Light and Glutamate-Induced Degradation of the Circadian Oscillating Protein BMAL1 during the Mammalian Clock Resetting

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Recently discovered mammalian clock genes are believed to compose the core oscillator, which generates the circadian rhythm. BMAL1/CLOCK heterodimer is the essential positive element that drives clock-related transcription and self-sustaining oscillation by a negative feedback mechanism. We examined BMAL1 protein expression in the rat suprachiasmatic nuclei (SCN) by immunoblot analysis. Anti-BMAL1 antiserum raised against rBMAL1 recognized 70 kDa mBMAL1b and detected a similar immunoreactivity (IR) as a major band in rat brains. Robust circadian BMAL1-IR oscillations with nocturnal peaks were detected in the SCN during a light/dark cycle and under constant darkness. A short duration light exposure at night acutely reduced BMAL1-IR in the SCN during photoentrainment. This might be attributable to the degradation of BMAL1 protein. Application of glutamate and NMDA to the SCN slices at projected night, a procedure mimicking photic phase delay shift, also acutely reduced BMAL1-IR in a similar manner. A rapid decrease of BMAL1 protein suggests that BMAL1 protein might be implicated in the light-transducing pathway within the SCN.

Key words: circadian clock; light; phase shift; glutamate; NMDA; suprachiasmatic nucleus

Circadian clocks controlling various biological timings are widely observed in eukaryotes and some prokaryotes. Recent progress has revealed an important part of the mechanism, a set of mammalian circadian clock genes localized within the site of the master circadian pacemaker in the hypothalamic SCN (Dunlap, 1999).

The molecular core of the circadian clock is thought to be the feedback loop with positive and negative limbs. The mouse *Clock* gene was first demonstrated to be an essential component of the mammalian circadian clock (Vitaterna et al., 1994; King et al., 1997). BMAL1 protein is a heterodimeric partner of CLOCK protein (Ikeda and Nomura, 1997; Gekakis et al., 1998). CLOCK/BMAL1 heterodimer is believed to bind E-box elements and drives and maintains circadian oscillations of mammalian orthologs of *Drosophila* period genes, i.e., *mPer1* transcript (Sun et al., 1997; Tei et al., 1997). *BMAL1* transcript exhibits evident circadian oscillation, whereas *Clock* transcripts do not (King et al., 1997; Honma et al., 1998). CYCLE, a *Drosophila* ortholog of BMAL1, is essential for the circadian rhythmicity (Rutila et al., 1998).

The negative limb in the circadian loop is believed to be composed of PER1, PER2, PER3, TIM, CRY1, and CRY2. These molecules, except TIM, show stronger circadian oscillation than that of *Clock/BMAL1* (Albrecht et al., 1997; Shigeyoshi et al., 1997; Sangoram et al., 1998; Zylka et al., 1998a,b; Kume et al., 1999; Okamura et al., 1999). *mCry1*, *mCry2,* and *mPer2* genes encode a functional component of the circadian clock (van der Horst et al., 1999; Zheng et al., 1999).

Light is the most powerful external stimulus for connecting and entraining the circadian clock to the environment. In rodents, even a single brief exposure to light in the early (subjective) night causes a phase delay shift, whereas a light pulse during late (subjective) night induces a phase advance shift. The *c-Fos* gene was first to be identified as one of the immediate responsive genes to light in the SCN (Rea, 1989; Rusak et al., 1990). Rodent *Per1* and *Per2* transcripts are also immediately induced (Albrecht et al., 1997; Shigeyoshi et al., 1997; Yan et al., 1999). *Drosophila* dCRY protein is an essential transducer in photic phase shift (Emery et al., 1998). Light-induced degradation of dTimeless protein correlates with behavioral entrainment (Myers et al., 1996; Zeng et al., 1996; Naidoo et al., 1999). However, no mammalian gene has been proved essential in photoentrainment, nor have hypothetical light-responsive elements (LREs) upstream of the light-responsive genes been identified.

The function of BMAL1 in the photoentrainment and maintaining of the circadian clock is not clear. To understand further how the putative BMAL1 functions in the circadian clock cells, we have generated a specific antiserum against rBMAL1 and used it for the immunoblot analysis of the temporal regulation related to the clock mechanism. In this report, we discuss photic downregulation of BMAL1 protein during the resetting of the circadian clock.

MATERIALS AND METHODS

Animals. Male Wistar rats (Nippon Bio-Supply Center, Tokyo, Japan) aged 5–7 weeks were maintained at 25°C on a 12 hr light/dark (LD) cycle [light: zeitgeber time (ZT) 0–12; dark: ZT12–24] for at least 10 d before use. The animals were then transferred to a dim light (<1 lux) condition, and their circadian locomotor activities were monitored using the far-infrared monitor system (Supermex System, Muromachi-Kikai, Tokyo, Japan). The experiments under constant darkness (DD) conditions were performed 2–3 d after the transition. For the light pulse experiment, rats were exposed to white light (1000 lux) for 30 min, then they were killed at the experimental time point. As controls, we analyzed the locomotor activities of a number of rats under the same sampling conditions and confirmed that the phase shifts occurred only by light exposure at subjective night.

Materials. A glutathione-Sepharose, HiTrap column and pGEX-5X vector DNA were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). *Escherichia coli* strain JM109 and pBluescript SK$^+$ were from Clontech (Tokyo, Japan). Affi-Gel10 was from Bio-Rad (Hercules, CA). Glutathione and complete protease inhibitor cocktail tablets were from Boehringer Mannheim (Tokyo, Japan). pGEM-T Easy vector DNA and TNT T7 Quick Coupled Transcription/Translation System were from Promega (Tokyo, Japan). Bovine serum albumin, glutamate, NMDA, and tetrodotoxin (TTX) were from Sigma-Aldrich (Tokyo, Japan). TiterMax Gold was from CytRx (Atlanta, GA). The BCA protein assay system was from Pierce (Rockford, IL). Immobilon P membranes were from Millipore (Bedford, MA). HISTOFINE Simple Stain PO (MULTI) system was Nichirei (Tokyo, Japan). Anti-actin antibody was from Chemicon (Temecula, CA). Anti-c-fos antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). HIBERNATE A medium and B27 supplement were

from Life Technologies (Tokyo, Japan). (*R,S*)-3,5-dihydroxyphenylglycine (DHPG) was from Tocris Cookson (Bristol, UK).

Preparation of recombinant BMAL1 proteins. A full-length of cDNA for mBMAL1b was amplified by PCR (94°C for 30 sec, 50°C for 1 min, 72°C for 2 min, 30 cycles), using mouse brain cDNA as a template. The PCR product was ligated into the plasmid pGEM-T Easy and analyzed. The construct was transformed into *E. coli* strain JM109. Recombinant mBMAL1b protein was produced from the construct by *in vitro* transcription and translation using the TNT T7 Quick Coupled Transcription/Translation System.

A fragment of cDNA for rBMAL1 (encoding amino acid 154–182; chosen as a region nonhomologous to rTIC, assumed to be a BMAL1 homolog or splice variant) was amplified by PCR (94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min, 30 cycles), using rat brain cDNA as a template. The PCR product was ligated into the plasmid pBluescript SK$^+$ and analyzed, and ligated into pGEX-5X1. The construct was transformed into *E. coli* strain JM109. Expression of glutathione *S*-transferase (GST)-rBMAL1 fragment fusion protein (GST-ΔBMAL1) was induced by a final concentration of 0.5 mM isopropyl-thiogalactoside for 12 hr at 25°C. The cells were harvested by centrifugation and disrupted by sonication in buffer A (in mM: 50 Tris-HCl, pH 8.0, 50 NaCl, 1 dithiothreitol, and 1 EGTA). After centrifugation at 10,000 × *g* for 20 min at 4°C, the supernatant was incubated with glutathione-Sepharose for 1 hr at 4°C. After extensive washing with buffer B (0.14 M NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, and 1.5 mM KH$_2$PO$_4$, pH 7.4, and 0.1% Tween 20), the bound proteins were eluted with buffer A containing 20 mM glutathione. The eluate was dialyzed against 1000-fold volume of 20 mM HEPES, pH 7.5, containing 25% glycerol, 0.01% NP-40, and 1 mM dithiothreitol for 6 hr at 4°C.

Preparation of the antiserum against rBMAL1. Primary immunizations were performed with purified GST-ΔBMAL1 using TiterMax Gold, and boosts (at 2 week intervals) used Freund's incomplete adjuvant. Obtained antiserum was used for immunoblot analyses.

Preparation of the tissue extracts. Animals were decapitated at each experimental time point. They were killed under dim light (<1 lux) during the DD cycle or the dark phase during the LD cycle. The brain was removed from the skull and quickly frozen in dry ice. As described previously, the SCN region was punched out with a 2-mm-diameter needle that was inserted to a 1 mm depth into the surface of the coronal plane, an area that included the SCN (Tamaru et al., 1999). Statistical significance was confirmed by using sufficient numbers of SCN. The sample was homogenized in 30 μl of buffer C: 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.4 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, 2 mM EGTA, 20 mM NaF, 0.5 mM Na$_3$VO$_4$, 0.1 mM Na$_2$MoO$_4$, 1 mM dithiothreitol, and 20 mM sn-glycerol 2-phosphate and complete protease inhibitor cocktail. Immunoblot analysis was performed using 10 μl of the extract. For nuclear fractionation, the SCN sample was homogenized in 20 μl of buffer D: 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.32 M sucrose, 1.5 mM CaCl$_2$, 10 mM KCl, 3 mM MgCl$_2$, 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 0.5 mM Na$_3$VO$_4$, 0.1 mM Na$_2$MoO$_4$, 1 mM dithiothreitol, and 20 mM sn-glycerol 2-phosphate and complete protease inhibitor cocktail. The homogenate was centrifuged at 1000 × *g* for 10 min. The pellet was solubilized with 20 μl of buffer C. The nuclear fraction was recovered in the supernatant after centrifugation at 10,000 × *g* for 20 min.

Brains and tissues were removed from rats during the day phase and homogenized using 5 volume buffer C, as described above. Protein concentration was determined using a BCA protein assay system, with bovine serum albumin as a standard. Twenty micrograms of the extracts were subjected to immunoblot analysis.

Immunoblot analysis. The samples were separated on SDS-10% polyacrylamide gels and transferred onto Immobilon P membranes. After blocking with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TTBS), membranes were incubated at 4°C for 16 hr with a 1:200 anti-BMAL1 antiserum dilution in TTBS containing 3% bovine serum albumin. After washing with TTBS, membranes were developed with a HISTOFINE Simple Stain PO (MULTI) system according to the manufacturer's protocol. Quantification of bands of interest was performed by densitometry in a computerized image analyzer system (AE-6920; ATTO, Tokyo, Japan). As shown in figure legends, the relative density of BMAL1-IR was normalized against actin-IR (1 = the bottom level in each experiment). Student's *t* test was used for the statistic analysis of the relative values.

Analysis using the rat SCN slices. Rats aged 4–5 weeks were killed at 9–10 hr after light onset (ZT9–10), and their brains were removed. A region containing SCN located immediately dorsal to optic chiasm was processed into two sections cut at 500 μm. SCN-containing sections were transferred to HIBERNATE A medium supplemented with B27 and placed in an incubator and slowly brought to a final temperature of 35°C. Experiments were performed in the continued presence of 1 μM TTX, which reportedly reversibly blocks electrophysiological output from the circadian clock, but does not affect the timing of the circadian clock itself (Shibata and Moore 1993) to discount intrinsically released glutamate. To examine dose-responsive effects on BMAL1-IR reduction, the slices were treated with glutamate (0, 3, 10, 30, 100, 300 μM) or NMDA (0, 3, 10, 30, 100, 300 μM) for 40 min at projected ZT14. Slices were also treated with glutamate (100 μM), NMDA (100 μM), DHPG (100 μM) or without for the indicated times, which are shown in the figures and figure legends (see Fig. 6).
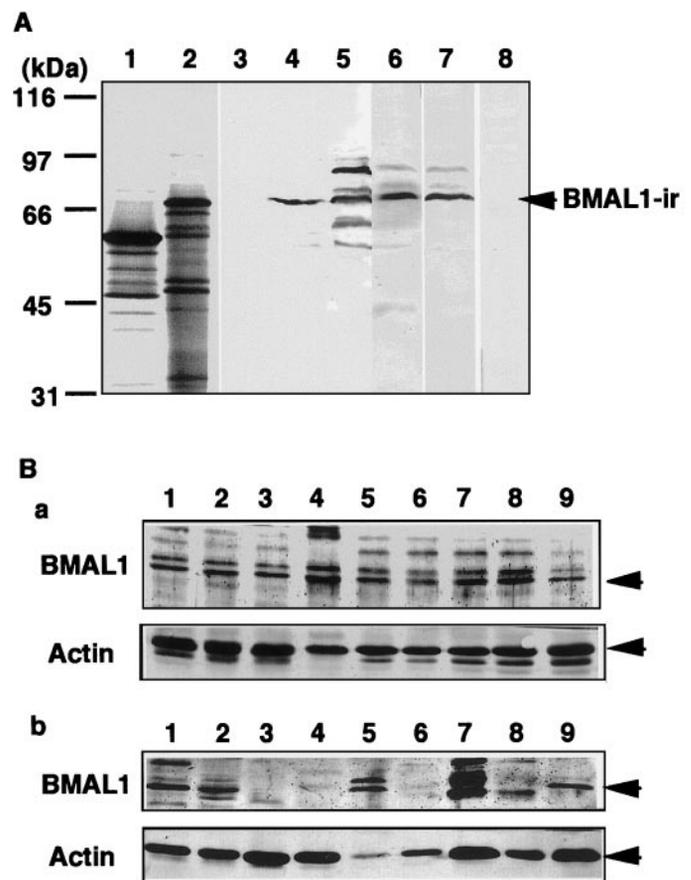

Figure 1. Characterization of anti-BMAL1 antiserum. *A,* Immunizing a rabbit with GST-ΔBMAL1 (amino acids 154–182) produced anti-BMAL1 antiserum. Recombinant mBMAL1b protein (*lanes 2* and *4*) and Luciferase (*lanes 1* and *3*) were produced by *in vitro* transcription and translation using methionine labeled with ³⁵S (*lanes 1* and *2*) and without (*lanes 3* and *4*). The lysates containing mBMAL1b protein and luciferase, mouse brain extract (20 μg of protein, *lane 5*), and rat brain extract (20 μg of protein, *lanes 6–8*) were subjected to immunoblot analysis using anti-BMAL1 antiserum. BMAL1-IR was specifically absorbed by addition of GST-ΔBMAL1 (10 μg/ml; *lane 8*) or not of GST (10 μg/ml; *lane 7*). *Ba,* Distributions of BMAL1-IR in rat brain. Lysates (20 μg of protein) of hypothalamus (*lane 1*), olfactory bulb (*lane 2*), hippocampus (*lane 3*), pineal body (*lane 4*), cerebral cortex (*lane 5*), basal ganglia (*lane 6*), thalamus (*lane 7*), superior colliculus (*lane 8*), and cerebellum (*lane 9*) were subjected to immunoblot analysis probing with anti-BMAL1 antiserum and anti-actin antibody. *Bb,* Distributions of BMAL1-IR in rat tissues. Lysates (20 μg of protein) of whole brain (*lane 1*), testis (*lane 2*), heart (*lane 3*), lung (*lane 4*), liver (*lane 5*), kidney (*lane 6*), skeletal muscle (*lane 7*), intestine (*lane 8*), and spleen (*lane 9*) were subjected to immunoblot analysis and probed with anti-BMAL1 antiserum and anti-actin antibody.

RESULTS

Characterization of anti-BMAL1-specific antibody

Polyclonal antibody was raised against recombinant GST-ΔBMAL1 protein by immunizing a rabbit. Specificity of anti-BMAL1 antiserum was then characterized. By immunoblot analysis, anti-BMAL1 antiserum cross-reacted with 70 kDa mouse BMAL1b protein, which was produced by *in vitro* translation from a cDNA of a full-length type of BMAL1 variant (Fig. 1*A*). In mouse and rat brains, similar BMAL1-like immunoreactivities of ~70 kDa were detected as a major band. BMAL1-IR in rat brain was specifically absorbed with GST-ΔBMAL1 antigen, but not with GST. Next, we examined the tissue distribution of BMAL1-IR. BMAL1-IR was detected in all regions of rat brain (Fig. 1*Ba*). BMAL1-IRs were highly detected in skeletal muscle, testis, liver, and spleen, as well as in brain. Brain and testis mainly contained ~70 kDa BMAL1-IR, whereas the spleen mainly contained one of ~71 kDa (Fig. 1*Bb*).
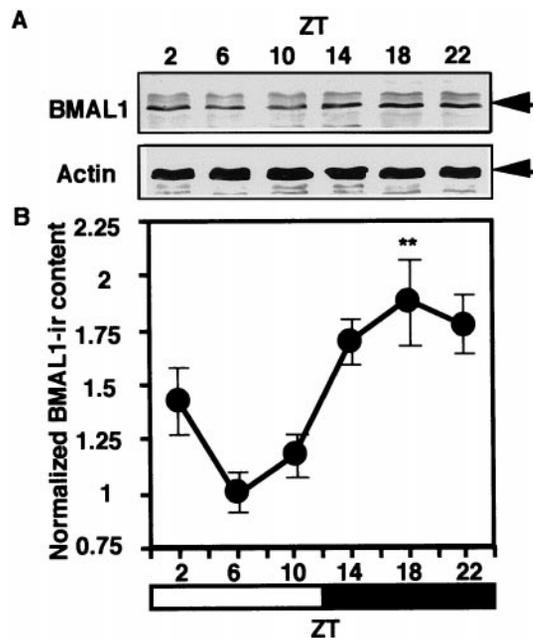
Figure 2. Circadian profile of BMAL1-IR in the rat SCN during the LD cycle. *A,* Male Wistar rats aged 5–8 weeks were maintained under a 12 hr light/dark regimen. Rats were killed at ZT2, 6, 10, 14, 18, or 22. The SCN extracts from five animals at each time point were combined and subjected to immunoblot analysis. *B,* The relative density of BMAL1-IR from the analysis for individual SCN was normalized against actin-IR and plotted (1 = the bottom level at ZT6). Each point represents the mean ± SEM. One-way ANOVA was used for statistical analysis. Statistical significance value: **$p < 0.001$.

Circadian oscillation of BMAL1-IR content in the SCN

To investigate the roles of BMAL1 in the circadian clock, we examined the circadian profile of BMAL1-IR content in the rat SCN. By immunoblot analysis, robust circadian oscillation of BMAL1-IR content was observed in the SCN under both LD (Fig. 2*A*) and DD conditions (Fig. 3*A*). A 70 kDa major band and a slightly larger minor band were detected. Normalizing BMAL1-IR content against actin-IR content as a constitutively expressed internal control was performed as the exact quantitative analysis. Circadian BMAL1-IR profiles during LD and DD cycles exhibited evident oscillation with a nocturnal peak (Figs. 2*B*, 3*B*). The peak/trough ratios for both cycles were ~2:1.

Light pulse-induced rapid reduction of BMAL1-IR in the SCN during the resetting of the clock

To investigate the roles of BMAL1 in the phase-shifting mechanism of the circadian clock by photic stimulus, we examined the profiles of BMAL1-IR in the rat SCN containing retinorecipient cells after light exposure of rats. Interestingly, a significant decrease in BMAL1-IR content in the SCN was observed within 30 min after 1000 lux light exposure delivered 2 hr after the onset of early night in the LD cycle (ZT14), a result known to cause behavioral phase delay shift (Fig. 4*A*). BMAL1-IR in the SCN also decreased after a 30 min 1000 lux light pulse delivered from ZT14, a stimulus sufficient to cause the phase shift (Fig. 4*B*). Without light illumination at ZT14, decrease of the basal level of BMAL1-IR was not observed from ZT14 to ZT18 (Fig. 2). A significant trough in BMAL1-IR content, 48–49% lower than basal level, was observed 1–2 hr after the exposure. BMAL1-IR content returned to near basal level 3 hr after the pulse began. A similar pattern of reduction in BMAL1-IR content was observed after a light pulse during subjective early night [circadian time 15 (CT15)] (data not shown). As positive controls, we detected an acute increase of c-fos protein after the stimulus (data not shown).
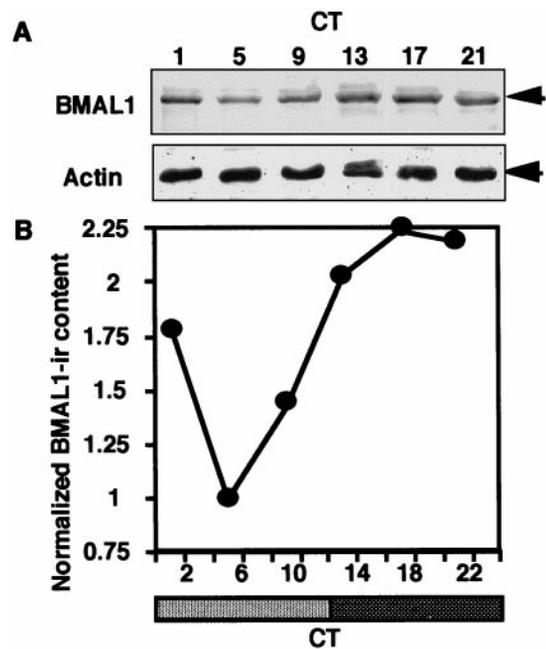

Figure 3. Circadian profile of BMAL1-IR in the rat SCN during the DD cycle. *A,* Male Wistar rats aged 5–8 weeks were maintained under a 12 hr light/dark regimen (light: ZT0–12; dark: ZT12–24), then transferred to dim light (<1 lux). Rats were killed at CT1, 5, 9, 13, 17, or 21. The SCN extracts from six animals at each time point were combined and subjected to immunoblot analysis. *B,* The relative density of BMAL1-IR was normalized against actin-IR and plotted (1 = the bottom level at CT5).

Nocturnal-specific reduction of BMAL1-IR

Because the circadian clock is entrained by photic stimulus only during subjective night, we examined the phase dependency of the photic reduction of BMAL1-IR. A number of rats ($n = 5$ at each treatment) were exposed to 30 min, 1000 lux light pulses delivered at early subjective night (CT15), late subjective night (CT21), and mid subjective day (CT6) (Fig. 5*A*). We examined the samples at 2 hr after light exposure, because the level of BMAL1-IR became trough at that time (Fig. 4). SCN from individual rats killed 2 hr after exposure were all subjected to immunoblot analysis. A clear phase dependency was revealed in the photic reduction of BMAL1-IR. A light pulse at CT6 induced no significant change in BMAL1-IR. BMAL1-IR was significantly lower after exposure at CT15 ($p < 0.001$; 56% of basal level) and modestly lower after exposure at CT21 ($p < 0.01$; 74% of basal level).

Glutamate and NMDA-induced rapid BMAL1-IR reduction in the SCN slices

To explore the mechanism of photic reduction of BMAL1-IR in the SCN, we performed *in vitro* pharmacological experiments on the rat SCN slices. Glutamate is believed to be a major neurotransmitter which transduces retinal photic information to the SCN via retinohypothalamic projection. Application of glutamate to the SCN can mimic the photic phase shift (Ding et al., 1994). NMDA alone substantially mimics photic phase shifts (Mintz et al., 1999). At projected ZT14, slices were treated with glutamate (0–300 μM) or NMDA (0–300 μM) for 40 min, and the dose-dependent effects of glutamate and NMDA on BMAL1-IR content in the SCN were examined. Glutamate and NMDA exhibited dose-responsive effects on BMAL1-IR reduction (Fig. 6*A*). The effective concentration of NMDA (>10 μM) revealed slightly more sensitive than glutamate (>30 μM). The maximum reducing effects were observed by using ~100 μM of glutamate and NMDA.

The precise temporal patterns of BMAL1-IR were analyzed after the application of glutamate receptor agonists. Similar to the photic reduction, rapid BMAL1-IR reduction in the SCN slices began within 10 min after the application of glutamate and continued to decrease for at least 1 hr (Fig. 6*B*). Evident reduction of
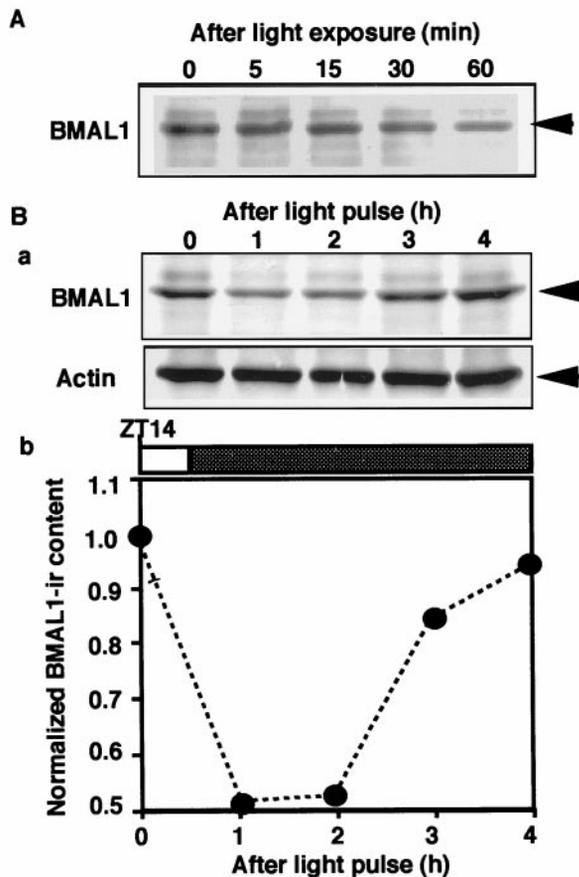
Figure 4. Temporal profile of BMAL1-IR in the rat SCN after light exposure at early night. *A,* Rats (*n* = 4 for each lane) were exposed to 1000 lux light delivered from ZT14, then killed at the indicated time. The SCN extract (10 μg of protein) from the rat was subjected to immunoblot analysis probing with anti-BMAL1 antiserum. *B,* Rats (*n* = 4 for each lane) were exposed to a 30 min 1000 lux light pulse delivered from ZT14 to ZT14.5, then killed at the indicated time. The SCN extract from the rat was subjected to immunoblot analysis probing with anti-BMAL1 antiserum and anti-Actin antibody (*a*). The relative densities of BMAL1-IR in the immunoblot membranes were normalized against actin-IR and plotted (1 = the basal level at ZT14) (*b*).

BMAL1-IR also was observed even 2 hr after the application of glutamate (data not shown). Acute c-fos protein induction was clearly detected after the application of glutamate, confirming the effect of glutamate on the SCN slices under our experimental conditions (data not shown). Treatment with NMDA also promptly reduced BMAL1-IR in the SCN nuclear fraction, in a pattern similar to the glutamate application (Fig. 6*C*). In addition to ionotropic NMDA receptors, metabotropic glutamate receptor subtypes are also clearly detected in the SCN (Gannon and Rea, 1994). In contrast, DHPG, an agonist for mGluR1 and mGluR5, did not significantly change BMAL1-IR content in the SCN nuclear fraction (Fig. 6*C*).

DISCUSSION

Using anti-BMAL1 antiserum raised against GST-ΔBMAL1, robust circadian oscillation in BMAL1-IR content in the SCN was detected under LD and DD conditions. BMAL1-IR content peaked during the night phase (but did not significantly fluctuate during the night phase) and reached a trough in the mid-period of the day phase. The peak/trough ratio was ~2:1. As reported, *rBMAL1* transcripts also exhibited evident circadian fluctuation in LD and DD conditions (Honma et al., 1998), with quite similar phase-angle and amplitude to our data for BMAL1 protein. These findings indicate that the change in BMAL1 protein content is a direct manifestation of the change in BMAL1 transcript, without
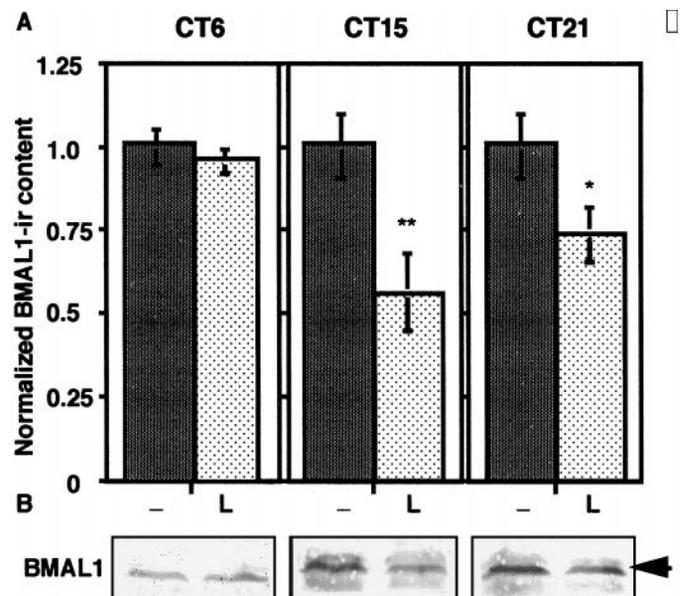

Figure 5. Phase-dependent effect of light exposure on BMAL1-IR in the rat SCN. *A,* Rats (*n* = 5 at each treatment) were exposed to 30 min 1000 lux light pulses delivered from CT15, CT21, and CT6 (*L*), then killed at the same time schedule (CT17). Control (−) rats were kept in darkness, then killed at 2 hr after the exposure. The SCN extracts from the rats were subjected to immunoblot analyses. The relative density of BMAL1-IR was normalized against actin-IR and plotted (1 = the basal level of control). Each column represents the mean ± SEM. Two-tailed Student's *t* test was used for statistical analysis. Statistical significance value: **$p < 0.001$; *$p < 0.01$. *B,* The SCN extracts from five animals at each time point were combined and subjected to immunoblot analysis. The figure represents the typical pattern for the phase-dependent effect of light exposure on BMAL1-IR in the SCN.

significant time lag and post-translational modulation of the protein content throughout the circadian cycle. CLOCK, the partner of BMAL1, is essential for maintaining circadian rhythm, but the *Clock* transcript shows no circadian change in the SCN (King et al., 1997). *Per* gene expression peaks during the day phase (CT4–8) when BMAL1 content reaches a trough (Albrecht et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998a). *Cry* gene expression peaks during the day/night boundary [*mCry1* at CT (ZT) 12; *mCry2* at CT (ZT) 8–16] (Okamura et al., 1999), when BMAL1 content is still rising to a peak. During this period, all PER and CRY protein levels are elevated (Hastings et al., 1999; Kume et al., 1999; Field et al., 2000). The current evidence does not permit a direct explanation of the generating mechanism of circadian oscillatory *BMAL1, Per,* and *Cry* expression. Additional mechanisms may be required for generating the circadian oscillation of the components in the core feedback loop. Nevertheless, our findings suggest that BMAL1 oscillation contributes to circadian oscillatory expression of some additional circadian clock-controlled genes in the SCN.

The specificity of anti-BMAL1 antiserum is discussed from several aspects. As reported, in mice, three splice variants, mBMAL1b (mArnt3), mBMAL1b′, and mBMAL1g′ encode 626, 636, and 222 amino acids, whose predicted molecular weights are 68.6, 69.4, and 25 kDa, respectively (Yu et al., 1999). The BMAL1b variant is assumed to be a full-length type and is used as a "standard" form because it contains all the functional regions, PAS A, PAS B, and basic helix-loop-helix, which are required for interaction with PAS-containing transcription factors like CLOCK and transcriptional activation via E-box. The rBMAL1 antigen was designed as a 28 residue peptide containing amino acids 154–182, corresponding to the C-terminal half of the PAS A domain in rBMAL1b. The antigenic peptide is completely included in mBMAL1b and mBMAL1b′, but not in mBMAL1g′. These variants are expressed widely and differentially in the tissues. A [^{35}S]-labeled band of ~70 kDa was determined to be a major band in the

A

Concentration(μM)

0   3   10   30   100   300

Glu

NMDA

B

After application (min)

0   10   20   40   60

Glu

0   60

–

C

After application (min)

0   10   20   40   60
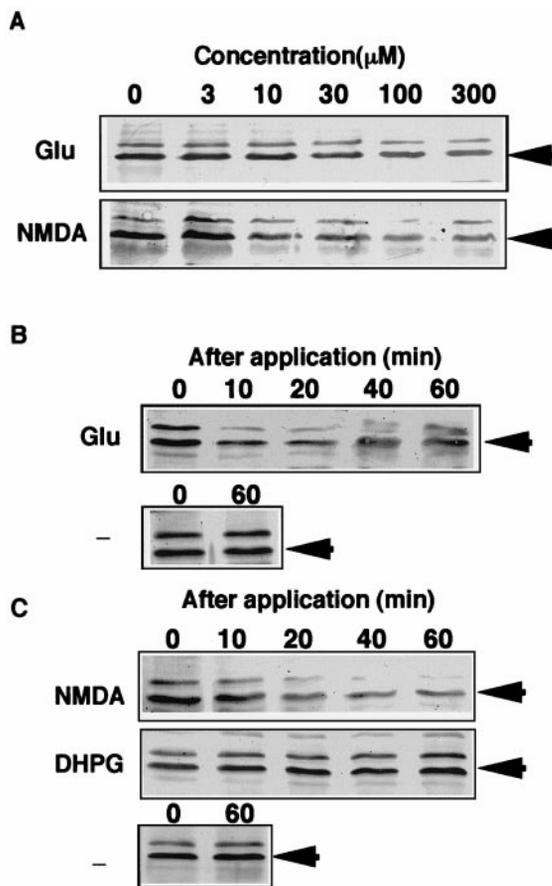
NMDA

DHPG

0   60

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Figure 6. Effects of glutamate receptor agonists on BMAL1-IR in the rat SCN slices. Rats (*n* = 4 at each treatment) aged 4 weeks were killed at ZT9 to ZT10. At projected ZT14, slices were treated with glutamate (*A*; 0–300 μM) or NMDA (*A*; 0–300 μM) for 40 min. Slices were also treated with glutamate (*B*; 100 μM), NMDA (*C*; 100 μM) or without (*B, C*; −) for the indicated time. SCN extracts (*A, B*) and nuclear (*C*) fractions containing 10 μg of protein were subjected to immunoblot analysis probing with anti-BMAL1.

lysate containing the reaction mixture for *in vitro* translation using mBMAL1b cDNA as a template and might therefore represent mBMAL1b protein. In the lysate, anti-BMAL1 antiserum almost uniquely recognized the 70 kDa band, demonstrating that anti-BMAL1 antiserum cross-reacts with mBMAL1b protein. In rats, two variants, rBMAL1b and rTIC encoding 626 and 590 amino acids (Honma et al., 1998; Wolting and McGlade, 1998), have predicted molecular weights of 68.5 and 64.7 kDa. rTIC does not include the antigenic peptide, and thus does not contain the functional PAS A domain. In the rat brain extract, a similar 70 kDa band was determined to be a major band. A band (71 kDa) slightly larger than the 70 kDa was clearly detected in certain brain regions such as the SCN. These bands selectively disappeared by the addition of GST-ΔBMAL1, but not of GST, indicating that the bands represent immunoreactivity for ΔBMAL1 but not for GST. These two evidently detected bands of ~70 kDa were also found in the mouse brain extract and were widespread in a number of the rat brain regions. We suspect that the 70 kDa protein represents BMAL1b, and 71 kDa represents BMAL1b′, but no evidence excludes the possibility that post-translational modifications caused such mobility shifts. In the SCN and retina, the major bands were detected at ~70 kDa. These results demonstrate that intensities of the immunoreactive bands of ~70 kDa detected with anti-BMAL1 antiserum might represent the expression levels of functional, typical BMAL1 protein. This has been given recent support by a report that describes the daily accumulation of a 70 kDa antigen in mouse liver detected with the anti-BMAL1 antibody raised against full-length hBMAL1 (Ripperger et al., 2000).

It is surprising that BMAL1-IR remarkably decreased promptly after a nocturnal light pulse. It has been reported that *BMAL1* transcript does not change for up to ~1 hr after light pulse (Abe et al., 1998). This evidence strongly suggests that acute BMAL1 protein reduction might be caused by degradation. This implies a certain evolutionary conserved similarity to the fly photoentrainment process accompanying dTimeless degradation (Myers et al., 1996; Zeng et al., 1996; Naidoo et al., 1999). Degradation of dTimeless appears to be one of the pivotal events for the resetting of the fly clock. Thus, BMAL1 reduction (presumably degradation) is also a potential light-sensitive target of the mammalian clock. The effect of light on BMAL1 also provides a potential explanation for entrainment to a 24 hr light/dark cycle. It can account for phase-shifting light pulses having three different time-dependent effects. (1) Light delays the BMAL1 protein cycle at CT15 or ZT14 by causing a reduction of BMAL1. In the early night, reduction of BMAL1 after a light pulse was observed more clearly than in late night. Although BMAL1 protein increases steeply at the boundary from day to night, reduction of BMAL1 at the boundary from night to day seems to be relatively moderate (Figs. 2, 3). Because the difference in increasing and decreasing pattern of BMAL1 protein, we can assume that phase delay shift needs a larger level of BMAL1 protein reduction than phase advance shift. This assumption can explain the reason why light exposure at CT15 cause a larger BMAL1 protein reduction than at CT21. Recovery of BMAL1 content is probably attributable to the existence of substantial transcript levels at these times, so the reduced BMAL1 can be replaced. (2) In the late night, reduction of BMAL1 after a light pulse was observed modestly, probably because the slight circadian change in BMAL1 content during night/day boundary does not require much change to shift into a newly advanced phase. This notion is supported by the fact that *BMAL1* transcript is also decreasing at these times. (3) During subjective day, no significant reduction of BMAL1 protein is consistent with no evident effect of light on phase shifting at these times.

We cannot exclude the possibility that other components of the system influence light response. Acute induction of *Per1* and *Per2* transcripts in the SCN by photic stimulation is assumed to be mediated by transactivation of BMAL1/CLOCK heterodimer on their E-box elements or another unknown activating pathway downstream of hypothetical LRE. Induced *Per1* and *Per2* transcripts peak after 1–1.5 hr of light onset, then rapidly decrease (Albrecht et al., 1997; Shigeyoshi et al., 1997; Yan et al., 1999). Our data reveal that the BMAL1 content in the SCN substantially decreases within ~2 hr after a light pulse. Experiments using hamsters and mice demonstrate that resetting of the circadian oscillator occurs within ~2 hr, indicating that the molecular photoentrainment process in rodents may be completed at ~2 hr after photic stimulation (Best et al., 1999). This circumstantial evidence suggests the following hypothesis: during the photoentrainment, acute BMAL1 reduction temporally releases the major part of the E-box elements upstream of *Per* genes from the control of the CLOCK/BMAL1 heterodimer for circadian expression. Simultaneously, another unknown LRE-binding factor replaces the major part of the controller upstream of *Per* genes, enabling the LRE to induce *Per* genes acutely. Our findings may shed light on unknown modulating pathways potentially important in the phase resetting of the mammalian circadian clock.

The biochemical mechanism responsible for light-induced BMAL1 reduction (presumably degradation) is largely elusive. Glutamate is considered a major neurotransmitter mediating photoentrainment into the SCN (Ding et al., 1994). Glutamate causes phase shifts on the SCN slice and induces LRE-controlled immediate early genes, i.e., *c-Fos* and *mPer1* (Akiyama et al., 1999), probably via intracellular signaling mainly downstream of an NMDA receptor (Mintz et al., 1999). Our results showed that application of glutamate and NMDA, but not DHPG, on SCN slices directly reduced BMAL1 protein content in a manner similar to the photic reduction *in vivo*. The reduction in BMAL1 protein appears to occur via certain unknown signal transduction pathways

probably including activation of some protein kinases and proteases in the nucleus downstream of the ionotropic NMDA-gated calcium channel. Our findings strongly suggest the existence of novel modulating mechanisms, potentially the primary light-responsive targets before the elevation of LRE-evoked transcription during the resetting of the mammalian biological clock.

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