate the number of animals used per condition. $^{*}P < 0.0001$ relative to all other conditions. No significant difference ($P > 0.15$) was observed between any of the other conditions. Error bars denote the SEM. Scale bar, 100 μm (A); 200 μm (C).

activation in the SCN. A region of U0126-mediated ERK inhibition was observed surrounding the 3rd ventricle (dashed outline, Fig. 4C). This outline defines the approximate dorsal and lateral extent to which U0126 diffused from the 3rd ventricle and suppressed MEK activation. Importantly, the disruption of MAPK activation blocked the capacity of light to trigger RSK-1 phosphorylation (Fig. 4A and B; $P < 0.05$). In conclusion, these results reveal the presence of a light-responsive MAPK/RSK signalling cassette in the SCN.

Discussion

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

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

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

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

stimulation to couple to RSK activation. Indeed, using an immunoprecipitation kinase assay we found that light triggered an increase in RSK-1 activity. These data suggest strong coupling between ERK, PDK-1 and RSK-1 in the SCN.

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

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

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

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

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

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

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Abbreviations

CRE, cAMP response element; CREB, CRE binding protein; CT, circadian time; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; PBS(T), phosphate buffered saline (1% Triton X-100); PDK-1, 3-phosphoinositide-dependent kinase-1; pERK, dually phosphorylated ERK; pRSK, phosphorylated RSK; RHT, retinohypothalamic tract; RSK, ribosomal S6 kinase; SCN, suprachiasmatic nucleus; ZT, zeitgeber time.

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