Supporting Information

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SI Materials and Methods

Animals. Animal experiments were conducted in accordance with the guidelines of the University of Tokyo. Newly hatched male chicks were purchased from a local supplier (Ohata Shaver) and maintained under 12-h light/12-h dark (LD) cycles for 7 d with the light provided by white fluorescent lamps (≈300 lx at the level of the heads of chicks). They were transferred to constant darkness thereafter. On day 8, they were exposed to a 1-h pulse of the 300-lx light from circadian time (CT) 2, CT6, CT10, CT14, CT18, or CT22. CT0 and CT12 correspond to the times of lights on and off, respectively, in the previous LD cycles. Control animals were kept in the dark during the irradiation period of 1 h. The pineal glands were isolated from the light-exposed and dark-kept (control) animals at CT3, CT7, CT11, CT15, CT19, or CT23 on day 8. All of the procedures during the dark period were performed under dim red light (>640 nm).

GeneChip and Quantitative RT-PCR Analyses. Total RNA was prepared from the isolated pineal glands by using TRIzol reagent (Invitrogen). Nine micrograms of total RNA from six pineal glands was used to synthesize biotinylated cRNA by using Affymetrix GeneChip one-cycle cDNA synthesis kit and IVT labeling kit (Affymetrix). Twenty micrograms of biotinylated cRNA was hybridized to GeneChip Chicken Genome Array (37,703 probe sets including >32,773 chicken genes) for 16 h at 45 °C. The arrays were washed and stained by using the Affymetrix Model 450 Fluidics Station and then scanned by the Affymetrix Model 3000 scanner according to the GeneChip Manual. The scanned array image was processed by GeneChip Operating Software Version 1.4 (Affymetrix) to calculate the signal intensity data for each probe set. Original data were deposited in Gene Expression Omnibus (GEO) (accession no. GSE21915). Quantitative RT-PCR analysis was performed as described (1) or by using QuantiTect SYBR Green PCR Kit (Qiagen) and GeneAmp 5700 (Applied Biosystems). The levels of spliced and total *Xbp1* (*Xbp1s* and *Xbp1t*, respectively) were analyzed by real-time PCR by modifying the method of Back et al. (2). The primers and optimal cycle numbers were summarized in Table S1.

Preparation of Protein Samples and Immunoblot Analysis. Thirty pineal glands and 0.5 mg of liver of 8-d-old male chicks were homogenized in 600 μL and 4.5 mL, respectively, of buffer I (10 mM Hepes-KOH at pH 7.6, 10 mM KCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 5 μg/mL pepstatinA, 2 μg/mL aprotinin, 10 μg/mL leupeptin, and 50 μg/mL *N*-acetylleucylleucylnorleucinal) by using a Dounce homogenizer, and the homogenate was centrifuged for 10 min at 1,000 × *g*. The resulting nuclear pellet was resuspended in 80 μL (for pineal gland) or 330 μL (for liver) of buffer II (20 mM Hepes-NaOH at pH 7.6, 0.5 M NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol, 5 μg/mL pepstatinA, 2 μg/mL aprotinin, 10 μg/mL leupeptin, and 50 μg/mL *N*-acetylleucylleucylnorleucinal), and the mixture was rotated for 60 min at 4 °C, followed by a centrifugation for 30 min at 22,000 × *g*. The supernatant was used as a nuclear extract. Protein concentration of each sample was measured by the Bradford method.

Proteins were separated by SDS/PAGE and transferred to a polyvinylidene difluoride membrane. The blot was incubated with a blocking solution [1% skim milk in TBS (50 mM Tris·HCl at pH 7.4, 200 mM NaCl, and 1 mM $MgCl_2$)] for 1 h at 37 °C and then incubated at 4 °C overnight with anti-SREBP-1 2A4 antibody (4 μg/mL; Santa Cruz Biotechnology), anti-cHSF1c, anti-HSF2-4, or anti-HSF3γ (3) (1:1,000 dilution). Immunoreactivities were visualized by enhanced chemiluminescence system (PerkinElmer Life Sciences) using a horseradish peroxidase-conjugated antibody against mouse or rabbit Ig (0.2 μg/mL; Kirkegaard & Perry Laboratories). The blot was reprobed with anti-TBP antibody (0.4 μg/mL; Santa Cruz Biotechnology).

Plasmid Construction. The coding regions corresponding to amino acids 1–461 of chicken SREBP-1 (GenBank accession no. NP_989457) and 1–454 of chicken SREBP-2 (CAC93938) were amplified by PCR from pineal cDNA and subcloned into pcDNA3.1-TOPO (Invitrogen) to yield expression plasmids nSREBP-1/pcDNA3.1 and nSREBP-2/pcDNA3.1, respectively. The nucleotide sequence corresponding to 5′ upstream region of chicken *E4bp4* gene was determined by direct sequencing of chicken liver genome. The determined sequence contained a region identical to the 5′ region of an EST clone isolated from the chicken liver (GenBank accession no. BG71112, nucleotides 7–199) that is highly similar to the first exon region of human *E4bp4* cDNA (NM_005384, nucleotides 1–223). The transcription initiation site (+1) was estimated from the sequence alignment between the EST clone and the genome sequence data. A DNA fragment corresponding to –2,033 to +101 of chicken *E4bp4* (deposited in GenBank; accession no. EF221611) was amplified by PCR from chicken genomic DNA and cloned into pGL3 basic vector (Promega) to yield *E4bp4*us2.0 reporter vector. Similarly, DNA fragments corresponding to –975 to +101, –517 to +101, –258 to +101, –207 to +101, –154 to +101, and –104 to +101 of *E4bp4* gene were cloned into pGL3 basic vector to create six kinds of reporter vectors termed *E4bp4*us1.0, *E4bp4*us0.5, *E4bp4*us0.25, *E4bp4*us0.2, *E4bp4*us0.15, and *E4bp4*us0.1, respectively. The reporter vectors containing human *HMG-CoA synthase* promoter and *Squalene synthase* promoter were described (4).

Transcriptional Assay. CHO-K1 cells were seeded on 24-well plates at a density of 1.6×10^5 cells per well in DMEM/Ham's F-12 supplemented with 10% FBS. After 24 h, the cells in each well were transfected by using Lipofectamine 2000 (Invitrogen) with various amounts of expression plasmid (total amount was adjusted to 200 ng by adding empty vector pcDNA3.1), 30 ng of firefly luciferase reporter plasmid, and 0.3 ng of *Renilla* luciferase reporter plasmid pRL-CMV (Promega) as an internal control. The cell lysates were prepared 48 h after the transfection and subjected to dual-luciferase assay by a luminometry (Promega). To investigate the effect of sterol application, the cells were seeded in the medium supplemented with 5% FBS. Four hours after transfection, the cells were washed with PBS, and the medium was changed to DMEM/Ham's F-12 supplemented with 5% lipoprotein-deficient serum, 1 μg/mL 25-hydroxycholesterol, and 10 μg/mL cholesterol.

Measurement of 7α-Hydroxypregnenolone Production by HPLC. The pineal glands, diencephalons, and adrenal glands were dissected from 7- to 8-d-old male chicks exposed to 1-h light pulse from CT14. Their homogenates (each 10 mg of wet weight tissue) were incubated at 40 °C in PBS containing 70 nM [7-^{3}H]pregnenolone [1×10^6 cpm (2×10^6 dpm)] and 0.24 mM NADPH. After incubation for indicated time periods, steroids were extracted by ethyl acetate and subjected to HPLC to analyze pregnenolone metabolites as described (5, 6).

Measurement of 7α-Hydroxypregnenolone Concentration by Gas Chromatography/Mass Spectrometry (GC-MS). The entrained male or female chicks were transferred to constant darkness on day 7, and they were exposed to a 20-min pulse of the 300-lx white fluorescent light from CT6, CT14, or CT22. Control animals were kept in the dark during the irradiation period of 20 min. The pineal glands were isolated from the light-exposed and dark-kept (control) animals and a pool (eight pineal glands in each well) were cultured in 450 μL of medium (Medium 199 supplemented with 10 mM Hepes-NaOH at pH 7.4, 100 U/mL penicillin, and 100 μg/mL streptomycin) in 6-well plates at 37 °C under 5% CO_2/80% O_2. After 3-h culture in the light or in the dark, steroids secreted into the medium were extracted by ethyl acetate and subjected to GC-MS analysis to measure 7α-hydroxypregnenolone concentrations as described (5, 6).

7α-Hydroxypregnenolone Administration and Behavioral Analysis. All surgery was performed under ketamine-xylazine anesthesia. Using a stereotaxic instrument, 5-d-old male chicks were chronically implanted with a 9-mm, 23 gauge steel guide cannula aimed at the lateral ventricle of the brain. Five days after the surgery, 7α-hydroxypregnenolone (0, 10, or 200 ng) dissolved in 10 μL PBS containing 0.2% DMSO was injected into the lateral ventricle via a 12-mm, 30 gauge stainless steel injector over a period of 30 s at Zeitgeber time (ZT) 14–16 (ZT0 and ZT12 correspond to the times of lights on and off, respectively, in the LD cycles) under dim red light. After the injection, chicks were placed individually in an open field apparatus (O'Hara & Co.) for locomotor activity measurement for 20 min under infrared light. The obtained data were analyzed by using Image OF1 software (O'Hara & Co.).

Measurement of Light Response of the Behavior. The entrained 8- to 12-d-old male chicks were transferred to constant darkness. On the next day, they were placed individually in an open field apparatus for locomotor activity measurement for 10 min under infrared light at CT6–8, CT14–16, or CT22–24. Immediately after the measurement, they were exposed to ≈300-lx white fluorescent light for 10 min and then placed individually in an open field apparatus for locomotor activity measurement for 10 min under white LED light (≈300 lx). Pinealectomy and sham operation were performed as described (7) with slight modification for 4-d-old male chicks under isoflurane anesthesia. After the surgery, they were further entrained for 15 d, and their locomotor activities were measured at CT14–16.

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2. Back SH, Schröder M, Lee K, Zhang K, Kaufman RJ (2005) ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. *Methods* 35:395–416.
3. Fujimoto M, Nakai A (2010) The heat shock factor family and adaptation to proteotoxic stress. *FEBS J* 277:4112–4125.
4. Inoue J, Sato R, Maeda M (1998) Multiple DNA elements for sterol regulatory element-binding protein and NF-Y are responsible for sterol-regulated transcription of the genes for human 3-hydroxy-3-methylglutaryl coenzyme A synthase and squalene synthase. *J Biochem* 123:1191–1198.
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6. Tsutsui K, et al. (2008) 7α-Hydroxypregnenolone mediates melatonin action underlying diurnal locomotor rhythms. *J Neurosci* 28:2158–2167.
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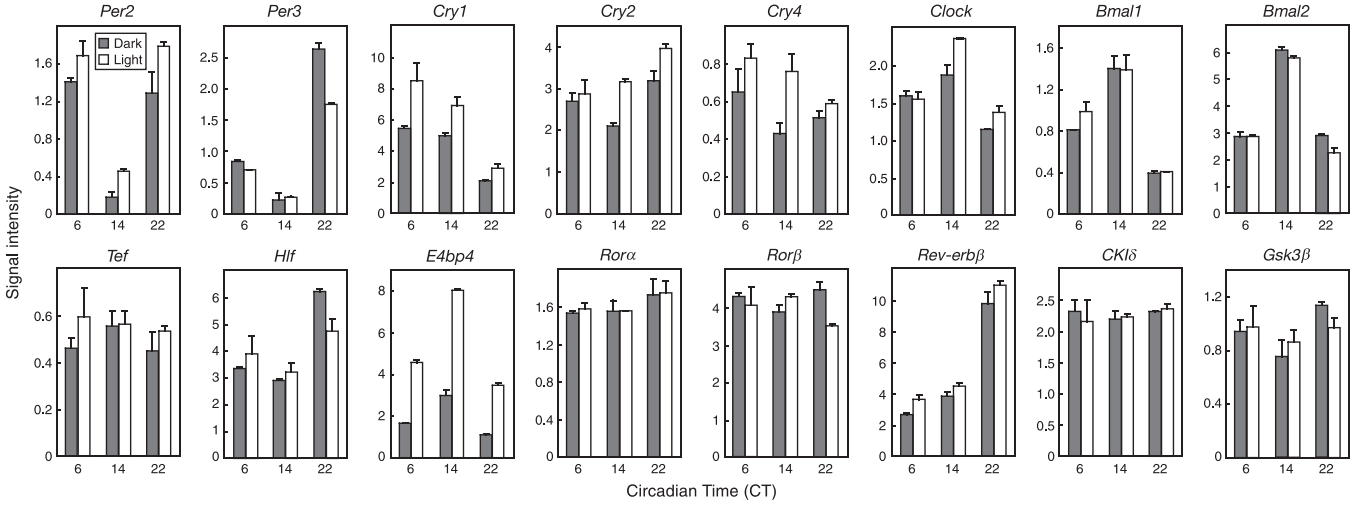

Fig. S1. Expression profiles of the clock genes in the pineal gland. Dark-kept animals were exposed to a 1-h light pulse (open bars) from CT6, CT14, or CT22, or kept in the dark as a control (solid bars). The GeneChip profiles of the clock genes are displayed (mean with variation, $n = 2$). The gene expression analysis program indicated that the signals for *Npas2* and *CK1ε* were "Absent", because their signals were too low and/or unreliable. No probe set was assigned to *Per1*, *Dbp*, *Rorγ*, and *Rev-erbα*.

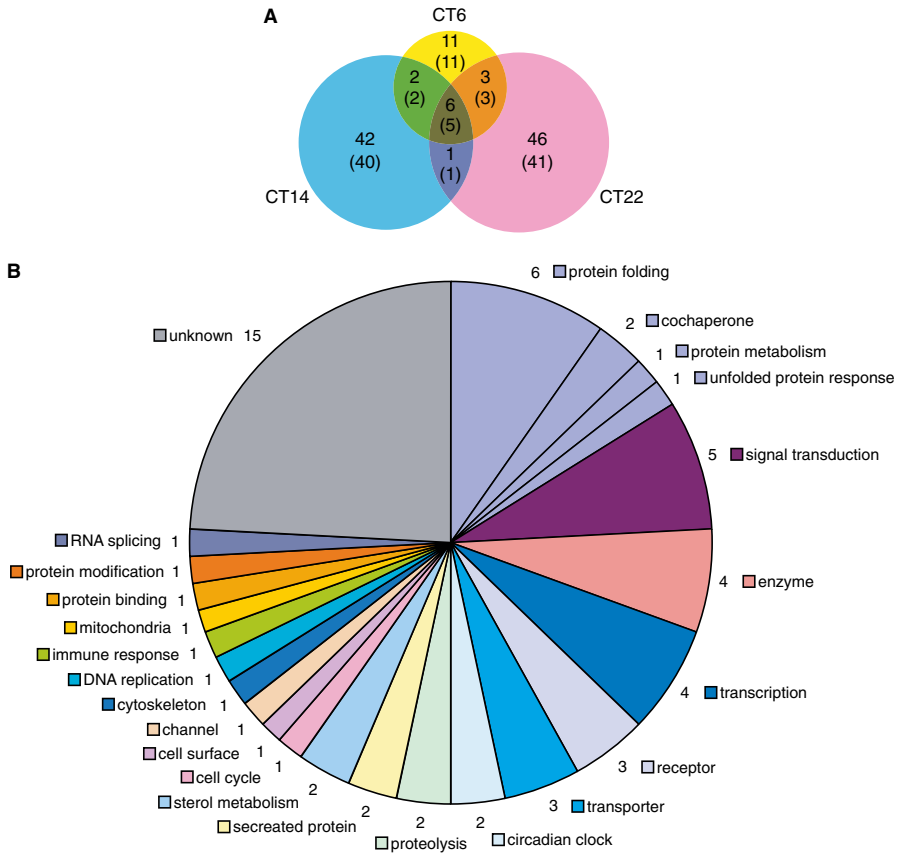

Fig. S2. Time-of-day dependence and functional classification of light-induced genes in the pineal gland. (*A*) Venn diagram showing time-of-day dependence of the light induction. One hundred eleven probe sets (corresponding to 62 genes and 40 ESTs) exhibited >2.5-fold increase of their signal intensities by the light pulse in at least a single time point. Shown are the numbers of probe sets or corresponding genes (in parentheses) induced at each time point. Two probe sets against the gene for heat shock protein 105 kDa were included in the list, and each of them belonged to different category (CT22 group and all time group). (*B*) Functional classification of the 62 light-induced genes.

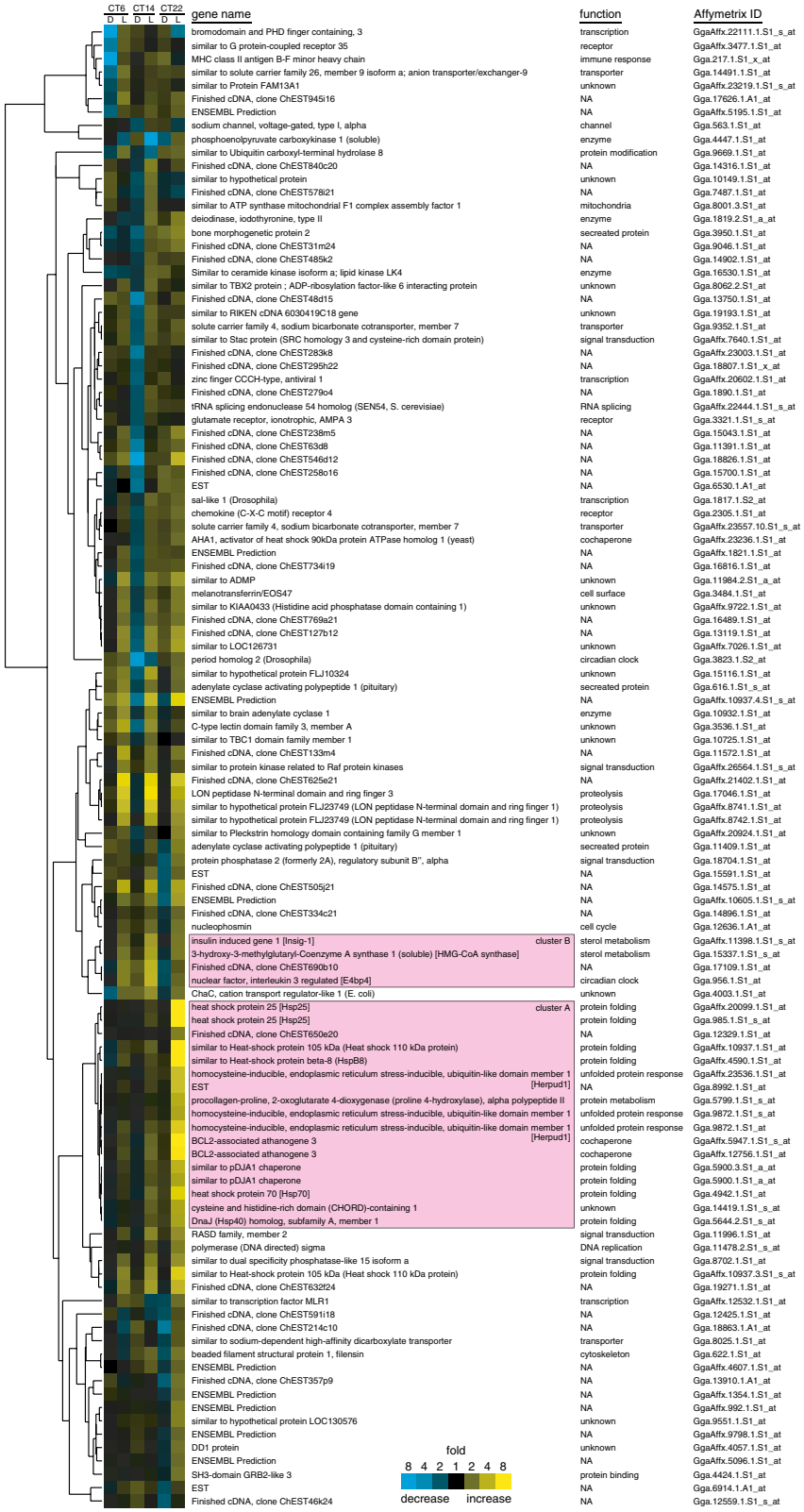

Fig. S3. Light-induced genes in the chicken pineal gland. See Fig. 1 legend for details. Gene clusters containing stress–response genes (cluster A), and *E4bp4* (cluster B) are highlighted by pink.

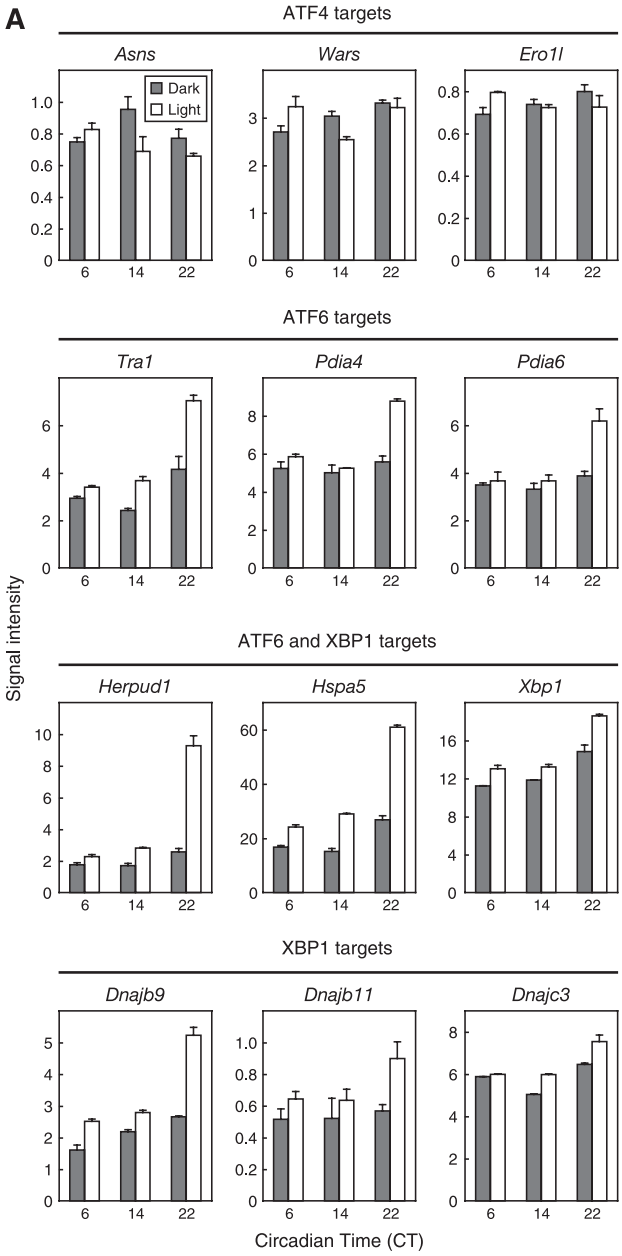

Fig. S4. (Continued)

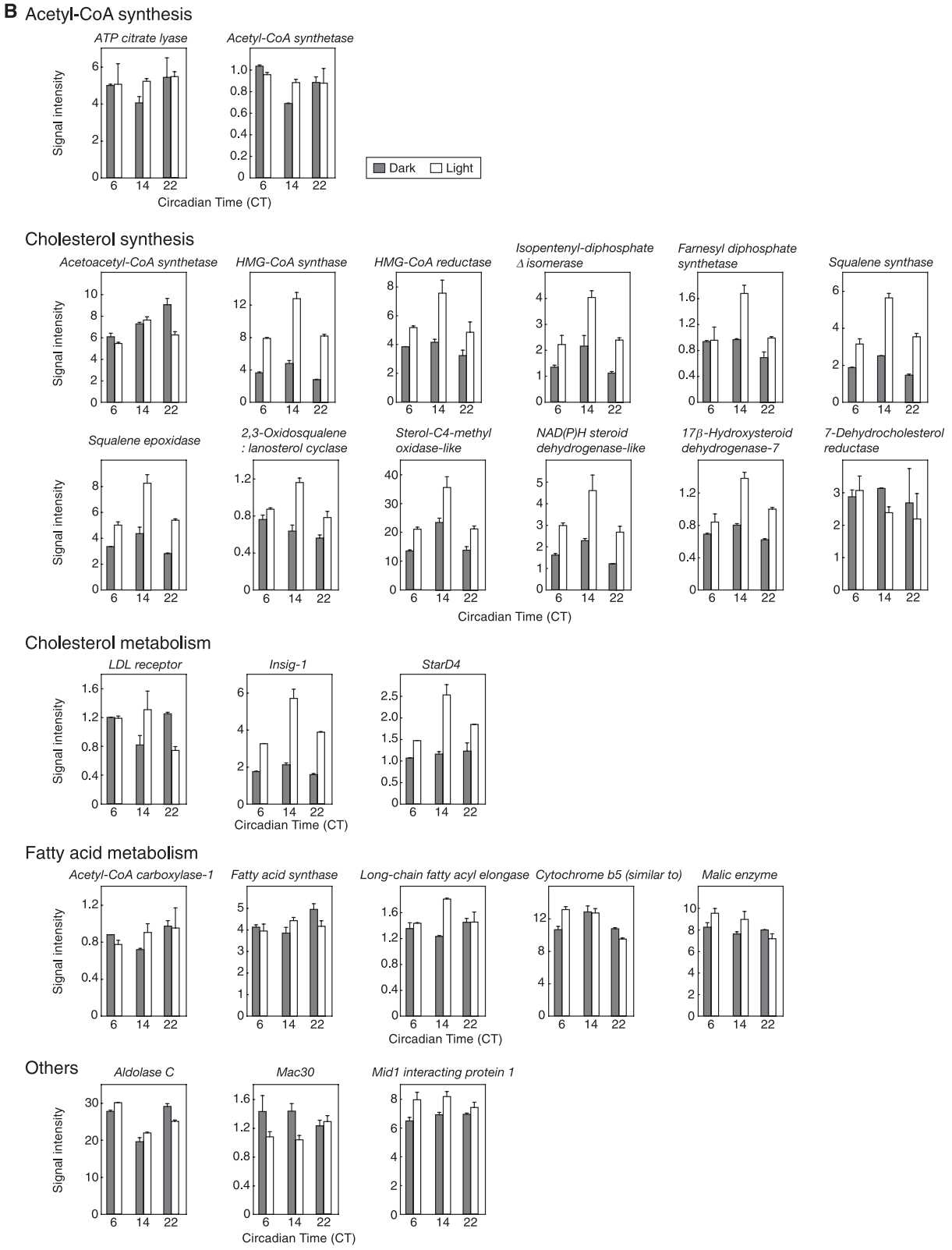

Fig. S4. Expression profiles of ATF4-, ATF6-, XBP1-, and SREBP-target genes in the pineal gland. (*A*) ATF4-, ATF6-, and XBP1-target genes. (*B*) SREBP-target genes. Dark-kept animals were exposed to the 1-h light pulse (open bars) from CT6, CT14, or CT22, or kept in the dark as a control (solid bars). The GeneChip profiles of the genes listed in Figs. 2*C* and 3*B* are displayed (mean with variation, $n = 2$).

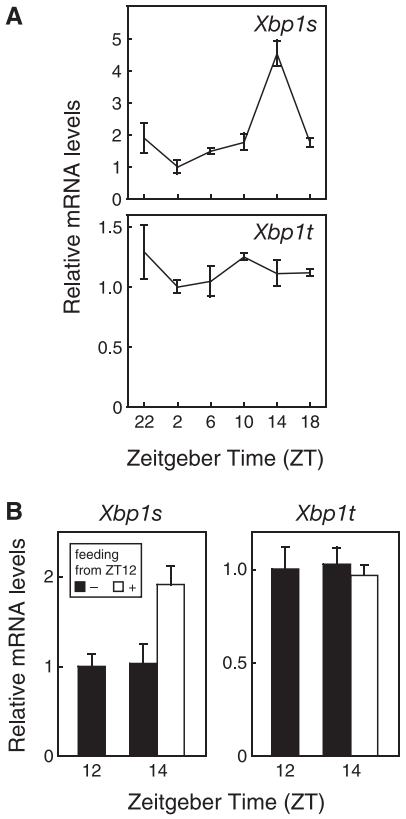

Fig. S5. Daily expression profile and feeding response of spliced *Xbp1* mRNA levels. (*A*) Daily expression profile. Mice were housed in 12-h light/12-h dark cycles with foods and water freely available, and the liver was isolated at the indicated time point. (*B*) Feeding response. Mice were housed in 12-h light/12-h dark cycles with water freely available and foods available only at nighttime (ZT12–24) for a week. On day 7, foods were provided (open bars) or not (solid bars) from ZT12, and the liver was isolated at the indicated time point. Relative mRNA levels of *Xbp1s* and *Xbp1t* were determined by RT-PCR analysis. The lowest value (*A*) or the value at ZT12 (*B*) was set to 1. Data are the mean ± SEM (n = 3).

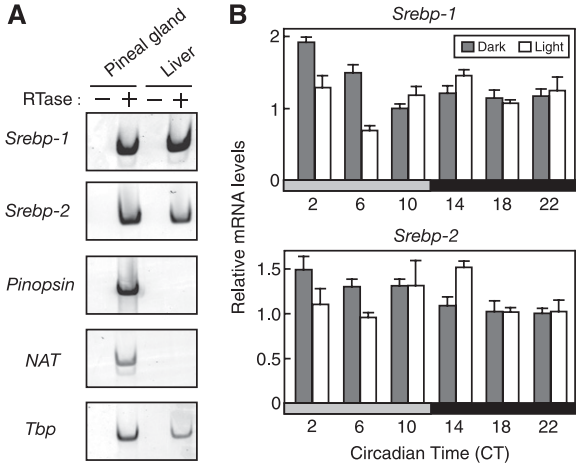

Fig. S6. Light-response of *Srebp-1* and *Srebp-2* genes in the pineal gland. (*A*) Expression of *Srebp-1* and *Srebp-2* in the pineal gland and liver. Total RNA extracted from the tissues were subjected to reverse transcription with (+) or without (–) reverse transcriptase (RTase), followed by PCR amplification of *Srebp-1*, *Srebp-2*, *Pinopsin*, *NAT*, and *Tbp*. *Pinopsin* and *NAT* were expressed in the pineal gland. (*B*) Effect of light on the mRNA levels of *Srebp-1* and *Srebp-2*. Dark-kept animals were exposed to a 1-h light pulse (open bars) or kept in the dark for 1 h (solid bars) at each time point, and the pineal glands were isolated for RT-PCR analysis as in Fig. 2*A*.

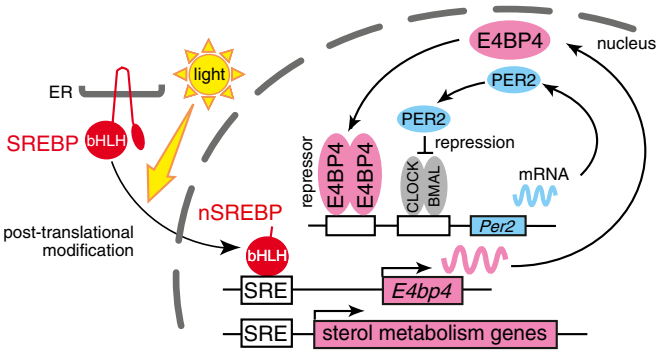

Fig. S7. A model for the role of SREBP in the light-input pathway of the pineal circadian clock. See text for details.

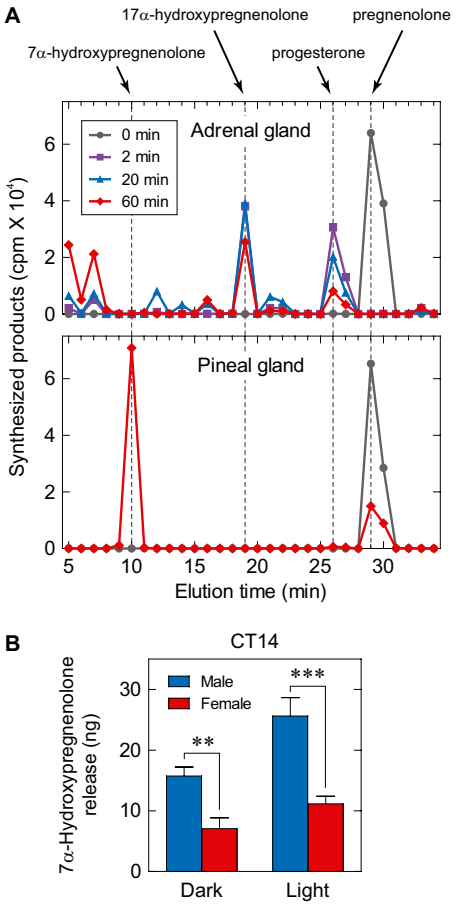

Fig. S8. Steroid production in the adrenal gland and 7α-hydroxypregnenolone release from the female pineal gland. (*A*) Production of steroids from pregnenolone in adrenal gland and pineal gland lysates. The homogenate from the adrenal gland or the pineal gland were incubated with [^{3}H]pregnenolone for indicated time periods, and each extract was subjected to the reversed-phase HPLC. The elution positions of metabolites are indicated by vertical broken lines. (*B*) Comparison of 7α-hydroxypregnenolone release from the pineal glands between male and female chicks. The chicks were exposed to 20-min light pulse (≈300 lx with white fluorescent lamps) from CT14, and then the pineal glands were isolated and cultured in 450 μL of medium as a pool (8 pineal glands per well) for 3 h under the light condition. For dark control group, all procedures were performed in the dark. The amounts of 7α-hydroxypregnenolone released into the medium were measured by gas chromatography/mass spectrometric (GC-MS) analysis. Data are the mean ± SEM (n = 4–5). $^{**}P < 0.01$ and $^{***}P < 0.005$ by Student's t test. Data for the male sample are reproduced from Fig. 5*E*.

Table S1. Primers and cycle numbers for PCR analyses

Gene	Forward primer sequence (5′->3′)	Reverse primer sequence (5′->3′)	PCR cycle numbers
Per2	GGAAGTCCTTGCAGTGCATAC	ACAGGAAGCGGATATGCAG	24
E4bp4	CCTTTCTCAGTTCAGGTGAC	TGAAATGACATCATGAGTCCAG	21
StarD4	TACCACAGCATCGCCGACAG	TCCTTAAGAGGGACGCAGAACC	24
Insig-1	TGCCCAAAGGAAGTAGATCG	AAAGCCCTGACTCAAACAGG	20
Srebp-1	GTACCTTCAGCTGCTCAACG	TCCTGCTTGCTCAACATGG	26
Srebp-2	CTAAGCAGTCTGGTTGACAACG	AGAGGCACAGGAATGTCAGG	24
Pinopsin	CTGAAGGGTTGAGGACATCG	CTGCAGTGACATCTGCATGG	24
NAT	ATGAGATCCGCCACTTCCTAAC	AATCCTCGCACATGAGCACG	21
StAR	AATCACTCAGCATCCTCGG	GGACCTGGTTGATGATGGTC	27
P450scc	TGCAGGTTGGTCTCTACGC	CTCCAGGATGTGCATGAGG	24
P4507α	ATGAACATTCGCATCAGCC	TCATCTCATTCATTGCGAGG	26
Tbp	GTCGAATATAATCCCAAGCG	TCTGCTCGAACTTTAGCACC	24
Hsp25	CCAAGGATGGAGCTGTCAGC	CGATGCAGACCGTTGTTCC	*
Hsp70	ACCGAAACCAGATGGCAGAG	TTGTGACAATCGGGTTGCAG	*
Herpud1	ACGTCGGTGAGCTTGAGTCC	CAGTGAAGGCCAGAGAAATGC	*
Hspa5	ATCAGAATCGGCTAACACCAGAG	TCCTCAGCAAACTTCTCAGCATC	*
Xbp1s	GAGTCCGCAGCAGGTG	ACTGCCATCAGAATCCATG	*
Xbp1t	AGTGCGAGTCTACGGATGTGAAG	CCGGTCACCAACCTGATGTC	*
Tbp	AGCAAGGAAGTACGCAAGAGTTG	AGCTGCCCACCATGTTCTG	*
Mouse *Xbp1s*	GAGTCCGCAGCAGGTG	GTGTCAGAGTCCATGGGA	*
Mouse *Xbp1t*	AAGAACACGCTTGGGAATGG	ACTCCCCTTGGCCTCCAC	*
Mouse *Gapdh*	TGCACCACCAACTGCTTAGC	ACAGTCTTCTGGGTGGCAGTG	*

*Real-time PCR.