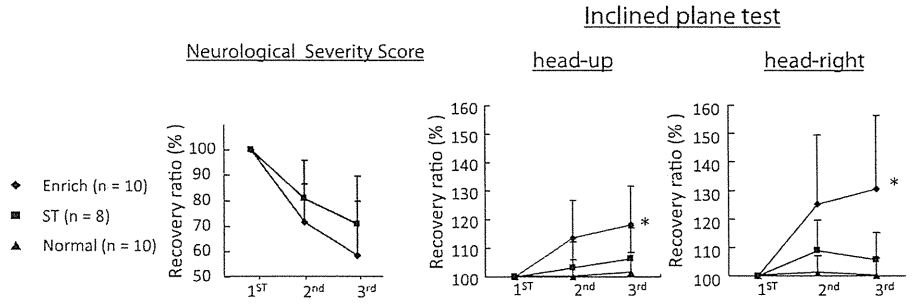

Fig. 2 – Changes of recovery after transient focal ischemia. There were no significant differences in the neurological severity score at 2 (the 2nd evaluation) and 4 weeks (the 3rd evaluation) after transient focal ischemia between the enriched and standard groups. To evaluate motor function, the inclined plane test was performed with the head in 2 different orientations: head-up and head-right. The rats housed in the enriched environment showed significantly better recovery compared with those in standard cages at 4 weeks (the 3rd evaluation) after transient focal ischemia. *$p < 0.05$; Enrich, enriched group; ST, standard group; Normal, normal group; 1st, the 1st evaluation; 2nd, the 2nd evaluation; and 3rd, the 3rd evaluation.

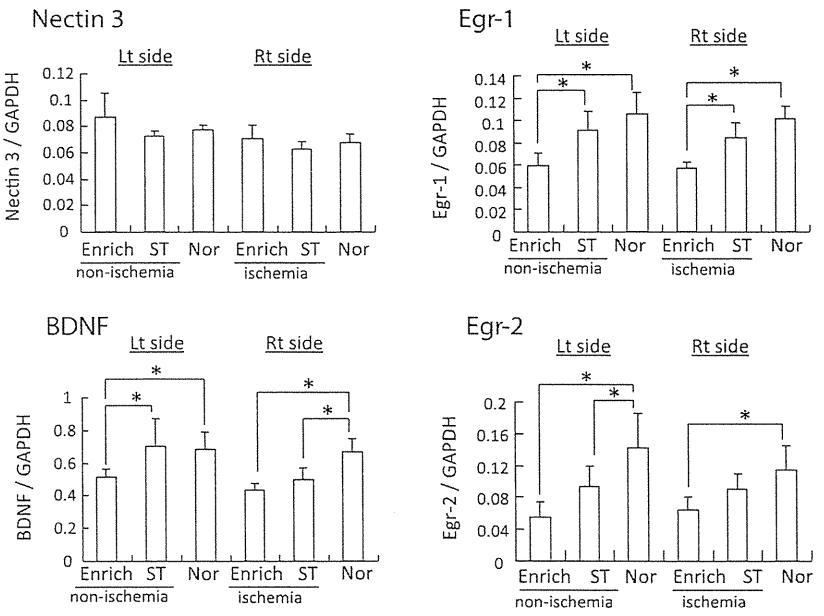

Fig. 3 – Comparison of gene expression in the whole brain among the 3 groups: ischemic rats in the enriched environment or standard cages, and non-ischemic rats. There were no significant differences in the mRNA expression of the four genes between the right and left sides. There were also no significant differences in nectin-3 mRNA expression for any condition. Egr-1 mRNA on both sides and BDNF mRNA on the left side levels were significantly decreased in the enriched group compared with the standard or normal groups. BDNF mRNA levels on the right side and Egr-2 mRNA levels on the left side were significantly decreased in the enriched and standard groups compared with the normal group. Egr-2 mRNA levels on the right side were significantly decreased in the enriched group compared with the normal group. *$p < 0.05$; Enrich, enriched group; ST, standard group; Nor, normal group; Lt, left; and Rt, right.

4. Conclusion

5. Experimental procedures

5.1. Animals and surgical procedures

5.2. Housing conditions and neurological evaluations

Table 1 – Results of the first neurological evaluation.

* p
** p

5.3. Brain preparation and histological examinations

5.4. Gene analysis

5.5. Real-time quantitative reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

Table 2 – Oligonucleotide primers used in real-time quantitative RT-PCR.

N

5.6. Data analysis

t p

Acknowledgments

Light-dependent and circadian clock-regulated activation of sterol regulatory element-binding protein, X-box-binding protein 1, and heat shock factor pathways

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The circadian clock is phase-delayed or -advanced by light when given at early or late subjective night, respectively. Despite the importance of the time-of-day–dependent phase responses to light, the underlying molecular mechanism is poorly understood. Here, we performed a comprehensive analysis of light-inducible genes in the chicken pineal gland, which consists of light-sensitive clock cells representing a prototype of the clock system. Light stimulated expression of 62 genes and 40 ESTs by >2.5-fold, among which genes responsive to the heat shock and endoplasmic reticulum stress as well as their regulatory transcription factors heat shock factor (HSF)1, HSF2, and X-box-binding protein 1 (XBP1) were strongly activated when a light pulse was given at late subjective night. In contrast, the light pulse at early subjective night caused prominent induction of *E4bp4*, a key regulator in the phase-delaying mechanism of the pineal clock, along with activation of a large group of cholesterol biosynthetic genes that are targets of sterol regulatory element-binding protein (SREBP) transcription factor. We found that the light pulse stimulated proteolytic formation of active SREBP-1 that, in turn, transactivated *E4bp4* expression, linking SREBP with the light-input pathway of the pineal clock. As an output of light activation of cholesterol biosynthetic genes, we found light-stimulated pineal production of a neurosteroid, 7α-hydroxypregnenolone, demonstrating a unique endocrine function of the pineal gland. Intracerebroventricular injection of 7α-hydroxypregnenolone activated locomotor activities of chicks. Our study on the genome-wide gene expression analysis revealed time-of-day–dependent light activation of signaling pathways and provided molecular connection between gene expression and behavior through neurosteroid release from the pineal gland.

The oscillatory mechanism of the circadian clock is constituted of clock genes and the encoded proteins to generate physiological and behavioral rhythms with daily periodicity (1–3). The intrinsic period length of the circadian clock deviates from 24 h, thus clocks are entrained to (reset by) the environmental changes, among which light is the most common and the strongest time cue. A light stimulus given at early or late subjective night induces phase delay or phase advance, respectively, whereas the light during the subjective daytime has little effect on the clock (4). This time-of-day–dependent response is conserved among species, but the underlying molecular mechanism remains to be elucidated.

Among vertebrate clock-containing cells, the chicken pinealocyte is unique in that it retains intrinsic phototransduction pathways for entrainment of the clock (5, 6). Hence, the chicken pineal clock cell provides a prominent platform for studies on the light-entrainment mechanism. We performed previously a differential display analysis of light-inducible genes in the chicken pineal gland and identified a limited number of transcripts that are induced by light selectively at either early or late subjective night (7). Notably, light at early subjective night caused remarkable induction of a transcript encoding a bZIP transcription factor E4BP4 that acts as a light-dependent suppressor of *Per2*, particularly in the phase-delaying process (8).

To identify transcription pathways that are light-activated at a particular time of day, we investigated light responses of the chicken pineal transcriptome by high-density oligonucleotide array (GeneChip) analysis. Our study revealed time-of-day–dependent activation of pathways mediated by sterol regulatory element-binding protein (SREBP), X-box-binding protein 1 (XBP1), and heat shock factor (HSF) transcription factors. SREBP activated the transcription of *E4bp4*, raising SREBP as a light-responsive immediate regulator of the clock phase. On the basis of the light activation of SREBP and the target genes for cholesterol biosynthesis, we found a substantial activity of the pineal gland to synthesize a neurosteroid, 7α-hydroxypregnenolone, that activates locomotor activities of chicks, connecting light-induced gene expression with behaviors.

Results

Light Pulse Induces a Subset of Pineal Genes in a Time-of-Day–Dependent Manner. To pursue time-of-day–dependent light responses of the pineal transcriptome, 12-h light/12-h dark cycle-entrained male chicks were transferred to constant darkness and exposed to light for 1 h from circadian time (CT) 6 (representing subjective day), CT14 (early subjective night), or CT22 (late subjective night). The pineal glands were isolated just after the 1-h light pulse or alternative 1-h dark period (as a control) and subjected to gene expression analysis by Affymetrix GeneChip containing 37,700 probe sets. The data from the dark-kept animals revealed circadian expression of the clock genes *Per*, *Cry*, *Clock*, and *Bmal* (Fig. S1, gray bars) with temporal profiles that are similar to those reported (9–11). Out of ≈21,000 probe sets that detected significant gene expression in the pineal gland, 111 probe sets (corresponding to 62 genes and 40 ESTs) exhibited >2.5-fold increase of the signal intensities when the 1-h light pulse was given at any of the three time points (Fig. S2*A*). Noticeably, most (89%) of the light-activated genes were strongly

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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE21915) and in the GenBank database, www.ncbi.nlm.nih.gov/nuccore (accession no. EF221611).

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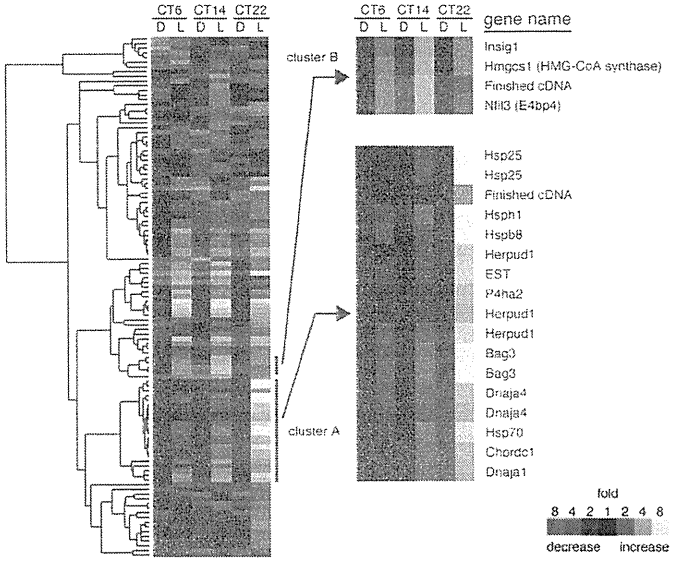

Fig. 1. Light-induced genes in the chicken pineal gland. Dark-kept animals were exposed to a 1-h light pulse (L) from CT6, CT14, or CT22, or kept in the dark (D). The pineal glands were isolated at CT7, CT15, or CT23 for GeneChip analysis (n = 2). (*Left*) Light-induced genes detected by 111 probe sets (>2.5-fold induction in at least a single time point) were subjected to the hierarchical clustering analysis. Signal intensities obtained from two independent samples were averaged for each probe set at each time point, and the average value of dark samples was set to 1. Genes clustered with stress–response genes (cluster A) and those with *E4bp4* (cluster B) are shown at *Right*.

induced only at a single time point, indicating strict circadian gating of the light induction.

Light Pulse at Late Subjective Night Induces Stress–Response Genes. The light-responsive genes detected by the 111 probe sets were first analyzed by a functional classification. Among a variety of functional groups, one related to protein folding formed a predominant group (Fig. S2*B*). Next, a hierarchical clustering analysis based on the light–response profiles (Fig. 1 and Fig. S3) revealed that most of the protein folding-related genes belonged to a single cluster showing prominent light induction almost specifically at CT22 (Fig. 1 and Fig. S3, cluster A). These genes are known to respond to either heat shock or endoplasmic reticulum (ER) stress (12–14), suggesting that these two pathways are activated specifically at late subjective night. We further explored circadian profiles of the light responses of heat shock–response genes (*Hsp25* and *Hsp70*) and ER stress–response genes (*Herpud1* and *Hspa5*) at 4-h intervals by reverse transcription (RT)-PCR analysis. The light induction of these genes was highly circadian-regulated with the largest response at CT22, whereas their basal expression profiles were less rhythmic in constant darkness (Fig. 2*A*). Heat shock–response genes are transcriptionally regulated mainly by a family of heat shock factor (HSF) transcription factors, and the activity of HSF is regulated posttranslationally by nuclear localization, oligomerization, and/or phosphorylation (13). We analyzed nuclear levels of HSF1–3 and found that HSF1 and HSF2 protein levels were up-regulated by the 1-h light pulse at CT22, whereas HSF3 level was almost unchanged (Fig. 2*B*). HSF1 and HSF2 are most likely the key regulators of the light response of the heat shock–response genes (*Discussion*). As for ER stress, it stimulates gene expression by activating three members of bZip transcription factors: ATF4, ATF6, and XBP1 (14). We found that ATF6- and XBP1-target genes but not ATF4-targets were prominently induced by light at CT22 (Fig. 2*C* and Fig. S4*A*). Activity of XBP1 is known to be regulated by frame-switch splicing, which produces a spliced form of *Xbp1* mRNA (termed *Xbp1s*) encoding active XBP1 (14). We found that the formation of pineal *Xbp1s* showed a striking peak of light response at CT22. In contrast, the total amount of spliced and unspliced forms of *Xbp1* (termed *Xbp1t*) showed no significant light response over the day (Fig. 2*D*). These results revealed light-dependent activation of HSF1, HSF2, and XBP1 pathways at late subjective night. In the mouse liver, HSF1 is implicated in the feeding-dependent regulation of rhythmic gene expression (15). Here, we found a robust daily rhythm of mouse liver *Xbp1s* mRNA level in ad libitum feeding condition (Fig. S5*A*) and the peak in the nighttime was attributed to food intake (Fig. S5*B*), whereas a recent report showed a low amplitude rhythm of *Xbp1s* during starvation (16). Because feeding is one of the most important time cues for entrainment

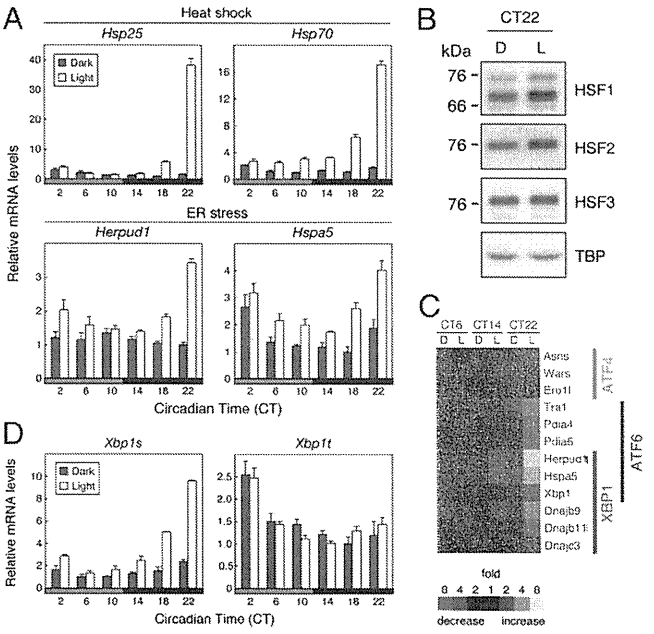

Fig. 2. Induction of stress–response genes by a light pulse at late subjective night. (*A* and *D*) Effect of light on the mRNA levels. Dark-kept animals were exposed to a 1-h light pulse (open bars) or kept in the dark for 1 h (solid bars) from each time point, and the pineal glands were isolated for RT-PCR analysis. The intensity of each amplified product was normalized to that of *Tbp*. The lowest value of the dark-kept animals was set to 1. Data are the mean ± SEM (n = 4). (*B*) Effect of light on nuclear HSF1, HSF2, and HSF3 levels. Dark-kept animals were exposed to a 1-h light pulse (L) from CT22, or kept in the dark (D), and then the pineal glands were isolated at CT23. Nuclear extracts (70 μg of protein for each lane) were subjected to immunoblot analysis. Data are the representatives of two independent experiments. (C) Light–response of ATF4, ATF6, and XBP1–target genes. ATF4, ATF6, and XBP1–target genes commonly identified in previous reports (36–39) were selected, and their expression profiles in the pineal GeneChip analysis are indicated as in Fig. 1. No probe set was assigned to *Chop* gene, which is a target of both ATF4 and ATF6.

of the liver clock (17), it is possible that HSF1 and XBP1 act as common regulators of rhythmic gene expression between the chick pineal gland and the mouse liver when the clock cells respond to resetting signals (*Discussion*).

SREBP-Target Genes and *E4bp4* Display Similar Light–Response Patterns. Among the clock genes, *E4bp4* was highly light-responsive in the pineal gland (Fig. S1). The hierarchical clustering analysis demonstrated that *E4bp4* formed a cluster with *Insig-1* and *HMG-CoA synthase* genes, all of which showed prominent light induction at CT14 (Fig. 1 and Fig. S3, cluster B). By applying our previous differential display analysis of the pineal genes (7), we also found that *E4bp4*, *StarD4*, and *Insig-1* transcripts were up-regulated by light at early subjective night (Fig. 3*A*, *Left*). In the mouse liver, Horton et al. (18) identified *StarD4*, *Insig-1*, and *HMG-CoA synthase* as the target genes of SREBP, a transcription factor activating expression of an array of genes responsible for synthesis and uptake of cholesterol and fatty acids in the liver (19). Giving attention to the SREBP-target genes, we found that genes participating in the cholesterol biosynthesis and metabolism were remarkably induced by the light pulse at CT14 (Fig. 3*B* and Fig. S4*B*). In contrast, another subset of SREBP-target genes responsible for synthesis of acetyl-CoA and fatty acids were almost unaffected by the light pulse given at any time point (Fig. 3*B* and Fig. S4*B*; see *Discussion*). Detailed analysis of circadian profiles of the light response of *StarD4* and *Insig-1* revealed that the transcript levels were markedly up-regulated by the light pulse given at around the day-night transition (CT10 and CT14), a temporal pattern that is very similar to that observed for *E4bp4* induction (Fig. 3*C*). These results suggest a common regulatory mechanism underlying the light induction of *E4bp4* and cholesterol-related genes through SREBP-mediated process.

Light Pulse at Early Subjective Night Activates SREBP. SREBP, a member of bHLH-Zip transcription factors, is encoded by two genes in vertebrates, *Srebp-1* and *Srebp-2*, each of which produces ≈120 kDa inactive precursor bound to ER membrane. The N-terminal bHLH domain of SREBP is proteolytically released from the membrane as a 68-kDa form, which translocates to the nucleus where it acts as an active transcription factor designated nSREBP (19). RT-PCR analysis revealed that *Srebp-1* and *Srebp-2* are expressed in the chicken pineal gland at levels comparable to those in the liver (Fig. S6*A*). Among available antibodies, anti-SREBP-1 (2A4) antibody reactive to mouse liver nSREBP-1 (Fig. 3*D*, lane 1) detected not only chicken nSREBP-1 expressed in CHO-K1 cells (Fig. 3*D*, lane 4) but also endogenous nSREBP-1 in the nuclear extract of the chicken liver (Fig. 3*D*, lane 6) with no detectable cross-reactivities to human/chicken nSREBP-2 (Fig. 3*D*, lanes 2 and 5). By using this antibody, we found that the 1-h light pulse at CT14 caused remarkable accumulation of proteolytically activated ≈68 kDa nSREBP-1 in the pineal cell nuclei (Fig. 3*E*). These results demonstrate light-induced posttranslational activation of SREBP-1, whereas RT-PCR analysis revealed marginal effects of light on the pineal transcript levels of *Srebp-1* and *Srebp-2* (Fig. S6*B*).

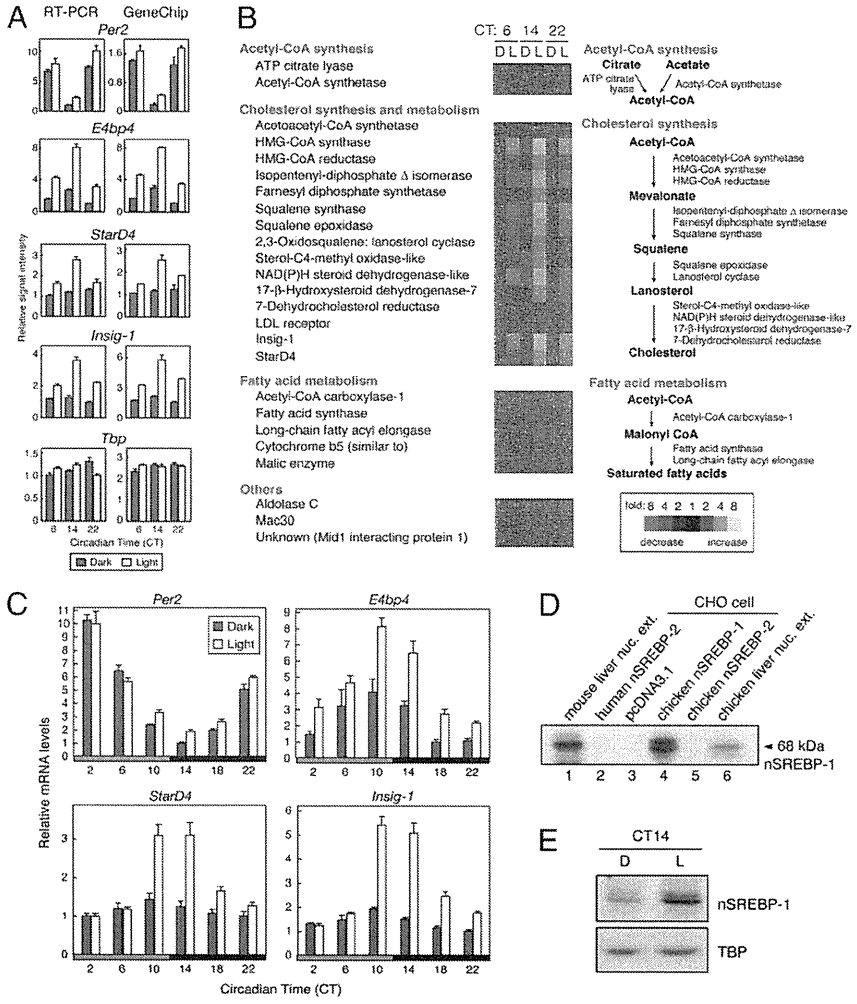

Fig. 3. Activation of SREBP pathway by a light pulse at early subjective night. (*A*) Time-of-day–dependent light-induction of *E4bp4*, *StarD4*, and *Insig-1* genes. Dark-kept animals were exposed to a 1-h light pulse (open bars) or kept in the dark as a control (solid bars) from each time point, and the pineal glands were isolated for RT-PCR and GeneChip analyses. Data are the mean ± SEM (RT-PCR; $n = 3$) or the mean with variation (GeneChip; $n = 2$). (*B*) Light responses of SREBP–target genes in the pineal gland. Expression profiles of SREBP–target genes reported by Horton et al. (18) were extracted from the pineal GeneChip analysis result and indicated as in Fig. 1. The gene expression analysis program indicated that the signals for *CYP51*, *Mevalonate pyrophosphate decarboxylase*, *Narc-1*, and *Srebp-1* were "Absent". No probe set was assigned to *Phosphomevalonate kinase*, *Sterol-C5-desaturase*, *Sterol C14-reductase-like*, and *SCALD*. (*C*) Effect of light on the mRNA levels. RT-PCR analysis was performed as in Fig. 2*A*. (*D*) Detection of chicken nSREBP-1 by immunoblot analysis. Liver nuclear extracts of mouse (lane 1) or chicken (lane 6) and lysates of CHO-K1 cells transfected with nSREBP-expression vector or empty vector (lanes 2–5) were subjected to immunoblot analysis with anti-SREBP-1 (2A4) antibody. None of available antibodies recognized chicken nSREBP-2 and, hence, we were unable to examine light activation of this protein. (*E*) Effect of light on nSREBP-1 level in the pineal gland. Dark-kept animals were exposed to a 1-h light pulse (L) from CT14 or kept in the dark (D), and then the pineal glands were isolated at CT15. Nuclear extracts were subjected to immunoblot analysis. Data are the representatives of three independent experiments.

SREBP Activates Expression of *E4bp4*. The effect of the active SREBP (nSREBP) on *E4bp4* promoter was explored by transcriptional assay in CHO cells. Transcription from a 2.0-kb promoter region of chicken *E4bp4* (*E4bp4*us2.0; Fig. 4*A*; deposited in GenBank, accession no. EF221611) was activated by nSREBP-1 and to a lesser extent by nSREBP-2 (Fig. 4*B*). Similar activation profiles were observed even with the shorter promoter regions, us1.0 and us0.5 (Fig. 4 *A* and *B*). In support of the idea that SREBP regulates *E4bp4* expression, the activity of the *E4bp4*us0.5 reporter was inhibited by treatment of the cells with a 1:10 mixture of 25-hydroxycholesterol and cholesterol (Fig. 4*C*), which is known to inhibit SREBP activation (19) as evidenced here by their inhibitory action on *HMG-CoA synthase* promoter activity (Fig. 4*C*). We found that the *E4bp4*us0.5 reporter contained multiple copies of potential SRE (sterol regulatory element) sequence (Fig. 4*D*, shaded boxes), a binding site of nSREBP (20). The nSREBP-1–mediated activation was largely reduced when the *E4bp4* promoter was further shortened from us0.2 to us0.15 and us0.1 (Fig. 4 *A* and *E*), in which one and two SRE sequences were eliminated, respectively (Fig. 4*D*). nSREBP-1 activated the transcription of *E4bp4*us0.5 reporter more potently than nSREBP-2 (Fig. 4*F*), contrasting with comparable activation by nSREBP-1 and nSREBP-2 on the promoter of *HMG-CoA synthase* or *Squalene synthase* (21, 22) (Fig. 4*E*). We conclude that SREBP-1 responds to light at early subjective night to operate as a transcriptional activator of *E4bp4*, a gene important for the photic-input pathway to the circadian clock (Fig. S7).

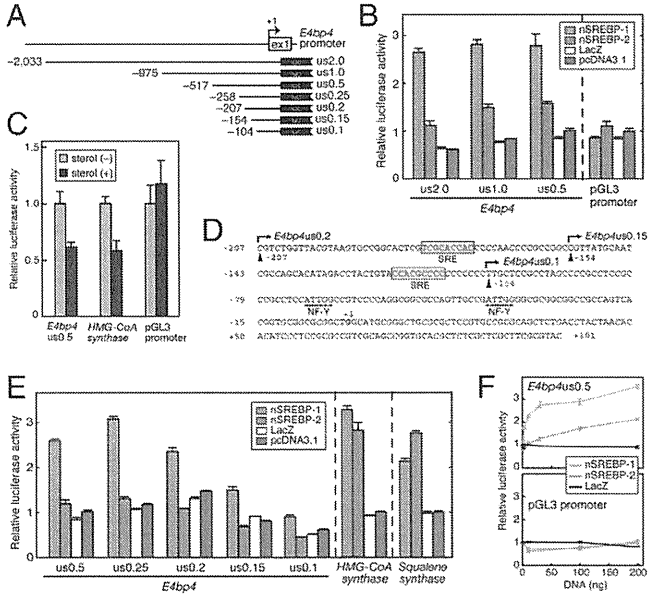

Fig. 4. The transcriptional activation of *E4bp4* promoter by nSREBP. (*A*) Schematic structure of chicken *E4bp4* promoter-luciferase reporter constructs. The putative transcription start site is indicated as +1. (*B*, *E*, and *F*) The transcriptional assays in CHO-K1 cells using luciferase reporters containing promoter region of *E4bp4*. The amount of SREBP-expression plasmid was 100 ng (*B* and *E*) or 1, 3, 10, 30, 100, and 200 ng (*F*). The luciferase activity derived from the firefly luciferase reporter was normalized to that from the *Renilla* luciferase reporter. The mean value obtained from the cells transfected with empty vector and *E4bp4*us0.5 (*B*, *E*, and *F*, *Upper*) or pGL3 promoter (*F*, *Lower*) was set to 1. Data are the mean ± SEM ($n = 3$). (*C*) Regulation of *E4bp4* promoter activity by cellular sterol levels. Four hours after transfection, CHO-K1 cells were treated with (+) or without (–) 1 μg/mL 25-hydroxycholesterol and 10 μg/mL cholesterol. After 48-h culture, the cells were subjected to luciferase assay. The mean value obtained from the sterol (–) cells was set to 1. Data are the mean ± SEM ($n = 6$). (*D*) Sequence of the promoter region of *E4bp4*us0.2 reporter vector. Sequences matched 8 of 9 bp with the SRE consensus (YCAYNYCAY, Y = pyrimidine; ref. 20) are indicated by gray boxes. The potential NF-Y binding sites (CCAAT) are underlined with broken lines (see *Discussion*).

Light Pulse at Early Subjective Night Activates 7α-Hydroxypregnenolone Secretion from the Pineal Gland. The light-dependent activation of SREBP-target genes for cholesterol biosynthesis (Fig. 3*B* and Fig. S4*B*) gave us the idea that light could stimulate pineal production of cholesterol and its derivatives such as neurosteroids, which act as signaling molecules in the brain (23). The rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone, a process that is facilitated by both mitochondrial cholesterol transporter StAR and cholesterol side-chain cleavage enzyme P450scc (24) (Fig. 5*A*). *StAR* gene was expressed at similar levels in a wide range of brain regions including the pineal gland, whereas *P450scc* transcript level was remarkably higher in the pineal gland than the other brain regions tested (Fig. 5*B*), suggesting active neurosteroidogenesis in the pineal gland. We examined potential neurosteroid biosynthesis by in vitro incubation of the pineal lysate with [^{3}H]-labeled pregnenolone as a substrate. HPLC-based separation of the reaction products demonstrated that pregnenolone was converted primarily to 7α-hydroxypregnenolone with the pineal lysate (Fig. 5*C*). The synthetic activity of 7α-hydroxypregnenolone was identified in the diencephalon of newts and quail (25, 26), whereas we found that the specific activity of the pineal lysate was ≈15-fold higher than that of the diencephalon of chicks (Fig. 5*D*). This pineal activity contrasts sharply with that of the adrenal gland showing no detectable activity to synthesize 7α-hydroxypregnenolone (Fig. S8*A*). We further explored a potential function of the pineal gland to synthesize and secrete 7α-hydroxypregnenolone in organ culture. The pineal glands isolated from the entrained chicks at CT6, CT14, or CT22 were exposed to light (or kept in the dark) under the cultured condition, which maintains the in vivo properties of the pineal gland (5). We found that a significant amount of 7α-hydroxypregnenolone was released from the pineal gland into the culture medium, and the release was stimulated by light at CT14 (Fig. 5*E*), consistent with the light response of SREBP-target genes for cholesterol biosynthesis (Fig. 3*B* and Fig. S4*B*).

Light Pulse at Early Subjective Night Strongly Stimulates Locomotor Activity in a Pineal Gland-Dependent Manner. In newts and quail, 7α-hydroxypregnenolone is reported to activate their locomotor activities (25, 26). In line with these observations, spontaneous locomotor activities of chicks were stimulated in a dose-dependent manner by intracerebroventricular injection of 7α-hydroxypregnenolone (Fig. 5*F*). The injected dose (10 or 200 ng) is equivalent to or in excess of 7α-hydroxypregnenolone content in the whole brain isolated after 1-h light pulse from CT14 [7.0 ± 1.2 ng ($n = 5$)]. We then analyzed the effect of light on locomotor activities at CT6, 14, and 22. Light exposure at CT14 strongly stimulated the activities to daytime level (Fig. 5*G*, *Left*), and light/dark ratio of the activities was highest at CT14 (Fig. 5*G*, *Right*) with a temporal profile very similar to that of the light response of 7α-hydroxypregnenolone release (Fig. 5*E*). We further investigated the effect of pinealectomy on light stimulation of locomotor activities at CT14 and found that the light response was attenuated by pinealectomy (Fig. 5*H*) to a level close to the light response of intact animals at CT6 and CT22 (Fig. 5*G*, *Right*). Collectively, these results revealed unique functions of the pineal gland, i.e., the biosynthesis and secretion of 7α-hydroxypregnenolone. Light-dependent activation of SREBP pathway in the pineal gland not only transactivates *E4bp4* transcription but also appears to contribute to stimulation of the locomotor activity by generating 7α-hydroxypregnenolone in a time-of-day–dependent manner.

Discussion

This study demonstrates that SREBP pathway in the pineal gland was activated within 1 h after the light exposure of chicks, and the light response was pronounced at the early subjective night (Figs. 1 and 3). This gating appears to play an important role in time-of-day–dependent phase delay by light because *E4bp4* expression was up-regulated by nSREBP (Fig. 4 and Fig. S7). It should be noted that the light pulse given at early subjective night remarkably ac-

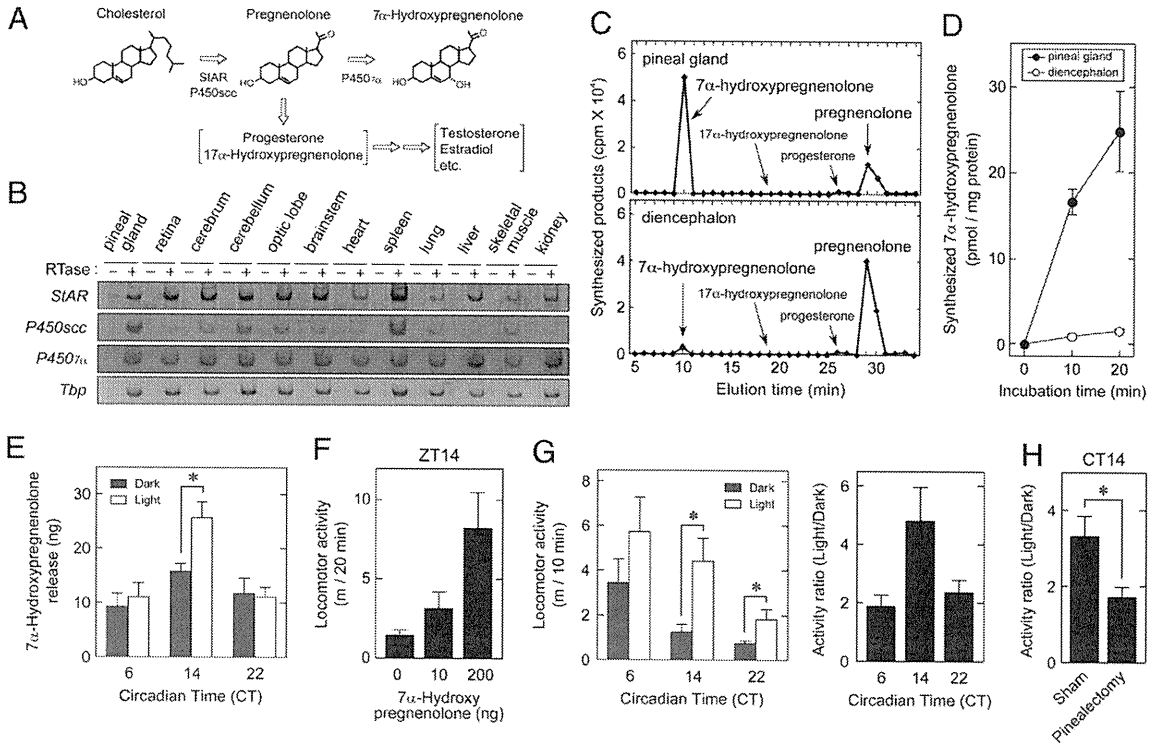

Fig. 5. Generation of 7α-hydroxypregnenolone in the pineal gland. (*A*) Biosynthetic pathway of 7α-hydroxypregnenolone from cholesterol. (*B*) Expressions of *StAR*, *P450scc*, and *P450$_{7\alpha}$* in the pineal gland. Total RNA extracted from the tissues at ZT6 and ZT18 were mixed and subjected to reverse transcription with (+) or without (–) reverse transcriptase (RTase), followed by PCR amplification. (*C* and *D*) Production of 7α-hydroxypregnenolone from pregnenolone in the pineal and diencephalon lysates. (*C*) The homogenate from the pineal gland or the diencephalon were incubated with [^{3}H]pregnenolone for 40 min, and each extract was subjected to HPLC with a reversed-phase column. (*D*) Radioactivity of 7α-hydroxypregnenolone fraction was plotted against incubation time. Data are the mean ± SEM ($n = 6$). (*E*) Effect of light on 7α-hydroxypregnenolone release from the pineal gland. The chicks were exposed to 20-min light pulse (≈300 lx; white fluorescent lamps) from CT6, CT14, or CT22, and then pineal glands were isolated and pooled (eight pineal glands) to culture for 3 h under the light. 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 ($n = 4$). (*F*) Effect of intracerebroventricular injection of 7α-hydroxypregnenolone on chick locomotor activity. 7α-hydroxypregnenolone (0 ng, $n = 14$; 10 ng, $n = 5$; 200 ng, $n = 9$) was injected at ZT14-16, and the locomotor activity of each individual was recorded for 20 min. Data are the mean ± SEM $P < 0.005$ by one-way ANOVA. (*G*) Effect of light on locomotor activity. Locomotor activity of individual chick was recorded for 10 min before (under infrared light) and after (under white LED light; ≈300 lx) the 10-min light exposure at CT6–8, CT14–16, or CT22–24 (*Left*). Data are the mean ± SEM ($n = 6$–8). Light/dark ratio of the activity of each chick was plotted in *Right* ($P < 0.05$ by one-way ANOVA). (*H*) Effect of pinealectomy on light-dependent stimulation of locomotor activity. Locomotor activity of pinealectomized ($n = 6$) or sham-operated ($n = 7$) chick was recorded for 10 min before and after the 10-min light pulse at CT14–16, and light/dark ratio of the activity was plotted as in *G*. *$P < 0.05$ by Student's *t* test.

tivated a subset of SREBP-target genes responsible for biosynthesis and metabolism of cholesterol, whereas it had only marginal effects on those involved in synthesis of acetyl-CoA and fatty acids (Fig. 3*B* and Fig. S4*B*). Intriguingly, it has been established that optimal transcriptional activation by nSREBP requires additional contribution of transcription factors such as Sp1 and nuclear factor Y (NF-Y), whose binding sites are positioned close to SRE (27). We searched for Sp1– and NF-Y–binding sites in mammalian SREBP-target genes in the literature (22, 28–30) and found that the NF-Y sites reside near SRE of the light-responsive cholesterol-related genes, such as *Farnesyl diphosphate synthase*, *HMG-CoA synthase*, *HMG-CoA reductase*, *Squalene epoxidase*, *Squalene synthase*, and *StarD4*. In contrast, the NF-Y site was not found in the light-insensitive genes such as *Acetyl-CoA synthetase*, *LDL receptor*, *Acetyl-CoA carboxylase-1*, and *Malic enzyme*. The presence of two NF-Y sites close to SRE in the chicken *E4bp4* promoter (Fig. 4*D*, broken lines) raises the possibility that NF-Y plays an additional role for the light induction of gene expression in a cooperative manner with SREBP.

In contrast to striking light activation of SREBP pathway at early subjective night, stress–response genes and their regulators were prominently induced by light at late subjective night (Figs. 1 and 2). It is known that nuclear accumulation and activation of HSF1 and HSF3 are stimulated by heat shock, whereas HSF2 is uniquely activated when the ubiquitin-dependent protein degradation machinery is inhibited (13). Our finding that HSF1 and HSF2 but not HSF3 accumulated in the pineal cell nuclei in response to light (Fig. 2*B*) suggests that the light activation of these factors is not simply due to a heat shock response such as changes in body temperature. Similarly, we found that light induced expression of the target genes of ATF6 and XBP1 but not those of ATF4 (Fig. 2*C* and Fig. S4*A*), whereas all of these genes are known to be induced by ER stress (14). It is possible that light activates an unidentified combination of upstream signaling pathways for the selective light response of these stress–response factors, rather than acts as a stress. Interestingly, SREBP (31), HSF1 (15), ATF6 (32), and also XBP1 (Fig. S5) are implicated in the feeding-dependent regulation of rhythmic gene expression in the mouse liver. These factors can be common regulators in various input pathways among clock cells, although the molecular link between the phase advance of the pineal clock and the signaling pathways of ATF6, XBP1, and HSF needs to be established, among which HSF1 is known as a regulator of the mammalian clockwork (15, 33). It is interesting to investigate whether SREBP, ATF6, XBP1, and HSF pathways show time-of-day–dependent responses also in the food-regulated clocks of the liver and in the central clock of the suprachiasmatic nucleus in mammals. Such a comparative genomic analysis will shed light on potential conservation of light-responsive pathways among tissues and/or species.

The brain is not only an important target site of steroid hormones but also capable of converting cholesterol to steroid hormones (neurosteroids) in several types of cells including oligodendrocytes, Purkinje neurons, and hippocampal neurons (23). Here, we found that the pineal gland has high activity to synthesize and secrete 7α-hydroxypregnenolone which enhances locomotor activities of chicks (Fig. 5). To the best of our knowledge, this is the first observation showing neurosteroid synthesis and secretion in the pineal gland in any vertebrate. In contrast, it is established that the canonical pineal hormone melatonin regulates sleep rhythms (34). Therefore, the pineal gland appears to participate in the regulation of sleep/wake state not only by circadian production of melatonin in the dark but also by synthesis and secretion of 7α-hydroxypregnenolone in the light. In this study, we used male chicks for the experiments. Our previous study on the quail diencephalon demonstrated much higher activity of 7α-hydroxypregnenolone synthesis in males than females, corresponding to relatively higher locomotor activities of males (26). Consistent with this observation, we found that the pineal gland of female chicks released far lower amount of 7α-hydroxypregnenolone than males (Fig. S8*B*), suggesting the conservation of the sex difference in 7α-hydroxypregnenolone system between the chick pineal gland and the quail diencephalon. Further study is necessary to reveal the mechanism underlying the sex difference.

In summary, our genome-wide gene expression analysis revealed multiple signaling pathways that were light-activated in a time-of-day–dependent manner to regulate gene expression. The SREBP-mediated pathway especially should be highlighted as the photic-input mechanism of the circadian clock through regulation of *E4bp4*, which acts as a light-induced suppressor of *Per2* particularly in the phase-delaying process. An intriguing idea is that the combination of these light-activated pathways determines the direction and degree of the time-of-day–dependent phase shift of the circadian clock.

Materials and Methods

Details are provided in *SI Materials and Methods*.

Animals. Animal experiments were conducted in accordance with the guidelines of the University of Tokyo. Newly hatched male chicks were maintained under 12-h light/12-h dark 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 and exposed to a 1-h light pulse at various time points. All procedures during the dark period were performed under dim red light.

GeneChip and Quantitative RT-PCR Analyses. Original analysis data using GeneChip Chicken Genome Array (Affymetrix) were deposited in GEO (accession no. GSE21915). Quantitative RT-PCR analysis was performed as described (35) or by using QuantiTect SYBR Green PCR Kit (Qiagen). The primers and optimal cycle numbers were summarized in Table S1.

ACKNOWLEDGMENTS. We thank Y. Nakajima, M. Doi, and T. Okano for sharing preliminary results and T. Ubuka, Y. Nobe, and K. Shimizu for technical assistance. M.H. and N.K. were supported by Japan Society for the Promotion of Science Research Fellowships. This work was supported in part by Grants-in-Aid for Scientific Research and by Global Centers of Excellence Program from Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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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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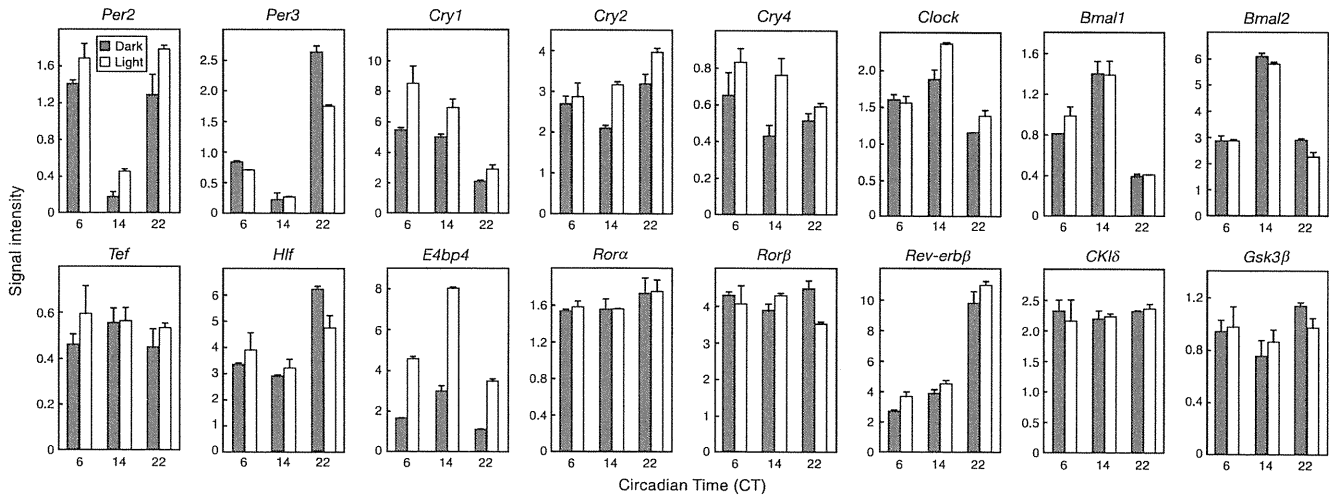

Fig. S1. Expression profiles of the clock genes in the pineal gland. Dark-kept animals were exposed to a 1-h light pulse (open bars) from CT6, CT14, or CT22, or kept in the dark as a control (solid bars). The GeneChip profiles of the clock genes are displayed (mean with variation, $n = 2$). The gene 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*.

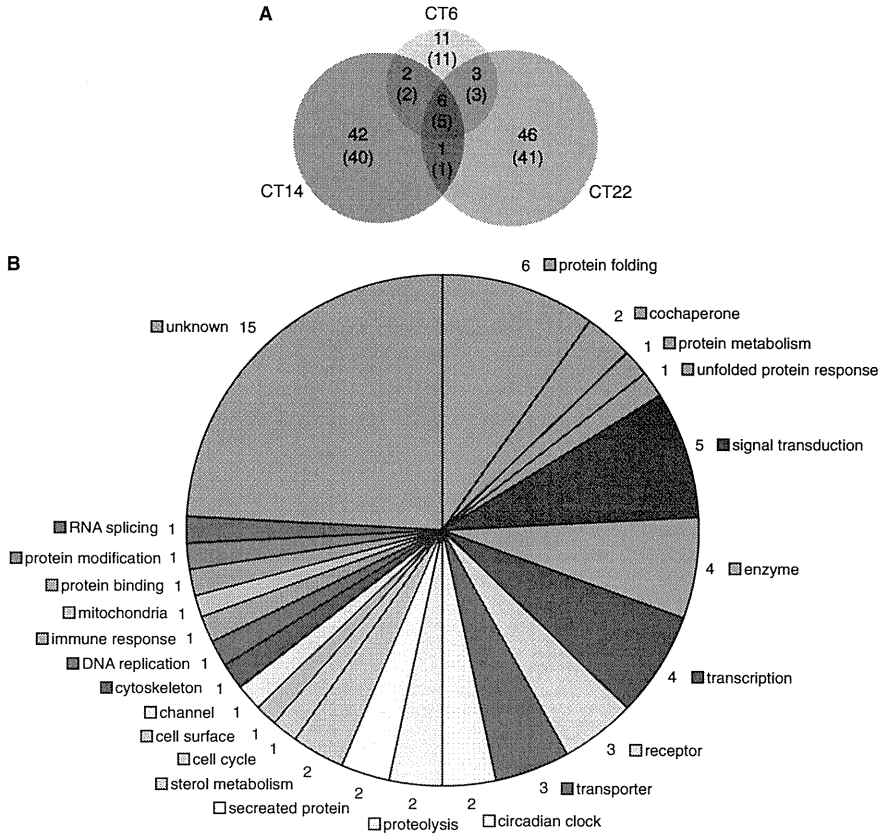

Fig. S2. Time-of-day dependence and functional classification of light-induced genes in the pineal gland. (*A*) Venn diagram showing time-of-day dependence of the light induction. One hundred eleven probe sets (corresponding to 62 genes and 40 ESTs) exhibited >2.5-fold increase of their signal intensities 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.

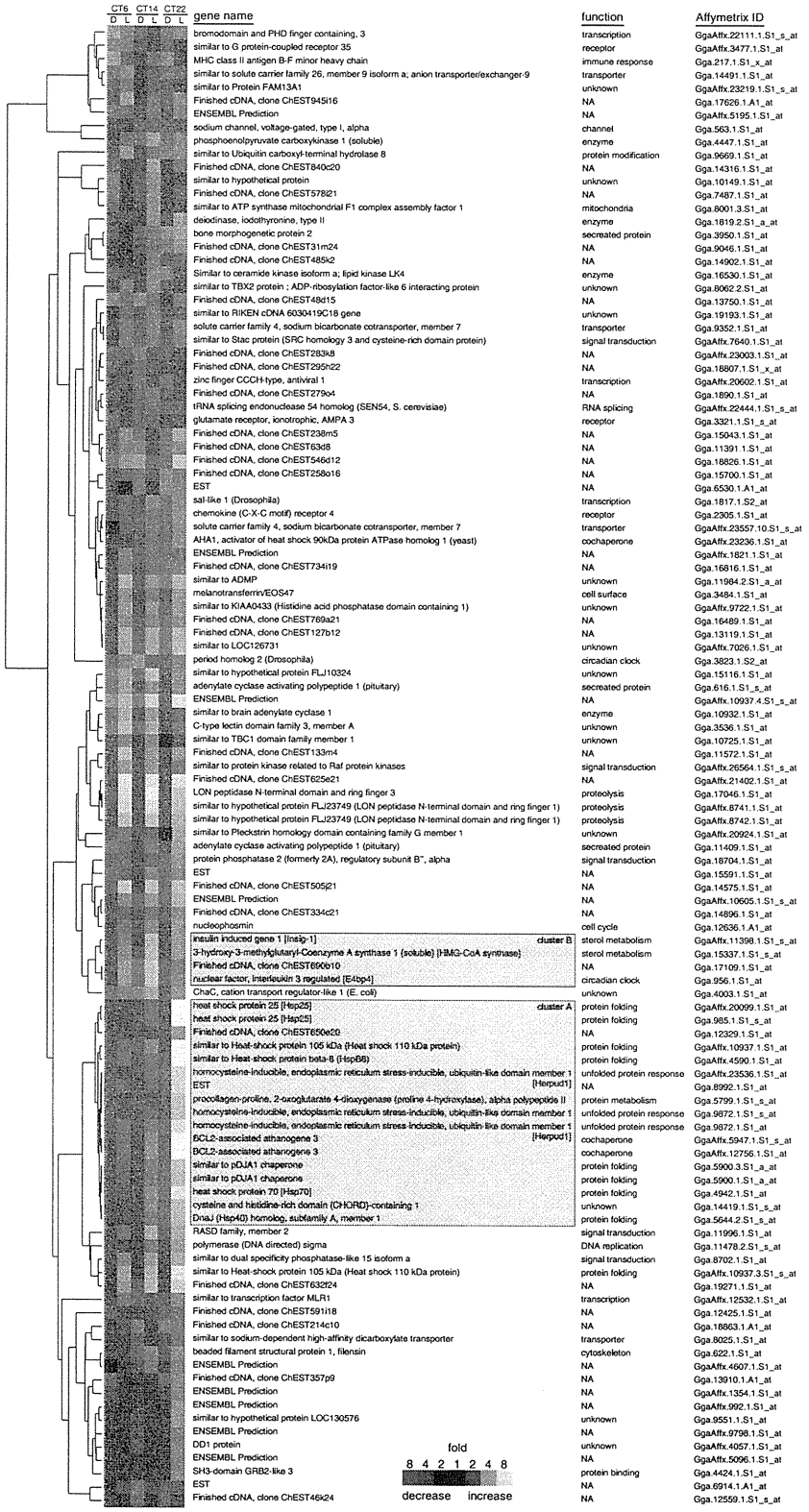

Fig. S3. Light-induced genes in the chicken pineal gland. See Fig. 1 legend for details. Gene clusters containing stress–response genes (cluster A), and *E4bp4* (cluster B) are highlighted by pink.

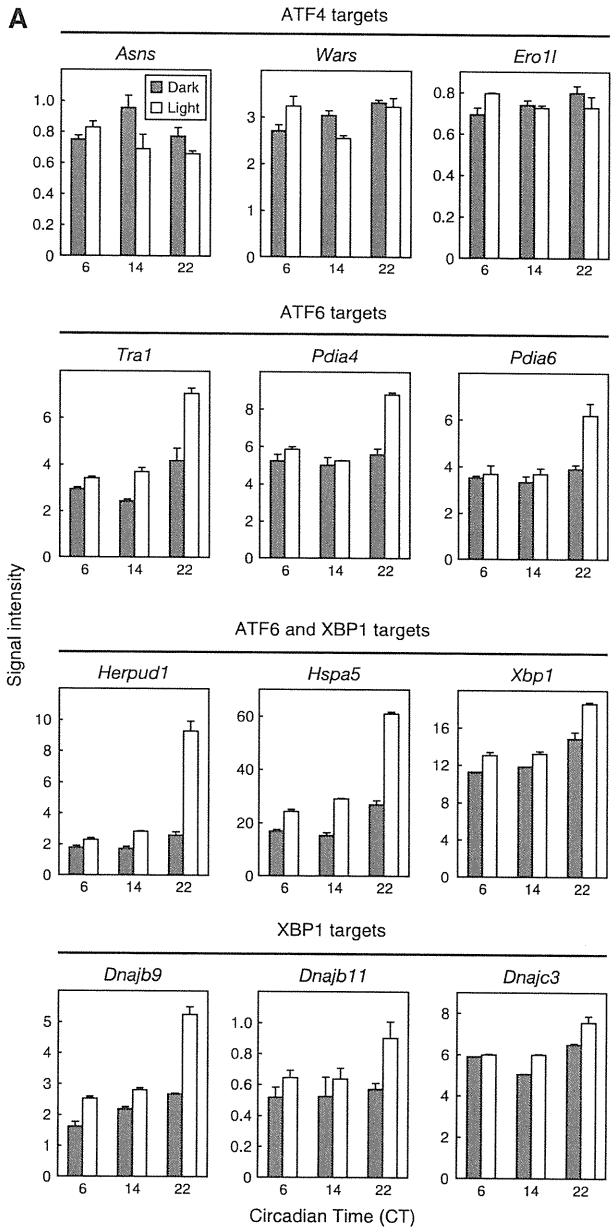

Fig. S4. (Continued)

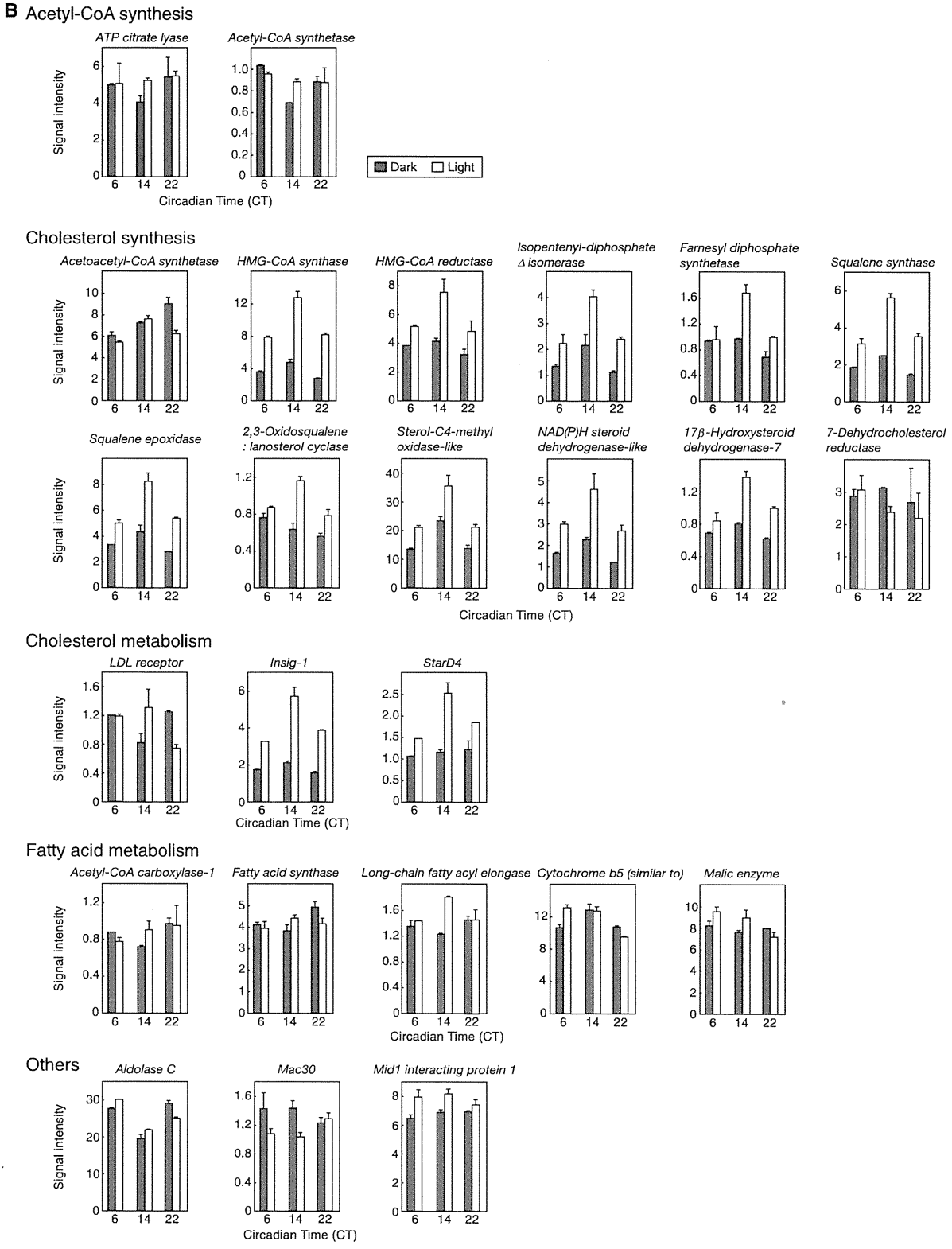

Fig. S4. Expression profiles of ATF4-, ATF6-, XBP1-, and SREBP-target genes in the pineal gland. (*A*) ATF4-, ATF6-, and XBP1-target genes. (*B*) SREBP-target genes. Dark-kept animals were exposed to the 1-h light pulse (open bars) from CT6, CT14, or CT22, or kept in the dark as a control (solid bars).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

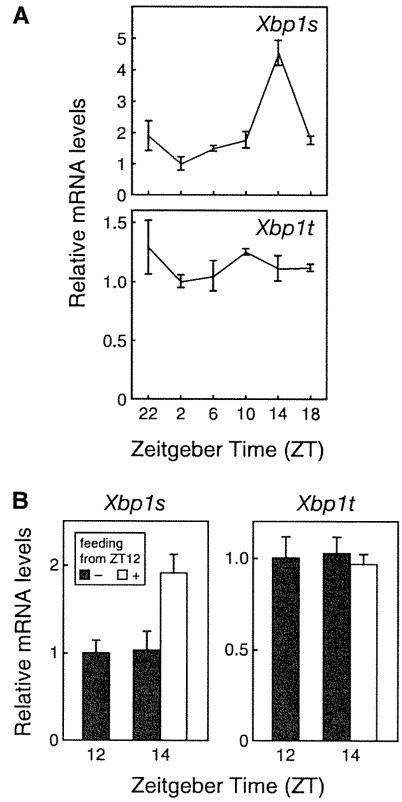

Fig. S5. Daily expression profile and feeding response of spliced *Xbp1* mRNA levels. (*A*) Daily expression profile. Mice were housed in 12-h light/12-h dark cycles with foods and water freely available, and the liver was isolated at the indicated time point. (*B*) Feeding response. Mice were housed 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).

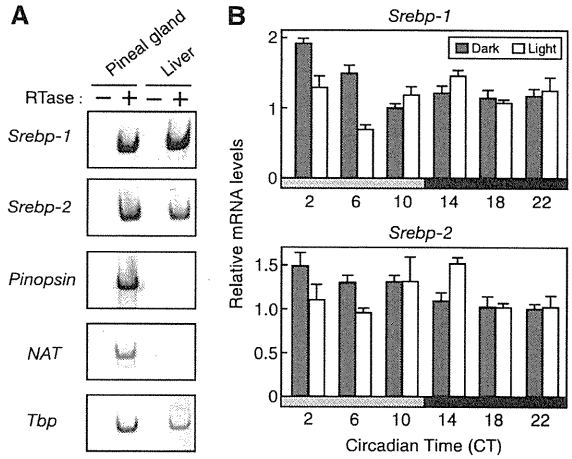

Fig. S6. Light-response of *Srebp-1* and *Srebp-2* genes in the pineal gland. (*A*) Expression of *Srebp-1* and *Srebp-2* in the pineal gland and liver. Total RNA extracted from the tissues were subjected to reverse transcription with (+) or without (–) reverse transcriptase (RTase), followed by PCR 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*.

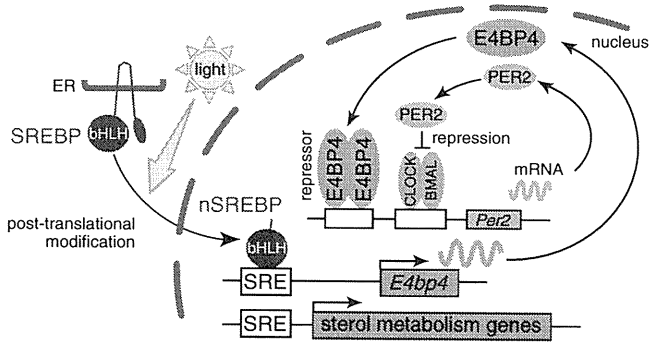

Fig. S7. A model for the role of SREBP in the light-input pathway of the pineal circadian clock. See text for details.

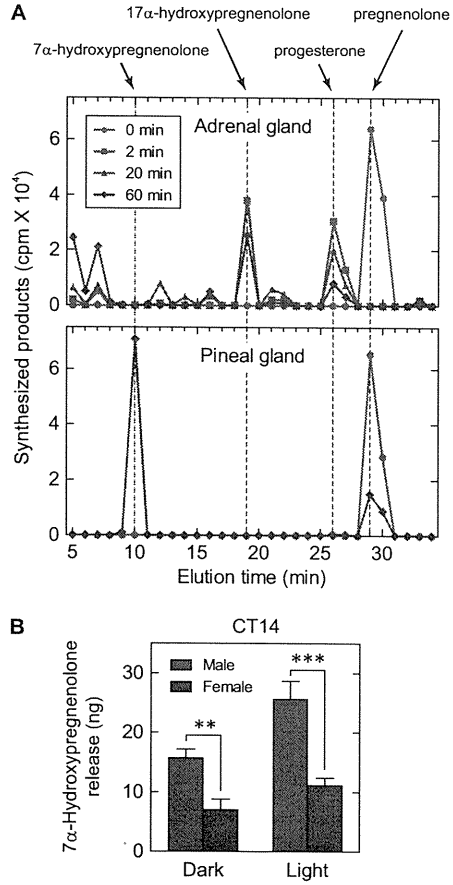

Fig. S8. Steroid production in the adrenal gland and 7α-hydroxypregnenolone release from the female pineal gland. (*A*) Production of steroids from pregnenolone in adrenal gland and pineal gland lysates. The homogenate from the adrenal gland or the pineal gland were incubated with [^{3}H]pregnenolone 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.

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Gene and protein analysis of brain derived neurotrophic factor expression in relation to neurological recovery induced by an enriched environment in a rat stroke model

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ABSTRACT

Although an enriched environment enhances functional recovery after ischemic stroke, the mechanism underlying this effect remains unclear. We previously reported that brain derived neurotrophic factor (BDNF) gene expression decreased in rats housed in an enriched environment for 4 weeks compared to those housed in a standard cage for the same period. To further clarify the relationship between the decrease in BDNF and functional recovery, we investigated the effects of differential 2-week housing conditions on the mRNA of BDNF and protein levels of proBDNF and mature BDNF (matBDNF). After transient occlusion of the right middle cerebral artery of male Sprague–Dawley rats, we divided the rats into two groups: (1) an enriched group housed multiply in large cages equipped with toys, and (2) a standard group housed alone in small cages without toys. Behavioral tests before and after 2-week differential housing showed better neurological recovery in the enriched group than in the standard group. Synaptophysin immunostaining demonstrated that the density of synapses in the peri-infarct area was increased in the enriched group compared to the standard group, while infarct volumes were not significantly different. Real-time reverse transcription polymerase chain reaction, Western blotting and immunostaining all revealed no significant difference between the groups. The present results suggest that functional recovery cannot be ascribed to an increase in matBDNF or a decrease in proBDNF but rather to other underlying mechanisms.

Functional impairment caused by stroke is a highly serious health problem throughout the world. Rehabilitation has been widely applied and has been shown to contribute greatly to neurological recovery. However, the mechanisms of the beneficial effects of rehabilitation remain unclear [3]. An enriched environment is a model of rehabilitation for rodents, in which multiple animals are housed together in a large cage equipped with toys. Enriched environments have been shown to enhance the recovery of neurological function impaired by experimental focal ischemia [13]. Brain-derived neurotrophic factor (BDNF), one of the neurotrophins, may be a key molecule in this effect, since it is central to many facets of the neural network, from differentiation and neuronal survival to synaptogenesis and activity-dependent forms of synaptic plasticity [9]. While an enriched environment increases BDNF expression in non-ischemic healthy animals [5], this is not the case with ischemic animals. The alteration of BDNF after ischemic stroke is not fully understood, although BDNF expression has been investigated in association with an enriched environment after experimental stroke. Zhao et al. demonstrated that BDNF mRNA

Abbreviations: BDNF, brain derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPT, inclined plane test; MAP-2, microtubular-associated protein 2; matBDNF, mature BDNF; MCA, middle cerebral artery; NSS, neurological severity scores; ROI, region of interest; RT-PCR, reverse transcription polymerase chain reaction; SYP, synaptophysin; tMCAO, transient MCA occlusion.

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