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Mitogen and stress-activated protein kinase 1 (MSK1) modulates photic entrainment of the suprachiasmatic circadian clock

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Abstract

The master circadian clock in mammals, the suprachiasmatic nucleus (SCN), is under the entraining influence of the external light cycle. At a mechanistic level, intracellular signaling via the p42/44 mitogen-activated protein kinase (MAPK) pathway appears to play a central role in light-evoked clock entrainment; however, the precise downstream mechanisms by which this pathway influences clock timing are not known. Within this context, we have previously reported that light stimulates activation of the MAPK effector mitogen stress activated kinase 1 (MSK1) in the SCN. In this study we utilized *MSK1*$^{-/-}$ mice to further investigate the potential role of MSK1 in circadian clock timing and entrainment. Locomotor activity analysis revealed that *MSK1* null mice entrained to a 12h light/dark cycle and exhibited circadian free-running rhythms in constant darkness. Interestingly, the free running period in *MSK1* null mice was significantly longer than WT control animals, and *MSK1* null mice exhibited a significantly greater variance in activity onset. Further, *MSK1* null mice exhibited a significant reduction in the phase delaying response to an early night light pulse (100 lux, 15 min), and, using an 8-hr phase-advancing "jet-lag" experimental paradigm *MSK1* knockout animals exhibited a significantly delayed rate of re-entrainment. At the molecular level, early night light-evoked CREB phosphorylation, histone phosphorylation and *Period1* gene expression were markedly attenuated in *MSK1*$^{-/-}$ animals relative to WT mice. Together, these data provide key new insights into the molecular mechanisms by which MSK1 affects the SCN clock.

Keywords

MAPK; ERK; CREB; SCN; entrainement; light

Introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus serves as the master circadian clock (Reppert and Weaver, 2002, Lowrey and Takahashi, 2011). Timing cues emanating from the SCN impart rhythmicity over a wide range of biochemical and physiological processes in the central nervous system. Further, via the endocrine system and peripheral nervous system, the SCN clock conveys phasing cues to peripheral organ oscillators, thus ensuring their proper, relative phase relationship.

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There has been significant interest in unraveling the intracellular signaling processes that couple light to clock entrainment (reviewed by Golombek and Rosenstein 2010). At the transcriptional level much of this work has been focused on the regulation of the CREB/CRE transcriptional pathway. The rationale for this is based on work showing that the CREB/CRE pathway is regulated by light in the SCN (Ginty et al., 1993; Obrietan et al., 1999), and that the CREB/CRE pathway couples extracellular entrainment cues to clock gene expression (Travnickova-Bendova et al., 2002; Tischkau et al., 2003). Several signaling pathways, including CaMK and PKG have been implicated in mediating photic induction of CREB phosphorylation at Ser 133 (Golombek and Ralph, 1995, Ding et al., 1997), a key event in CREB/CRE transcriptional activity (Sands and Palmer, 2008). In addition, work from our lab and other groups have identified the p42/44 mitogen-activated protein kinase (MAPK) pathway as a major regulator of the CREB/CRE pathway and light-evoked clock entrainment (Dziema et al., 2003; Obrietan et al.,1998; Schurov et al., 2002; Coogan and Piggins 2003; Cheng et al. 2006; Pizzio et al., 2005).

The MAPK pathway is formed by linear three kinases (i.e., RAF, MEK, ERK), where ERK functions as the effector kinase (Raman et al., 2007, Adams and Sweatt, 2002). ERK affects a range of physiological processes through directly targeting end effectors, and by activating second-order kinase signaling pathways. Consistent with this idea, ERK has been shown to affect CREB phosphorylation via mitogen-stress activated kinase 1 (MSK1; Deak et al., 1998;Arthur and Cohen, 2000; Wiggin et al., 2002; Arthur et al., 2004; Vermeulen et al., 2009). MSK1 and its homologue MSK2 are nuclear-enriched kinases that function downstream of both the ERK/MAPK pathway and the p38 MAPK cell-stress pathways (Arthur 2008; Vermeulen et al., 2009). In addition to regulating the transactivation of a number of transcription factors (e.g., CREB, ATF1, STAT3), MSKs have been shown to phosphorylate histone H3 at Ser-10 and Ser-28 and tophosphorylate the chromosomal binding protein HMGN1, which, in aggregate, are thought to facilitate transcriptional activity by reducing chromatin compaction (Arthur 2008; Vermeulen et al., 2009).

Our prior work revealed that light triggers ERK-dependent MSK1 phospho-activation in the SCN, and, using cell culture reporter gene assays, that MSK1 stimulates *Period1* gene expression (Butcher et al., 2005). However, the role of MSK1 in circadian clock function has not been examined *in vivo*. In this study, we utilized a *MSK1* null mouse line to address the role of MSK1 in clock timing and entrainment. Here, we report that MSK1 deficient mice exhibit a decreased capacity to entrain to a new light cycle, a lengthened free running period and significant variance in activity onset. At the molecular level, light-induced CREB phosphorylation, histone phosphorylation and *Period1* expression were significantly attenuated during the early night. These data reveal a role for MSK1 in key functional features of the SCN clock.

Materials and Methods

Animals

Generation of *MSK1* and *MSK2* knockout animals has been described elsewhere (Wiggin et al., 2002). Mouse genotypes were confirmed by PCR-based genotyping of tail biopsy DNA samples. For $MSK1^{-/-}$ genotyping, the following three primers were used: primers CACTTCGCCCAATAGCAGCCAGTCCCTTCC (targeted), TCCGCAGCTCGTGCTTGACAGTAAGGAGC (wild type), and AATAGCGCTGGTGGCTCAGGGCTGT (targeted or wild type), which gave a fragment of 870 bp from the targeted gene (for *MSK1* knockout) and 350 bp from the wild-type allele. The PCR program consisted of a hot start at 94°C, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. Mice were bred into a C57/Bl6 line for > 10

generations. Wild-type (WT) mice used in this study were derived from *MSK1*$^{-/-}$ *and* *MSK2*$^{-/-}$ crosses used to generate a *MSK1*$^{-/-}$,*2*$^{-/-}$ line (which is only briefly described here).

Behavioral rhythm and clock resetting

For wheel-running recording, adult (8 ~ 12 week-old) male *MSK1* null mice and WT animals were individually housed in polycarbonate cages equipped with running wheels. Wheel rotation was detected via the closure of a magnetic switch and recorded as 5 min binned periods using ActiView software (MiniMitter, Bend, Oregon). For the light pulse experiment, animals were entrained to a 12 h/12h Light/Dark (LD) cycle (100 lux) for 14 d and transferred to continuous darkness (DD). Short white light treatment (100 lux, 15 min) was applied at CT15 and CT22. Mice were then returned to their home cages for at least 10 days to record their post-light wheel-running rhythms. For the 'jet-lag' experiment, animals were entrained to a 7 lux LD cycle for 14 days. Then the lightening period was abruptly advanced 8 h. Fourteen days later, the light cycle was shifted back to the original cycle (i.e., 8 h delay of the cycle). For the light-evoked masking paradigm, mice were maintained on a 12/12 LD cycle, and exposed to light (1, 3, 7 and 30 lux) for 120 min starting at ZT 15. Average wheel rotations for the 'masking' periods were compared to wheel rotations under dark conditions (averaged over 3 days prior to masking), and the percent suppression of locomotor activity was calculated for each animal.

Assessment of circadian behavioral phenotypic characteristics

The free-running period (τ) of circadian locomotor activity was determined with the assistance of the ActiView software program. For τ estimation, linear regression analysis using a line drawn through activity onset was used to approximate the free-running period. Regression analysis was performed on 10 to 14 days of data, following stabilization of the free-running rhythm, which typically occurred 4 to 6 days following DD transition. Variance in activity onset was defined as number of days in DD (out of 14 days) and LD (out of 10 days) that the activity onset time differed from the anticipated onset time by ≥ 20 min. In LD, variance was calculated by determining the number of days when the first bout of activity onset occurred either 20 min before or after lights went off at ZT12. In DD, "variance in activity onset" reflects the difference between the activity onset time and the "predicted" activity onset, as time determined via the noted regression analysis. For this analysis, the initiation of the night (and subjective night) activity period was defined as a 15 min period of consecutive wheel-running with an average number of revolutions that were equal to or greater than 1/2 of the maximum revolutions (per 5 min binned period) during the entire recording period.

Light-evoked phase shifting of the clock was analyzed using the linear regression method described by Daan and Pittendrigh (1976). To this end, the least-squares method was used to determine the difference in activity onset before and after the day of light exposure. Regression analysis was performed on at least 6 days of locomotor actogram data preceding light treatment and on 6 days of data and starting 3 days after the light exposure. The phase shift was the difference in the projected versus the actual activity onset after light treatment. Values are presented as mean phase shift ± SEM. Significance was assessed via a one-way ANOVA followed by the Student–Newman–Keuls (SNK) test. A value of $p < 0.05$ was accepted as statistically significant.

Brain tissue processing

Mice were entrained and transferred to total darkness for two consecutive 24 h cycles. Animals then received a single light exposure (7 or 100 lux, 15 min) at CT 15 and were sacrificed via cervical dislocation under dim red light (Kodak series 2 filter <5 lux at cage level; Eastman Kodak, Rochester, NY) at the time points noted in the Results section. Of

note, in control experiments where animals were exposed to red light (10 min at CT15), neither kinase activity, nor clock phasing (as accessed via wheel running activity) was affected. Brains were harvested, placed in ice-cold oxygenation media, and then cut into 1.5 mm coronal slices with a vibratome (OTS 2000; Electron Microscopy Sciences, Fort Washington, PA). Tissue was then fixed in 4 % paraformaldehyde for 6 hr at room temperature and then transferred into cryoprotective 30% sucrose (w/v, with 2 mM sodium azide and 3 mM NaF) overnight at 4 °C. All procedures involving live animals were in accordance with Ohio State University animal welfare guidelines and approved by the Institutional Animal Care and Use Committee.

Immunohistochemistry

Coronal brain sections containing the SCN were thin cut (40 μm) using a freezing microtome and placed in PBS containing 2 mM sodium azide and 3 mM NaF, pH 7.4. For the immunohistochemical staining, sections were first treated with 0.3 % H_2O_2 and 20 % methanol in PBS for 10 min to deactivate endogenous peroxidase-like activity and permeabilize the tissue and then blocked for 1 h in 10% goat serum/PBS and incubated in one of the following antibodies: rabbit anti-phospho-MSK1 (Ser-360, 1:1000; Cell Signaling Technology, Danvers, MA), rabbit anti-MSK1 (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal phospho-CREB (Ser-133) (1:1000; Cell Signaling Technology, Catalog number: 9191), rabbit anti-phospho-ERK (pERK, Thr-202, Tyr-204) (1:2000; Cell Signaling Technology, Catalog number: 9101), rabbit polyclonal anti-c-Fos (1:3000; Calbiochem, Catalog number: PC38) or rabbit polyclonal anti-phospho-Histone H3 (Ser10) antibody (1:1000; Millipore, Catalog number: 06-570) overnight at 4°C. Next, tissue was incubated for 1.5 h in biotinylated anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) at room temperature and then placed in an avidin/biotin/HRP complex for 1 h (Vector Laboratories). Sections were washed in PBS (three times, 10 min per wash) between each labeling step. The signal was visualized using nickel-intensified DAB substrate (Vector Laboratories) and sections were mounted on slides with Permount media (Fisher Scientific, Houston, TX).

For immunofluorescent labeling, tissue was permeabilized with PBST (PBS with 1 % Triton X-100) for 30 min, blocked as described above and then incubated (overnight, 4° C) in 5% goat serum/PBS with chicken polyclonal anti-GFP antibody (to detect the Venus transgene, 1:2000; Abcam, Cambridge, MA) or with a rabbit polyclonal anti-vasopressin antibody (1:1000 Chemicon, Temecula, CA). The following day, sections were incubated (3 h, room temperature) with an Alexa Fluor-488-conjugated goat antibody directed against the IgG domain of the primary antibody (1:500; Molecular Probes, Eugene, OR). Sections labeled for vasopressin were also incubated (5 min) with the DNA stain DRAQ5 (1:10,000; BioStatus Limited, UK). Sections were mounted on slides with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

Photomicrographic bright-field images were acquired using a 16 bit digital camera (Micromax YHS 1300; Princeton Instruments, Trenton, NJ) driven by Metamoph software (Molecular Devices, Sunnyvale, CA). The camera was mounted on an inverted Leica microscope (DM IRB; Nussloch, Germany). A Zeiss 510 confocal microscope (Oberkochen, Germany) was used to capture fluorescent images. All confocal settings (e.g., pinhole, contrast, brightness) were held constant for each experimental data set.

Materials

Unless otherwise indicated, reagents were obtained from Sigma.

Densitometry data analysis

Photomicrographic data sets were statistically analyzed using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA). For the pERK, pCREB and Venus intensity analysis, SCN were digitally outlined and the mean pixel values were determined (0-255 unit scale). Next, a digital oval (150×200 pixels) was placed on the adjacent lateral hypothalamus and this mean value determined, and subtracted from the SCN signal value to provide a normalized, background, corrected SCN intensity value. Of note antigenicity within the lateral hypothalamus was not affected by the experimental condition. For the c-Fos and phospho-histone H3-positive cell counting, an intensity threshold filter was initially applied to eliminate nonspecific background labeling, and then the number of signals detected above the filter threshold (defined as positive cells) were counted for each SCN.

Statistical analysis

For all histological data analyses, three central SCN sections were used from each animal to generate a mean value. These mean values were averaged for the four treatment groups (WT No light; WT Light; *MSK1-/-* No Light and *MSK1-/-* Light). Values are presented as a fold-change relative to the 'WT No light' condition, which is normalized to a value of 1. For all behavioral data analysis, animals were divided into two groups: WT and *MSK1-/-*. All data are expressed as the mean ± standard error of mean (SEM). Numbers of animals used for each group are described in the figures or figure legends. The mean values of different groups were compared via one-way ANOVA followed by the Student–Newman–Keuls (SNK) post-tests. $P < 0.05$ was accepted as statistically significant. All statistical analysis was performed using SPSS software (Version 13.0, SPSS Inc, Chicago, IL).

Results

MSK1 knockout animals

For our analysis of MSK signaling in the SCN, we utilized a genetically-modified mouse strain in which the *MSK1* gene was deleted (Fig. 1A, Wiggin et al., 2005). To confirm that the targeted gene disruption led to a loss of protein expression, coronal brain sections from WT and $MSK1^{-/-}$ mice were immunohistochemically-labeled for MSK1 expression. As shown in Figure 1B, expression of MSK1 was found throughout the SCN of WT mice, whereas MSK1 protein was not detected in $MSK1^{-/-}$ mice. As a further confirmation of *MSK1* disruption, WT and *MSK1* null mice were exposed to a brief light pulse (15 min: 100 lux) at zeitgeber time 15 (ZT 15: 3 hours into the dark-period), and animals were immediately sacrificed and SCN tissue was processed for MSK1 phosphorylation at Ser-360, a marker of kinase activity (McCoy et al., 2005). Consistent with our prior work (Butcher et al., 2005), light triggered an increase in phospho-MSK1 expression in WT mice; conversely Ser-360 phosphorylation could not be detected in MSK1 knockout animals (Fig. 1E), thus further supporting a complete loss of MSK1 signaling. Histological analysis using cresyl violet staining revealed that the SCN was grossly normal in the MSK null line (Fig. 1C). Likewise, the overall expression pattern of the SCN peptidergic neuronal marker arginine vasopressin (Abrahamson and Moore, 2001) was indistinguishable between *MSK1* null mice and WT littermates (Fig 1D). Further, *MSK1* null mice were fertile, and, we did not detect major health issues in young adult mice (6-12 week ages), which were used in this study. Consistent with these observations, the total daily amount of locomotor activity (accessed via wheel running activity under a standard 12 hr light/dark cycle) of MSK1 null mice was indistinguishable from WT littermates (Table 1, Parameter 4).

Finally, to control for possible effects of MSK deletion on SCN photic responsiveness, we examined the MAPK cascade, a key signaling pathway which couples light to SCN clock resetting (Obrietan et al., 1998; Butcher et al., 2002). To this end, the activation state of

ERK was profiled via immunolabeling for the Ser-202 and Thr-204 phosphorylated form of ERK1/2 (a marker of MAPK pathway activation: Obrietan et al., 1998; Butcher et al., 2005). For this experiment, mice (MSK1 null and WTs) were dark-adapted for 2 days, and then exposed to light pulses (15 min) at CT 15, and then immediate sacrificed. Quantitative densitometric analysis revealed that low (7 lux) and high (100 lux) level illumination led to marked ERK activation in *MSK1* nulls, which was not significantly different from the level of ERK activity detected in WT mice (Fig. 2C). Of note, the masking effects of a two hour light pulse (1-30 lux) at ZT 15 were not significantly different between WT and MKS1 null mice (Fig. 2C). In total, these data reveal that the genetic deletion of *MSK1* does not profoundly alter the light-responsiveness of the SCN or other non-image forming photic input pathways.

Circadian phenotype of MSK1 knockout animals

For our assessment of the circadian phenotype, *MSK1*$^{-/-}$ mice and WT littermates were entrained to a 12h LD cycle for 10 d and then transferred to total continuous darkness (DD) and clock phasing was monitored via wheel running activity. Of note, both lines entrained to the 12 LD cycle, and, as noted above, there was no difference in overall level of wheel-running activity in LD (Table1, Parameter 4) between *MSK1*$^{-/-}$ and their WT littermates. The animals were then switched to constant dark conditions so that the inherent periodicity (i.e., τ) of the SCN pacemaker would be revealed. Under this free-running paradigm, *MSK1*$^{-/-}$ mice exhibited a modest, yet significant lengthening of τ relative to WT mice (Table 1, Parameter 1). Interestingly, *MSK1*$^{-/-}$ mice also exhibited a significant relative decrease in wheel-running activity in DD (Table 1, Parameter 5).

We then examined the entraining effects of short light pulses (100 lux, 15 min) administered during the early (CT 15) and late subjective night (CT 22). This Aschoff type I paradigm allowed us to probe the resetting effects of an acute phase delaying (early night) and phase advancing (late night) light stimulus (Aschoff, 1965). Compared to WT mice, the early night light-induced phase delay was significantly decreased (> 2-fold) in *MSK1*$^{-/-}$ mice (Figure 2A and Table 1, Parameter 10). Conversely, the late night (CT 22) light-induced phase advance was not significantly changed in *MSK1*$^{-/-}$ mice (Table 1, Parameter 11). Mock light exposure (i.e., handling controls) at CT15 and CT22 did not significantly affect clock phasing in WT and *MSK1*$^{-/-}$ mice (data not shown). Together, these data reveal an important role for MSK1 in coupling light to clock resetting during the early night.

To further this line of inquiry, we tested the rate at which mice re-entrain to an 8 hr advance and an 8 hr delay of the LD cycle. In this experiment, lighting conditions were set to 7 lux. The purpose in using the low intensity illumination was to identify a potentially subtle phenotype. In the 8-hr phase advance 'jet-lag' paradigm, *MSK1*$^{-/-}$ mice exhibited a marked reduction in the rate of re-entrainment relative to WT mice. Hence, WT mice took ~ 4 days to fully entrain to the new LD cycle, whereas *MSK1*$^{-/-}$ were not fully entrained even after 8 days (Table 1, Parameter 8). As shown in Figure 2B, *MSK1*$^{-/-}$ mice exhibited a slow, incremental (~ 1 hr/per day), resetting of the clock, whereas WT mice exhibited a relatively rapid (1-2 days) shift in the initial bout of activity, which was consistent with the beginning of the new dark phase, and by 4 days, the mice had a consolidated/extended bout of activity during the early night. After 14 days in the new cycle, the LD cycle was shifted back to the original L/D cycle (equivalent to an 8 h delay in the light cycle). Under this condition, *MSK1*$^{-/-}$ mice did not exhibit a significantly slower rate of entrainment, than WT littermates (Figure 2B and Table 1 Parameter 9). Of note, the interpretation of the data from the jet lag/ phase delay paradigm may be complicated by the masking effects of light. In total, data from the light pulse and jet lag experiments indicate that, *MSK1*$^{-/-}$ mice have compromised light entrainment capacity.

Notably, both in LD and DD, *MSK1*$^{-/-}$ mice showed larger variance in activity onset than WT mice. (Table 1, Parameter 2 and 3). Hence, *MSK1*$^{-/-}$ mice exhibited significantly more bouts of sustained activity that occurred either ≥ 20 min before or after the predicted time of locomotor activity onset. An increase in activity onset variance suggests that the stability and accuracy of the SCN oscillator is compromised in *MSK1*$^{-/-}$ mice.

Light-induced CREB phosphorylation is decreased in MSK1$^{-/-}$ mice

To gain insight into the molecular mechanisms by which MSK1 could be affecting clock physiology, we first examined light-induced CREB phosphorylation at Ser133 (pCREB). CREB phosphorylation is a critical event in the induction of CRE-mediated gene transcription (Sakamoto et al., 2011). Notably, CRE-mediated gene expression is of particular interest in the timing field, where a number of studies have shown that light stimulates robust CREB phosphorylation and CRE-mediated transcription (Ginty et al., 1993; Obrietan et al., 1999; Ding et al., 1997). Further, multiple light-responsive genes, including the core clock genes *period1* and *period2* contain CRE-like elements in their regulatory regions (Travnickova-Bendova et al., 2002), and the abrogation of CRE-mediated transcription decreases light-responsiveness of the clock (Travnickova-Bendova et al., 2002, Tischkau et al., 2003). Given this, and previous work showing that MSK1 is a potent CREB kinase (Arthur et al., 2004, Arthur and Cohen, 2000), we were interested in examining light-inducible CREB phosphorylation in *MSK1* null mice.

To pursue this line of inquiry we focused our analysis on the early night (CT 15) time point, and employed a similar light pulse paradigm to the one used to reveal a phase-delay phenotype in MSK1 null mice. Given that MSK1 null mice did not exhibit a significant late night phenotype using the Aschoff type 1 paradigm, a parallel analysis of signaling during the late night was not performed. For these studies, animals were dark-adapted for two days, and then, at CT 15, exposed to a short light pulse (100 lux, 15 min), returned to darkness for 15 min, and then sacrificed, and tissue was labeled with an antibody against the Ser-133 phosphorylated form of CREB (Oster et al., 2003; Cao et al., 2008). In WT mice, we detected a significant light-evoked increase in pCREB expression in the SCN, relative to control mice (no light: Figure 3). In contrast, relative to the induction observed in WT mice, light-evoked a significantly lower level of pCREB in *MSK1*$^{-/-}$ mice (F=8.420, P=0.001). Together, these data indicate that MSK1 plays a key role in coupling photic activation of the MAPK pathway to the phosphorylation of CREB during the early night.

MSK1 regulates Histone H3 phosphorylation

To further pursue the molecular mechanisms by which MSK1 regulates clock entrainment, we examined the phosphorylation state of Ser-10 on histone H3. Interest in this event is based on work showing that that phosphorylation of this site is associated with transcriptional activation of proximal genes (Gutièrrez-Mecinas et al., 2011; Brami-Cherrier et al., 2007; Crosio et al., 2003). Further, Crosio et al (2000) reported that photic stimulation leads to Ser-10 phosphorylation of histone H3 in the SCN. Based on these findings, we profiled light-induced histone H3 phosphorylation in *MSK1* null animals. For this study, dark-adapted mice were exposed to light (100 lux, 15 min) at CT15, and then immediately sacrificed and tissue was immunolabeled with an antibody an antibody against the Ser-10 phosphorylated for of histone 3 (Liu et al., 2011; Viktorin et al., 2011). In WT SCN, light evoked a significant increase in histone phosphorylation (Figure 4). A light-induced increase in histone H3 phosphorylation was also detected in the *MSK1*$^{-/-}$ animals, however, the level of induction in the *MSK1* null mice was significantly lower than that observed in the WT animals (F=18.120, P=0.001). Together, these results indicate that MSK1 plays a central role in coupling light to histone H3 phosphorylation in the SCN during the early night.

Light-induced immediate early gene (IEG) expression is decreased in MSK1$^{-/-}$ mice

To gain insight into the role of MSK1 in gene expression, we profiled the inducible expression of the canonical light-evoked IEG c-FOS in the SCN. For these experiments, animals were dark-adapted for 2 days, and then exposed to a short light pulse (100 lux, 15 min) at CT 15, and animals were sacrificed 45 min later and tissue was labeled with a c-Fos antibody (Hale et al., 2011; Stratford et al., 2011). Compared to wild-type littermates, light-induced c-Fos expression in *MSK1*$^{-/-}$ mice was significantly decreased (~30%), although significant residual c-Fos expression was detected in the SCN (Fig. 5) (F=6.222, P=0.028). These data indicate that MSK1 contributes to light-evoked rapid response gene expression in the SCN.

Light-induced Period1 expression is decreased in MSK1$^{-/-}$mice

Light-induced *Period1* gene expression is considered to be one of the key mechanisms by which photic input resets the SCN clock. Given that light-evoked *Period1* expression is thought to be mediated via a CREB/CRE-dependent process, and that MSK1 regulates CREB phosphorylation, we logically extended our analysis to the inducible expression of *Period1* in the MSK1 null mouse line. To this end, we crossed *MSK1*$^{-/-}$ mice with our *Period1*-Venus transgenic reporter mouse strain. In this transgenic mouse strain, a nuclear-targeted, destabilized, version of the yellow fluorescent protein derivative Venus was inserted into the coding region of the *Period1* gene via BAC recombination (Cheng et al., 2009). Prior work has shown that the neuroanatomical expression and circadian regulation of the *Period1*-regulated Venus transgene parallels the expression pattern of the endogenous gene (Cheng et al., 2009, Cao et al., 2011). For these experiments, mice (*Period1*-Venus and *Period1*-Venus::*MSK1* null) were dark-adapted for 2 days, and then exposed to a short light pulse (100 lux, 15 min) at CT 15. Mice were then returned to darkness and sacrificed 4 h later. Relative to control *Period1*-Venus animals not exposed to light, photic stimulation triggered an ~ 2-fold increase in Venus expression in the SCN (Fig 6). Both the induction level and neuroanatomical expression pattern of light-evoked Venus is consistent with our prior reports (Cheng et al., 2009, Cao et al., 2011). In comparison, the inducible expression of Venus in MSK1 null mice was markedly attenuated. Along these lines, in *MSK1* null mice, light evoked a relatively modest, ~ 30% increase in Venus expression relative to *MSK1* null control mice (no light) (F=34.012, P=0.001). Of note, the general neuroanatomical expression pattern of the light-evoked Venus signal in MKS1 null mice was similar to the WT pattern, but it appeared that the cellular induction level was blunted. Together, these data indicate that the MAPK/MSK signaling cassette couples light to the expression of the core clock gene *Period1* during the early night.

Discussion

A central finding of the results presented here indicates that MSK1 plays a key role in clock entrainment. This conclusion is based on data showing that the genetic deletion of *MSK1*, A) markedly attenuates the phase shifting effect of light during the early night, B) slows the rate of re-entrainment to an advance of the LD cycle, and C) during the early night, uncouples light from transcriptional control mechanisms (i.e., CREB phosphorylation, and H3 phosphorylation and inducible *Period1* gene expression. These findings, placed within the context of studies showing that its upstream effector, the ERK/MAPK pathway, couples light to clock entrainment, strongly support a role for MSK1 in light-evoked clock resetting.

MSK1 and clock entrainment

The data reported here builds on our prior work examining light-evoked MSK1 activation in the SCN (Butcher et al., 2005). In the noted study, we found that MSK1 phospho-activation is tightly regulated by light in a phase restricted manner. Hence, exposure to light during the

night time domain led to a marked increase in MSK phospho-activation, whereas exposure to light during the subjective daytime did not affect MSK activity. These data parallel the activation parameters of the ERK/MAPK pathway and, indeed abrogation of MAPK signaling led to a complete block of MSK1 activity. Here we showed that the genetic deletion of *MSK1* potently suppressed early night light pulse-evoked entrainment, whereas the phase-advancing effect of late night light was not affected. On their own, these data suggest that MSK1 plays a central role in light-evoked phase-delaying, but not phase-advancing of the SCN clock. However, *MSK1* null mice exhibited a marked deficit in the rate of re-entrainment to an 8 hr advance of the L/D cycle, thus suggesting that clock coupling to light at both the early and late night time domains is compromised. Consistent with this idea, our prior work showed that MSK exhibits robust phosphorylation during both the early and late night (Butcher et al., 2005).

MSK1 and CREB

A molecular analysis of *MSK1* null mice logically led us to examine CREB phosphorylation. As noted above, CREB appears to play a central role in photic entrainment of the SCN clock. Along these lines, abrogation of CREB/CRE mediated transcription has been shown to repress clock gene (i.e., *Period1*) expression, and phase shifting of the clock (Travnickova-Bendova et al., 2002; Tischkau et al., 2003). Here we provide data implicating MSK1 in light-evoked CREB phosphorylation at Ser-133. Along these lines, relative to WT littermates, *MSK1* null mice exhibited a marked deficit in light-evoked pCREB expression. Although disruption of *MSK1* expression decreased light-evoked CREB phosphorylation, residual, statistically significant light-evoked pCREB expression was detected. These data clearly indicate that MSK1 is not the only light-evoked CREB kinase. With respect to MAPK signaling, residual CREB phosphorylation could be mediated by RSK, which is also light responsive, and has been shown to stimulate CREB phosphorylation, although its level of CREB kinase activity is much lower than MSK1 (Anjum, and Blenis, 2008; Arthur et al., 2004; Xing et al. 1996; Frödin and Gammeltoft, 1999). In addition, MSK2 could also couple the MAPK pathway to CREB in the SCN. However, our preliminary, unpublished, data on *MSK1*$^{-/-}$/*MSK2*$^{-/-}$ null mice revealed that the double knockout clock phenotype was not distinct from the *MSK1*$^{-/-}$ phenotype. Hence, this would suggest that MSK2 does not play a significant role in the SCN clock. Lastly, a number of signaling pathways in addition to the MAPK pathway have also been shown to contribute to inducible pCREB expression in the SCN (Golombek and Ralph, et al., 1995; Ding et al.,1997; Meijer and Schwartz, 2003; Schurov et al., 2002; Schurov et al., 1999).

MSK1 and histone phosphorylation

Inducible histone phosphorylation in the SCN was initially characterized by Crosio et al (2000). In that study, light exposure during the night triggered the rapid phosphorylation of histone H3 at Ser-10 in neurons, which occurred in parallel with *Period1* induction. Given these observations, and a number of studies showing that in post-mitotic cells, MSKs phosphorylate histone H3 at Ser-10 (Drobic et al., 2010; Thomson et al., 1999; Soloaga et al., 2003), we examined whether MSK1 null mice exhibit a deficit in light-evoked histone H3 phosphorylation. The data presented here reveal a role for MSK1 in this process: hence, light-evoked H3 Ser-10 phosphorylation was significantly repressed in MSK1 null mice. In something of a parallel to our findings, Brami-Cherrier et al (2005) reported cocaine-induced Ser-10 phosphorylation in the striatum was disrupted in *MSK1* knock-out mice. The phosphorylation of histone H3 at Ser-10 was suggested to destabilize nucleosomes and in turn facilitate transcriptional induction from proximal promoters (Brami-Cherrier et al., 2005). These data raise the interesting prospect that MSK1 regulates light-evoked gene expression in the SCN via the coordinate regulation of chromatin structure, and CREB/CRE-mediated gene expression.

MSK1 and transcriptional regulation

Here it is worth noting that coordinate MAPK-dependent regulation of transcription factors and epigenetic modulation (e.g., histone phosphorylation and acetylation) at sequence-specific regulatory regions has been well established in a number of model systems (Clayton and Mahadevan, 2003; Vicent et al., 2006). Along these lines, MSKs appear to form a complex with ERK and, upon nuclear translocation of activated ERK, the complex is recruited to the promoter regions of actively transcribed genes, where MSK stimulate H3 Ser-10 phosphorylation and CREB phosphorylation. Interestingly, one possible mechanism by which ERK/MSK1 directs acetylation is through CREB phosphorylation at Ser-133, which leads to the recruitment of p300/CBP, which functions as a transcriptional co-activator and histone acetyltransferases (Korzus et al., 1998; Ait-Si-Ali et al., 2000; Yuan and Gambee, 2001). Hence, MSK1 appears to actuate a multi-step phosphoacetylation process which contributes to rapid and robust transcriptional response (Clayton and Mahadevan, 2003). In line with these observations, our lab is currently working on the functional effects of MSK1 at distinct genetic loci in the SCN.

Interestingly, *MSK1* deletion had relatively modest effects on light-evoked c-Fos expression. This may, in part, result from the complex, multifaceted transcriptional regulation of *c-Fos* (Lucibello et al., 1991). Along these lines, the *c-Fos* promoter region contains a diversity of response elements, including and SRE, AP1, SIE and CRE. Hence, if we assume that *c-Fos* transcription is mediated by the combinatorial actions of multiple transcription factor families, then disruption of MSK1, which would likely function principally through the CREB/CRE, may only have minor effects. Here it is worth noting that the ERK/MAPK regulated transcription factor Elk-1 is activated by light in the SCN (Piggins et al., 2002; Coogan and Piggins, 2003). In turn, Elk-1 transactivation would likely play a substantial role in SRE induction of *c-Fos* expression (Xia et al., 1996).

MSK1 and Period1 gene expression

Ultimately, for MSK1 to effect clock entrainment, it would need to modulate the expression of a state-variable of the clock. The most parsimonious route by which MSK1 would affect the clock is via the regulation of light-evoked *Period1* gene expression. As noted, a number of studies have shown that light-induced Period1 expression plays a major role in clock resetting (Albrecht et al.,1997; Shigeyoshi et al.,1997; Akiyama et al., 1999; Albrecht et al., 2001; Wakamatsu et al., 2001), and the *Period1* gene has several CRE sequences in its promoter region (Travnickova-Bendova et al., 2002). These observations combined with our prior work in cell culture showing that MSK1 stimulates *Period1* reporter gene expression, led us to pursue this question *in vivo*. Here, we found a relative reduction in light-evoked *Period1*-Venus expression in *MSK1* null mice. These data suggest that MSK1 plays a central role in inducible *Period1* gene expression, potentially via the regulation of CREB.

MSK1 in SCN clock timing

Recent work has revealed a role for both the ERK/MAPK pathway and CREB in endogenous SCN clock timing (Lee et al., 2010; Akashi et al., 2008). Given that MSK1 is a kinase intermediate that couples ERK to CREB, we hypothesized that *MSK1* null mice might exhibit a profound deficit in inherent clock timing. Here we found a modest, yet significant effect on clock period length and marked variability in the circadian gating of activity onset. Further work using *ex vivo* bioluminescence recording will likely be required to gain a deeper understanding of how MSK signaling affects core clock timing, clock cell communication, and/or clock output pathways.

Conclusion

In conclusion, the data presented here provide new insights into the signaling events through which photic input engages the core clock timing mechanism. To the authors, the picture that is emerging places the ERK/MAPK pathway as a nodal point for the intracellular propagation of photic entrainment information. As such, the MSK-pCREB signaling cassette appears to be one of several downstream effectors through which the MAPK pathway engages the SCN oscillator (Fig.7).

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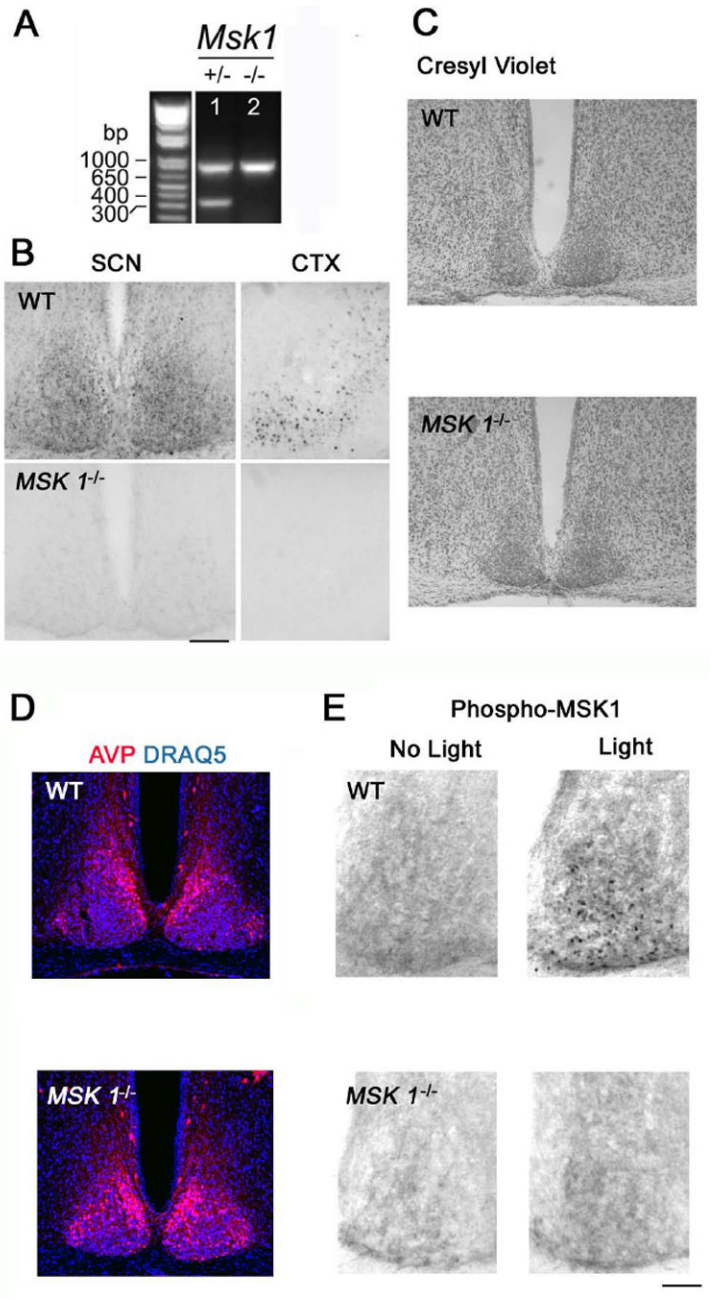

Figure 1. *Msk1* knockout animals

A) Genotyping of *MSK1* knockout animals. Agarose gel electrophoresis results of PCR genotyping of *MSK1* heterozygotic (+/-) and homozygotic (-/-) mice. An 870 bp amplicon indicates *MSK1* gene deletion and a 350 bp amplicon indicates a wild-type allele. Each lane denotes the genotype of a single mouse. B) MSK1 expression was completely eliminated in *MSK1*$^{-/-}$ animals. Representative photomicrograph images of immunohistochemical staining for MSK1 in the suprachiasmatic nuclei (SCN) and piriform cortex (CTX) of wild-type (WT, top) and *MSK1*$^{-/-}$ animals (bottom). C) Cresyl violet histology of SCN tissue from WT and *MSK1*$^{-/-}$ animals. Of note, in *MSK1*$^{-/-}$ animals, the gross neuroanatomical features of the SCN appeared to be normal. D) Representative fluorescent confocal images of SCN tissue immunolabeled for arginine vasopressin (AVP: red) and stained with the nuclear

marker DRAQ5 (blue). E) Light-induced phospho-MSK1 (Ser-360) expression was abolished in *MSK1*$^{-/-}$ animals. Animals were dark-adapted for 48 hrs and then given a light flash (100 lux, 15 min) at circadian time 15 (CT 15). Note the absence of immunoreactivity in the light-treated *MSK1*$^{-/-}$ animal. Scale bar: 100 microns.

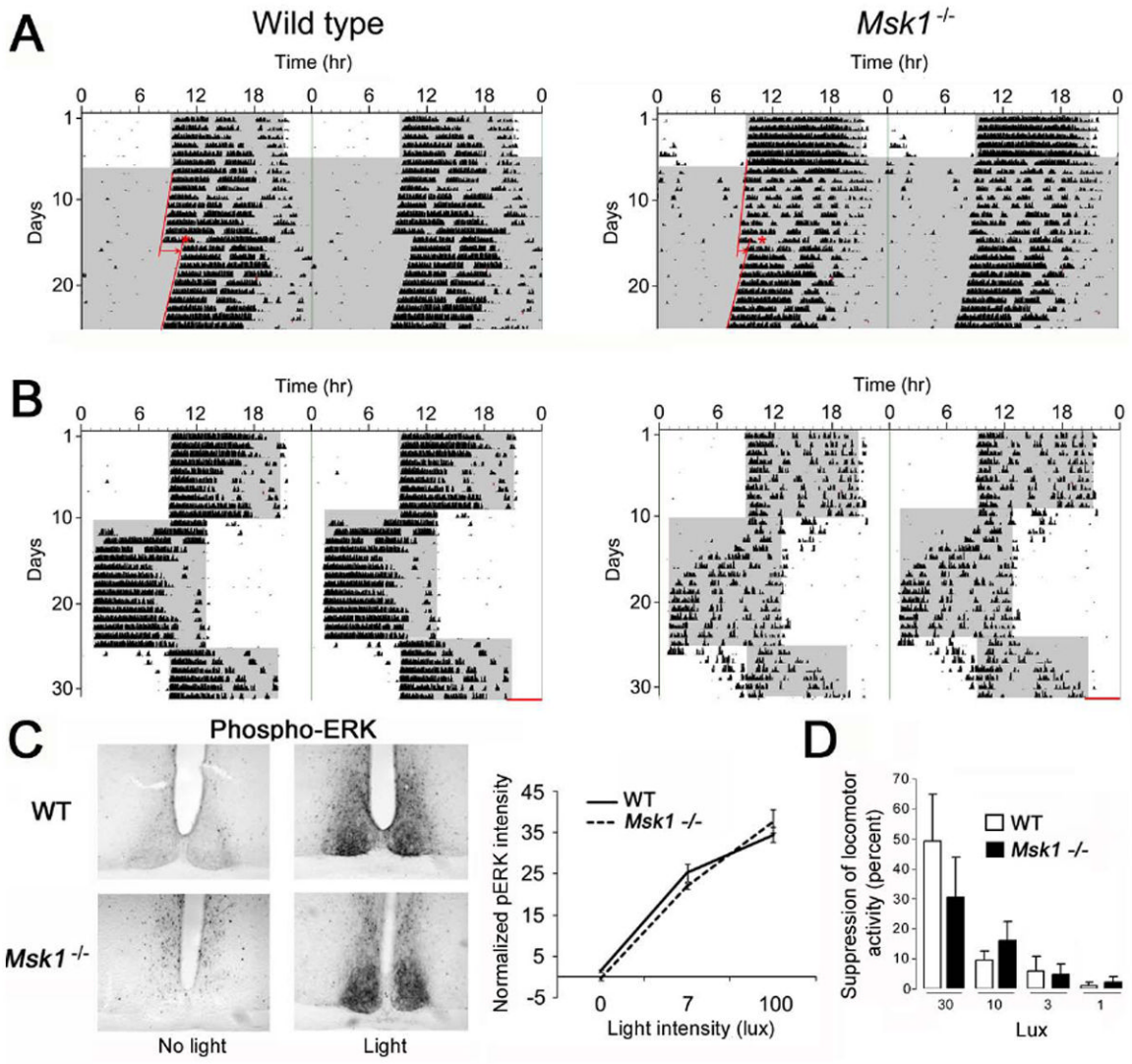

Figure 2. Light-evoked clock entrainment is compromised in *MSK1*$^{-/-}$ mice

A) Representative double-plotted actographs of wheel-running activity. Initially, mice were entrained on a 12 h LD cycle and then transferred to total darkness. After ~10 days under DD, mice were exposed to light (100 lux, 15 min) at CT15 (asterisks). Regression lines and arrows approximate the phase-delaying effects of light. Gray area indicates the periods when light was off. B) *MSK1*$^{-/-}$ mice required a longer time than WT mice to entrain to a new LD cycle. Representative double-plotted actographs of wheel-running activity. Initially, *MSK1*$^{-/-}$ and WT mice were entrained on a 12 h LD cycle and then transferred to an 8 h phase-advanced LD cycle. Mice were kept in the new LD cycle for 14 d and then transferred back to the original LD cycle. For this experiment, light levels were maintained at 7 lux. The horizontal bar at the bottom of the traces denotes an 'off-line' period when data were not recorded. C: Left) Light-evoked ERK activation was not altered in *MSK1* null mice. Representative immunohistochemical labeling for phospho-ERK (pERK) under control conditions, and following a 100 lux light pulse (15 min) administered at CT 15. Note that robust light-evoked pERK was detected in WT and *MSK1*$^{-/-}$ animals. C: Right) pERK quantitation for the control condition (0 lux) and following 7 lux and 100 lux light pulses. Significant differences in basal and evoked pERK levels were not observed between the WT and *MSK1*$^{-/-}$ animals. Error bars denote the SEM. The pERK expression in the WT SCN

under “no light” condition was normalized to a value of 1 and expression levels in all other conditions are reported as relative values of 1. Data were averaged from 5 animals per condition. D). Masking is not altered in *MSK1*$^{-/-}$ mice. Mice were exposed to light for 120 min starting at ZT 15, and the average suppression ± the SEM of wheel running activity was quantitated. No significant differences between *MSK1*$^{-/-}$ null and WT mice were detected; please see the Methods section for a detailed description of this experiment.

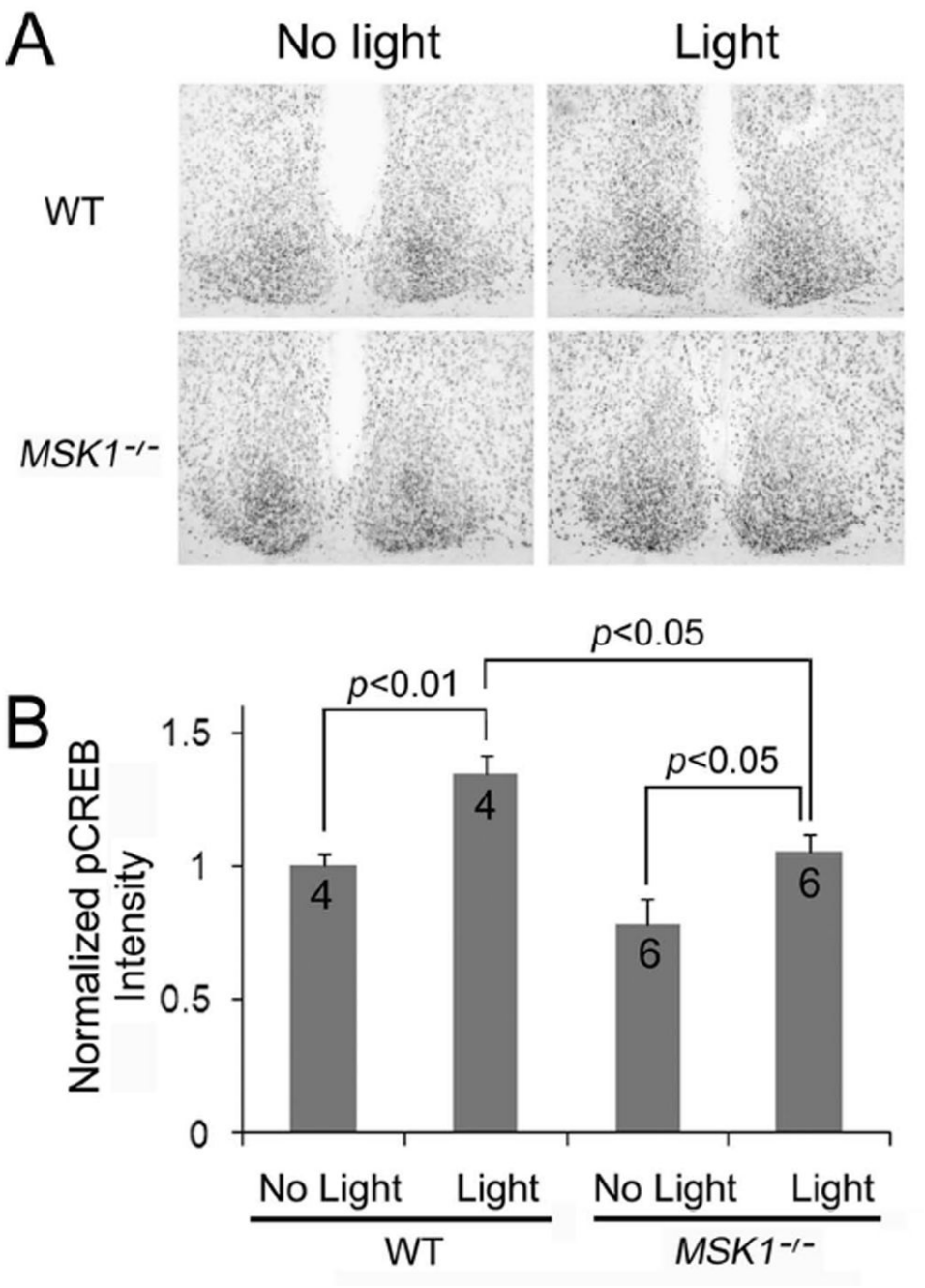

Figure 3. Light-induced phospho-CREB expression is decreased in *MSK1*$^{-/-}$ animals

A) Representative photomicrographs of SCN tissue immunohistochemically- labeled for phospho-CREB (pCREB). For this experiment, animals were dark-adapted for 2 days, exposed to light (100 lux, 15 min) at CT15 and sacrificed 15 min after light treatment. Relative to the control (no light) condition, photic stimulation evoked an increase in pCREB expression in the SCN of wild-type (WT) and *MSK1*$^{-/-}$ animals. B) Quantitative analysis of light-induced phospho-CREB expression in wild-type and *MSK1*$^{-/-}$ animals. The pCREB expression in the SCN under the "no light" condition was normalized to a value of 1 in wild-type animals and expression levels in all other conditions are reported as relative values of 1. Of note, in both WT and *MSK1*$^{-/-}$ animals, light induced a significant increase in pCREB

expression in the SCN. However, the total level of induction was significantly lower in *MSK1*$^{-/-}$ animals compared to WT mice. Numbers in bars denotes the number of animals tested for that condition.

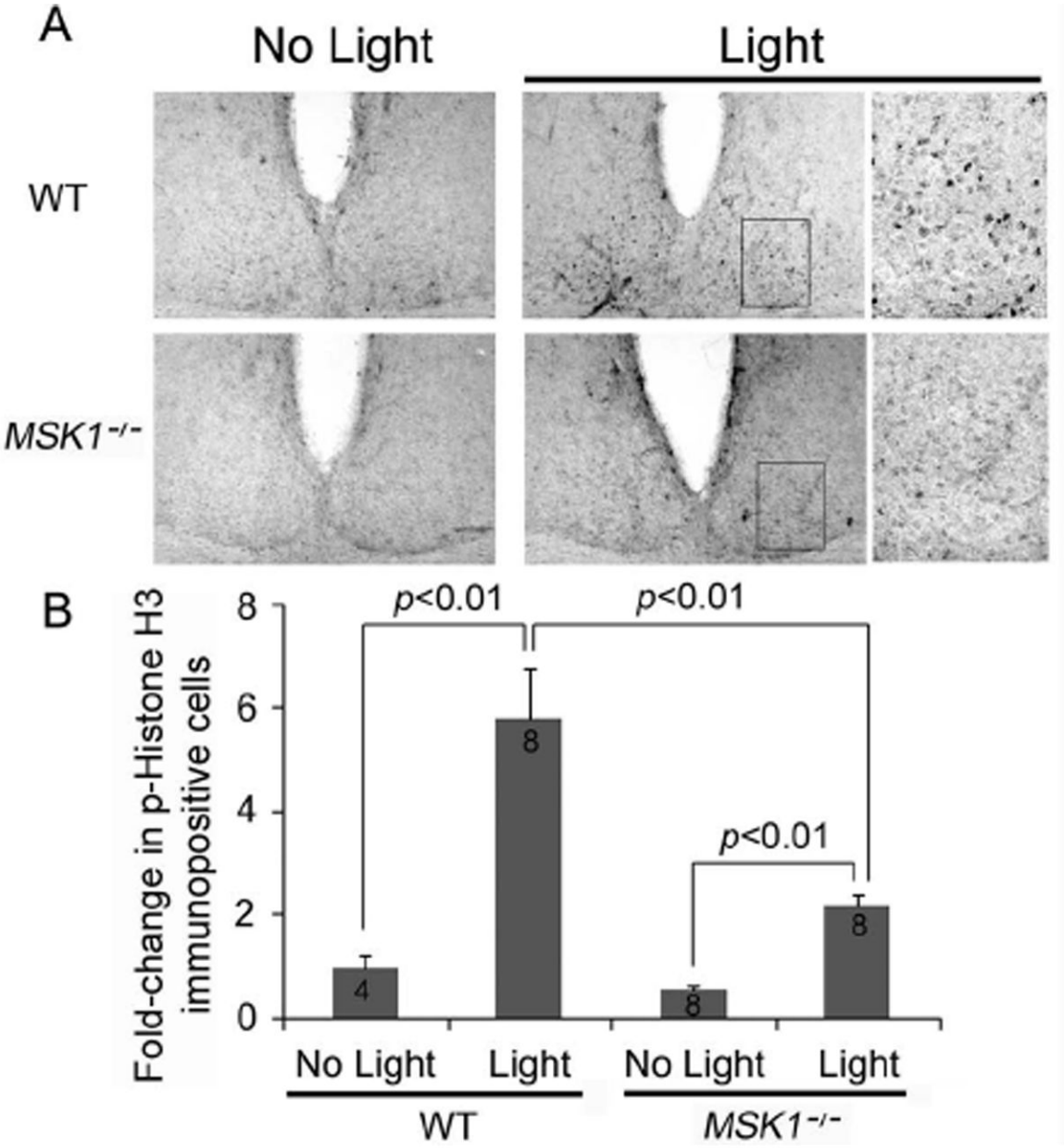

Figure 4. Light-induced phospho-Histone H3 expression is decreased in *MSK1*$^{-/-}$ animals

A) Representative photomicrographs of immunohistochemical staining for phospho-histone H3 (at Ser-10) expression in the SCN. Animals were dark-adapted for 48 hrs, then pulsed with light (100 lux, 15 min) at CT15 and immediately sacrificed. In WT mice, a light-evoked increase in phospho-histone H3 expression was detected in the ventral and central SCN. In the *MSK1*$^{-/-}$ animals, the light-induced phospho-histone H3 expression was blunted, relative to WT animals. Boxed regions are magnified to the right. B) Statistical representation of phospho-histone H3 expression in wild-type and *MSK1*$^{-/-}$ animals. Data are presented as fold-change in p-Histone H3 immunopositive cells relative to the no-light exposure WT animal group, which was set to a value of 1. Of note, both WT and *MSK1*$^{-/-}$ animals exhibited a significant light-evoked increase in phospho-histone expression. However, compared to WT mice, the light-evoked increase in phospho-histone expression

was significantly attenuated in *MSK1*$^{-/-}$ animals. Numbers on the histograms indicate the number of animals used for that condition.

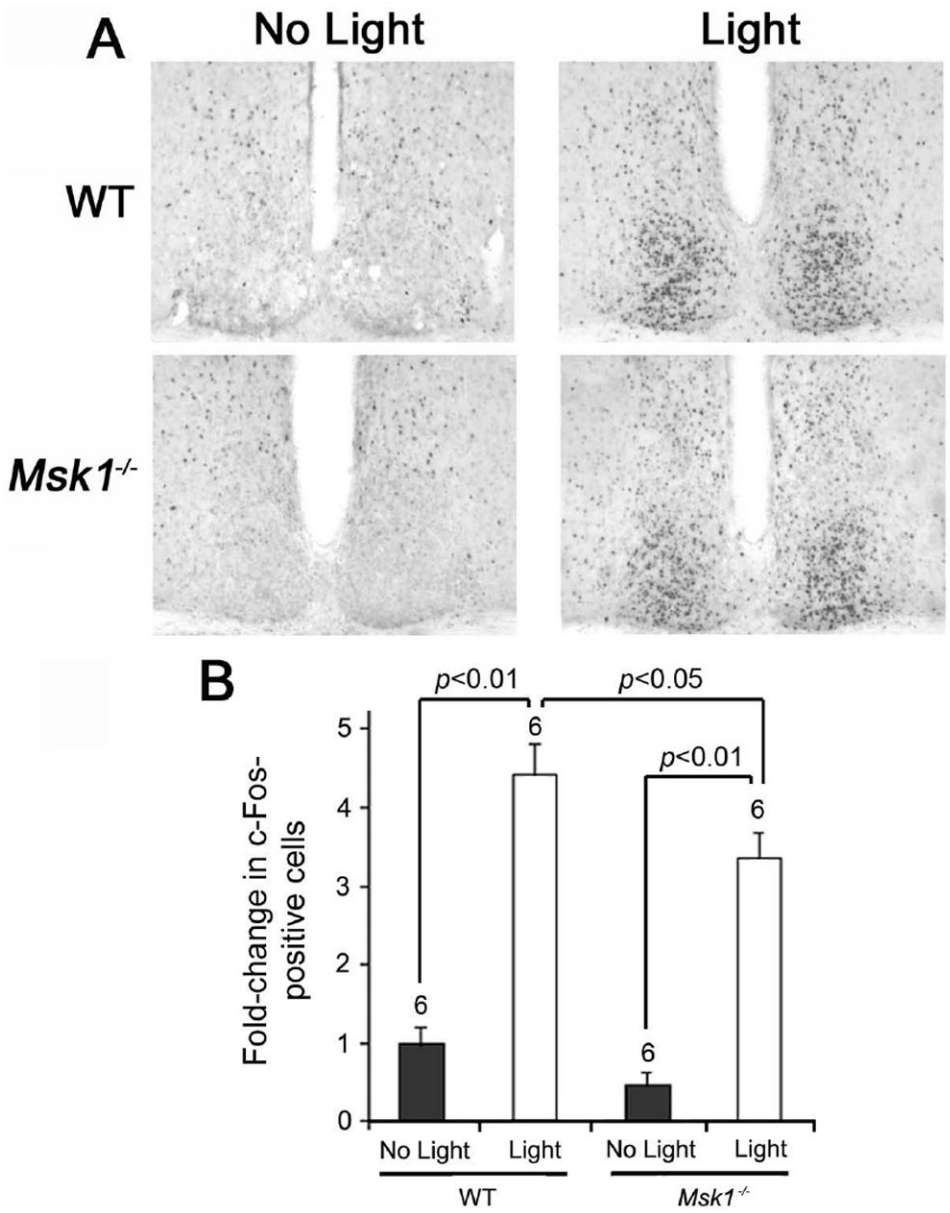

Figure 5. Light-induced c-Fos expression is decreased in *MSK1* knockout animals

A) Representative photomicrographs of c-Fos expression in the SCN. Dark-adapted mice were pulsed with light (100 lux, 15 min) at CT15, and sacrificed 45 min later. Relative to control ('No Light') animals, photic stimulation led to a demonstrable increase in c-Fos expression. B) Statistical analysis of light-evoked c-Fos expression in wild-type and *MSK1*$^{-/-}$ animals. The number of c-Fos expressing cells in the "No Light" condition was normalized to a value 1 in wild-type animals and expression levels in all other conditions are reported as relative values of 1. Of note, compared to WT mice, the light-evoked increase in c-Fos expression was significantly attenuated in *MSK1*$^{-/-}$ animals. Data were collected from 6 animals per condition.

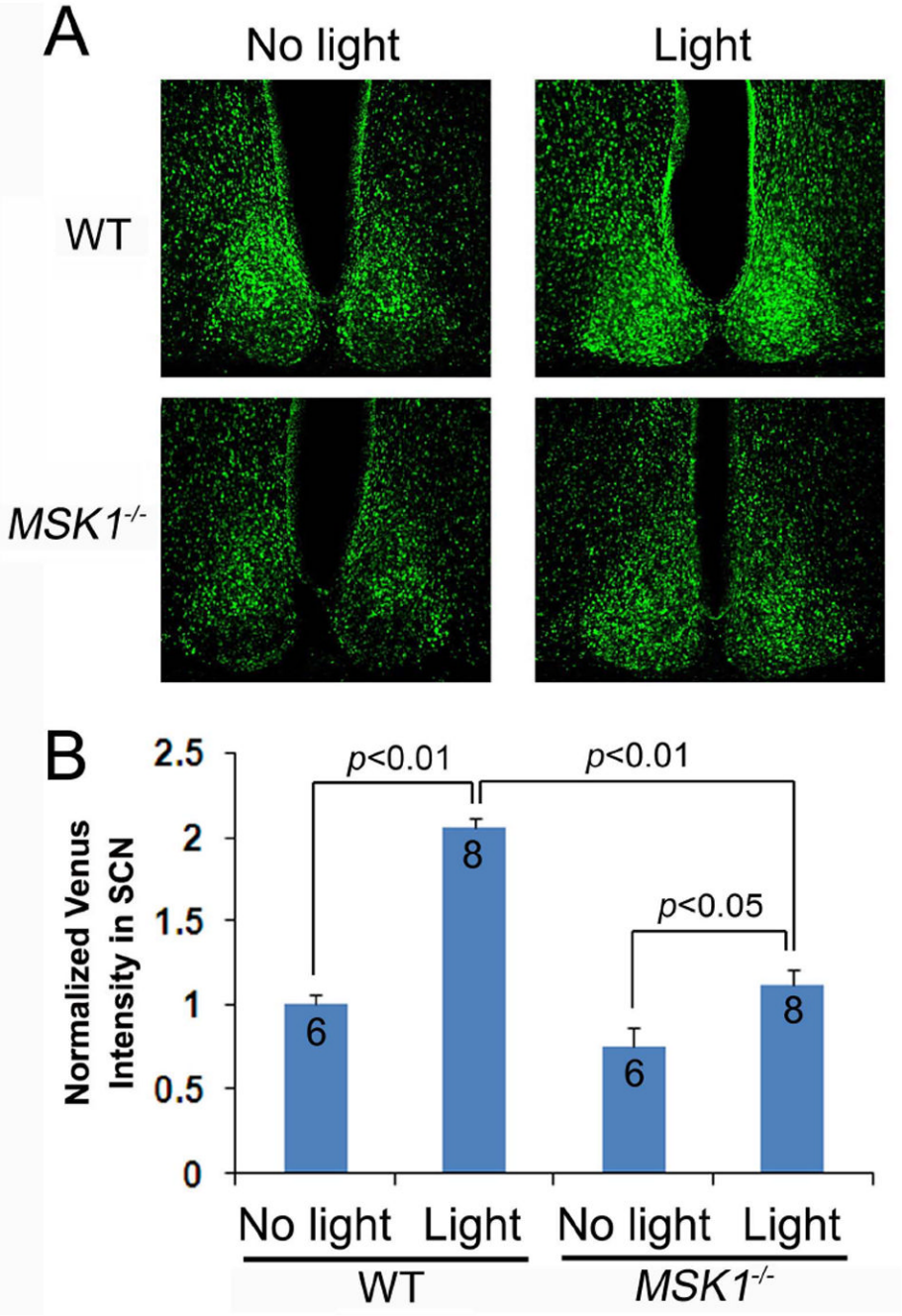

Figure 6. Light-induced *Period1*-Venus expression is decreased in *MSK1*$^{-/-}$ animals

A) Representative confocal images of Venus expression in the SCN. *MSK1*$^{-/-}$ null and WT mice were dark-adapted for 48 hrs and given a light flash (100 lux, 15 min) at CT15. Animals were sacrificed 4 hr later (CT19) and tissue was immunolabeled for Venus antigenicity. These images reveal a robust light-evoked increase in Venus expression in WT animals, and a more modest induction in *MSK1*$^{-/-}$ null mice. B) Quantitation of light-induced Venus expression in WT and *MSK1*$^{-/-}$ animals. The Venus expression in the SCN under "no light" condition was normalized to a value of 1 in the wild-type animals and expression levels in all other conditions are reported as relative values of 1. Light induced significant Venus expression in the SCN in both WT and In *MSK1*$^{-/-}$ animals. However, the

level of induction in the *MSK1*$^{-/-}$ null mice was significantly reduced relative to the WT animals. Numbers in bars denotes the number of animals examined for that condition.

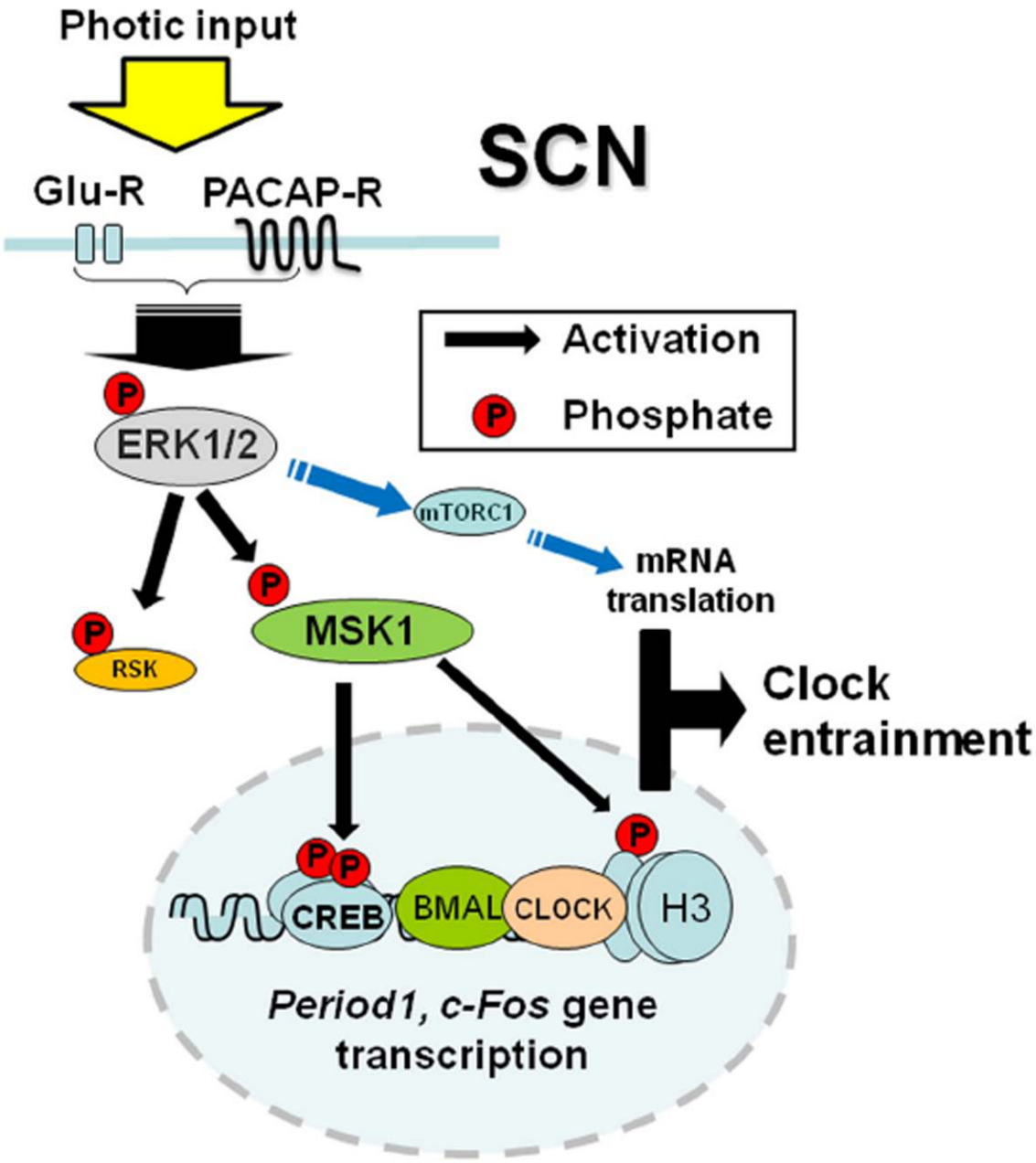

Figure 7. Schematic overview of MAPK/MSK1 signaling pathway in the SCN circadian clock

Photic input from the RHT drives the release of the excitatory amino acid glutamate and the neuropeptide PACAP. Postsynaptic receptors trigger actuation of the MAPK signaling cassette, ultimately leading to the phospho-activation of the effector kinase ERK. Three principal ERK-mediated signaling events are depicted: activation of MSK1, RSK and mTORC1 signaling. MSK1 stimulates activation of the transcription factor CREB, which in turn promotes CRE-dependent transcription of rapid response genes, including *c-Fos*, and the clock gene *Period1*. MSK1 may also facilitate clock gene transcription via epigenetic mechanisms such as histone H3 phosphorylation. These mechanisms work together to mediate photic entrainment of the SCN clock.

Table 1

Characterization of circadian and clock entrainment phenotype in *MSK1*$^{-/-}$ mice

Data are presented as the mean ± SEM for all values. For parameter 2,“variance in activity onset”, refers to the variability of the daily phase angle. In DD (parameter 3), when phase angle is no longer applicable, “variance in activity onset” reflects the difference between actual activity onset time and the “predicted” activity onset time determined by regression analysis. For parameters 2 and 3, data analysis was performed on 10 (LD) or 14 (DD) days of data. Please see the Methods section for a more detailed presentation of the analysis approach. For parameters 4 and 5, “overall activity” refers to the mean wheel-running activity values per binned 5 min period. Data were averaged over a 10-14 day period. For parameter 6, mean wheel-running activity values per binned 5 min period during the light and dark periods are presented. For parameters 7-9 “days to re-entrain” is defined as the number of days needed for animals to be entrained to the new light/dark cycle. The day when entrainment is completed is defined as the first day when the time between dark onset and activity onset is less than 20 min and stabilizes for the next three days. “Day 1” and “Day 2 lag in onset" is defined as mean time between light-off and activity onset on the first/second day following the shift in the light cycle. The photic phase shifting analysis approach is described in the Methods section. Significance was assessed via a one-way ANOVA followed by the Student–Newman–Keuls (SNK) test.

Parameter (mean ± standard error)	Wild-Type (n=11)	*Msk1*$^{-/-}$ (n=11)	*p* Value
1. Circadian period (τ) in DD (hr)	23.58±0.06	23.79±0.07	0.041 *
2. Variance in activity onset in LD (d)	0.55±0.21	3.08±0.42	0.001 **
3. Variance in activity onset in DD (d)	1.27±0.41	4.00±0.44	0.001 **
4. Overall activity in LD (rounds/5 min)	57.2±5.19	62.7±7.50	0.560
5. Overall activity in DD (rounds/5 min)	67.89±5.16	52.42±5.27	0.049 *
6. Activity during the light (L) period(rounds/5 min)	1.13±0.34	6.92±2.15	0.017 *
7. Activity during the dark (D) period(rounds/5 min)	104.27±12.92	108.56±21.15	0.861
8. Days to re-entrain to 8h advancing LD cycle(d)	3.55±0.53	8.00±1.50	0.011 *
9. Days to re-entrain to 8h delaying LD cycle(d)	2 82±0.54	3.29±0.47	0.555
10. Photic phase delay at CT15 (hr)	2.28±0.17	1.36±0.24	0.005 **
11. Photic phase advance at CT22 (hr)	0.64±0.11	0.49±0.11	0.342

Note: Significant differences between WT and *MSK1* null mice are highlighted with

*(p<0.05) and

**(p<0.01) denotations.