Establishment of TSHβ real-time monitoring system in mammalian photoperiodism

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Organisms have seasonal physiological changes in response to day length. Long-day stimulation induces thyroid-stimulating hormone beta subunit (TSHβ) in the pars tuberalis (PT), which mediates photoperiodic reactions like day-length measurement and physiological adaptation. However, the mechanism of TSHβ induction for day-length measurement is largely unknown. To screen candidate upstream molecules of TSHβ, which convey light information to the PT, we generated Luciferase knock-in mice, which quantitatively report the dynamics of TSHβ expression. We cultured brain slices containing the PT region from adult and neonatal mice and measured the bioluminescence activities from each slice over several days. A decrease in the bioluminescence activities was observed after melatonin treatment in adult and neonatal slices. These observations indicate that the experimental system possesses responsiveness of the TSHβ expression to melatonin. Thus, we concluded that our experimental system monitors TSHβ expression dynamics in response to external stimuli.

Introduction

Many organisms adapt their physiological functions and behaviors to seasonal environmental changes by measuring day length, a biological process known as photoperiodism (Dawson et al. 2001; Ebling & Barrett 2008; Revel et al. 2009). Integration of external light information and endogenous time mediated by circadian clock genes (clockwork genes) is important for the photoperiodic responses (Hamner 1963; Pittendrigh & Minis 1964).

The pars tuberalis (PT) of the pituitary gland may be responsible for the photoperiodic responses because of the high expression levels of the clockwork genes and melatonin receptor 1a (MT1) in the PT (Morgan & Williams 1996; Lincoln et al. 2003;
Ikegami & Yoshimura 2012). In mammals, it is suggested that nocturnal melatonin conveys darkness information to the PT, but the molecules conveying light information remain unknown (Hoffman & Reiter 1965; Morgan & Williams 1996). In a recent study, thyroid-stimulating hormone beta subunit (TSHβ) gene was found to be rapidly induced by long-day stimulation in the PT and the product of TSHβ, thyroid-stimulating hormone (TSH), has a functional role in seasonal testicular growth in birds (Yoshimura et al. 2003; Nakao et al. 2008). This TSHβ induction is conserved even in mammals including melatonin-proficient mice (Hanon et al. 2008, 2010; Ono et al. 2008; Dardente et al. 2010; Dupre et al. 2010; Masumoto et al. 2010; Yasuo et al. 2010). Therefore, TSHβ is a key factor for day-length detection and controls seasonal physiological changes.

Delivery of light information from the retina to the PT is an important pathway for inducing TSHβ expression and regulating photoperiodic responses. Indeed, removal of the eyes, the suprachiasmatic nucleus or the pineal gland disrupts testicular responses in hamsters (Hoffman & Reiter 1965; Rusak & Morin 1976; Stetson & Watson-Whitmyre 1976). In the mouse PT, long-day stimulation induces a transcription cofactor eyes absent 3 (Eya3) (Masumoto et al. 2010). This molecule regulates TSHβ expression with DNA-binding transcription factor sine oculis-related homeobox 1 (Six1) and several clockwork genes such as thyrotroph embryonic factor (Tef) and/or hepatic leukemia factor (Hlf) (Dardente et al. 2010; Masumoto et al. 2010). These molecular mechanisms have also been found in sheep (Dardente et al. 2010; Dupre et al. 2010). On the other hand, darkness information is mediated by melatonin that suppresses Eya3 and TSHβ expression in sheep and mice, respectively (Ono et al. 2008; Unfried et al. 2009; Dardente et al. 2010; Yasuo et al. 2010). Thus, the regulation of Eya3 by external light conditions is critical to control TSHβ expression for photoperiodism. However, the intracellular and extracellular signals that transfer the external light information to the PT to regulate Eya3 and TSHβ expression remain unknown.

In this study, we focused on how TSHβ expression in the PT is regulated by long-day stimuli. To accomplish this, we established a real-time monitoring system for measuring TSHβ expression dynamics for the purpose of screening candidate light information-transferring signals in the PT. We generated a genetic mouse model in which TSHβ expression dynamics in the PT can be visualized by bioluminescence. Treatment of cultured PT slices with melatonin decreased bioluminescence activities, and these results showed that PT slices can respond to melatonin in our culture condition. Thus, our mouse model could be used to search for molecules that relay external light signals in the PT.

Results
Generation of TSHβLac mice
To monitor TSHβ expression in the PT, we applied a knock-in approach in which 5′ exon of TSHβ-coding sequence was knocked out and replaced with a firefly Luciferase (Luc) gene (Fig. 1A). In this scheme, the luc activity represents direct transcription from the endogenous TSHβ promoter. Southern blot analysis confirmed that the desired mutation was successfully introduced (Fig. 1B, left), and genotypes of offspring were routinely analyzed by PCR (Fig. 1B, right). Animals were backcrossed and maintained on a CBA/N background because unlike the more common C57BL/6 strain, CBA/N mice maintain melatonin proficiency (Ebihara et al. 1986; Nakahara et al. 2003).

To verify the loss of TSHβ transcript, we performed quantitative RT-PCR (qPCR) to measure the expression levels of TSHβ and its related genes in the pituitary, where the highest TSHβ expression level in the brain was observed (Kasukawa et al. 2011). By using primers aligned within exon 4, which was deleted by the targeting (Fig. 1A), TSHβ mRNA was undetectable in homozygous TSHβLac mice. On the other hand, only 12% decrease in TSHβ expression levels was observed in heterozygous TSHβLac mice compared with wild-type mice (Fig. 1C). The expression levels of common glycoprotein α-subunit (Cga), the alpha subunit of TSH, were not different between wild-type and heterozygous TSHβLac mice, but there was a four-fold increase in homozygous TSHβLac mice (Fig. 1C), suggesting antirepression of negative feedback loop due to the disruption of the TSH heterodimer. Luc expression levels were observed in heterozygous TSHβLac mice, significantly increased in homozygous TSHβLac mice and undetectable in wild-type mice, also suggesting the disruption of the TSH heterodimer (Fig. 1C).

One hundred and thirty-seven offspring from heterozygous matings were examined. 30 animals were wild-type (21%), 85 heterozygous (53%) and 35 animals homozygous TSHβLac mice (26%). These results are consistent with single-gene Mendelian
in homozygous TSHβ\(^{lac}\) mice was recovered by supplementation with 100 ppm thyroid powder, consistent with the observation in TSH receptor (TSHR) knockout mice (Marians et al. 2002) (Fig. 1D). From these observations, we concluded that phenotypes of heterozygous TSHβ\(^{lac}\) mice are comparable with those of wild-type mice.

**Relationship between TSHβ and Luc expression in TSHβ\(^{lac}\) mice**

Next, we checked whether the knock-in Luc mice respond to changes in day length similar to endogenous photoperiodic genes in the PT. TSHβ, Luc and Cga expressions in the PT of wild-type and heterozygous TSHβ\(^{lac}\) mice under different day-length conditions were analyzed by radioisotope (RI) in situ hybridization. After 3 weeks of exposure to short-day [light/dark = 8:16 hour (L8 : D16), zeitgeber time 0 (ZT0; ZT0 was defined as the time of lights-on) = lights-on, ZT8 = lights-off] or long-day (L16 : D8, ZT0 = lights-on, ZT16 = lights-off) conditions, brain samples from each light condition were collected 4 h before light-off that corresponds to the same circadian phase under both conditions (Masumoto et al. 2010) (Fig. 2A). TSHβ, Luc and Cga were detected only in the PT under both conditions, and the TSHβ expression increased 2.5-fold under long-day conditions in wild-type and heterozygous TSHβ\(^{lac}\) mice. Although Luc expression was almost undetectable in heterozygous TSHβ\(^{lac}\) mice under short-day conditions and not detected in wild-type
mice, its expression was clearly detected in heterozygous TSHβLuc mice under long-day conditions (Fig. 2B). We also observed that Cga expression pattern was comparable in wild-type and heterozygous TSHβLuc mice. These results indicate that the Luc expression represents the physiological photoperiodic responses of TSHβ in the PT and does not affect the expressions of endogenous TSHβ and Cga.

**PT-specific signals of the TSHβ-Luc reporter**

To detect bioluminescence derived from the TSHβ promoter in the PT region, we prepared brain slices including regions from TSHβLuc mice. First, we monitored the spatial pattern of the bioluminescence activity in the slice with a macrozoom microscope and a CCD camera. As expected, bioluminescence activity was observed only in the putative PT region surrounding the median eminence of the slices from 5- to 6-week-old (adult) heterozygous TSHβLuc mice kept under long-day conditions for 2–3 weeks (Fig. 2C). These spatial bioluminescence patterns are compatible to signals of Luc mRNA (Fig. 2B,C). By contrast, no bioluminescence was observed in the slices from wild-type mice (Fig. 2C). Therefore, bioluminescence activity measured in the slice can be attributed to luc activity in the PT. In addition, similar bioluminescence pattern in the slices from adult mice was observed in the slices from postnatal 6- to 8-day-old (neonate) heterozygous TSHβLuc mice kept under long-day conditions (Fig. 2C). These observations are consistent with the TSHβ expression in vivo (Schulze-Bonhage & Wittkowski 1990; Japon et al. 1994) and thus imply the possibility of slice usage from both adult and neonatal PT.
Real-time bioluminescence monitoring of the TSHβ-Luc reporter

Next, we developed a real-time monitoring system for TSHβ-Luc bioluminescence by a photomultiplier tube (PMT). Because Luc appears to be specifically expressed in the PT of the slice (Fig. 2C), photon counts by PMT measurements represent a mean bioluminescence of the tissue. First, the bioluminescence signals of slices from adult heterozygous TSHβ-Luc mice kept under short- or long-day conditions for 2–3 weeks (adult short- or long-day slices) were measured (Fig. 3A,B). Significantly higher bioluminescence signals were detected from adult long-day slices than from adult short-day slices and wild-type slices. We further checked whether the slices have in vivo-like responsiveness to photoperiodic signals. We treated adult long-day slices with 10 nM melatonin from subjective ZT16 (ZT16 is the start of the culture and subjective night) (Fig. 3C). In agreement with previous in vivo studies (Ono et al. 2008; Unfried et al. 2009; Yasuo et al. 2010), a decrease in luc activity was observed by melatonin treatment. These results suggest that the experimental system keeps TSHβ response to melatonin even in our culture condition.

One important application of this real-time monitoring system is to screen molecules that convey light signals under long-day conditions and induce the TSHβ expression. Thus, we examined the possibility of using of neonatal tissues instead of adult tissues because of some advantages: (i) higher viability of tissues, (ii) ease of slice preparation and (iii) significant signal levels even in L12 : D12 conditioned animals rather than long-day conditioned animals (Fig. 2C). To check whether neonatal tissue can be used for the monitoring assay, luc activity in the PT slices of neonatal heterozygous TSHβ-Luc mice kept under short- or long-day conditions for 2–3 days (adult short- or long-day slices) were measured (Fig. 3A,B). Significantly higher bioluminescence signals were detected from adult long-day slices and in vivo.

To independently confirm that the observed dynamics of bioluminescence is correlated with endogenous TSHβ expressions in neonatal PT slices, the expression levels of TSHβ, Eya3 and Qqa, which are known to be decreased by melatonin (Ono et al. 2008; Unfried et al. 2009; Dardente et al. 2010; Yasuo et al. 2010), were measured by qPCR. Cultured PT tissues with vehicle or 10 nM melatonin treatment for 48 h after the start of the culture were collected and subjected to qPCR analysis. The values of sine oculis-related homeobox 5 (Six5) and potassium voltage-gated channel, subfamily Q, member 5 (Kcnq5) were used for measuring the relative PT amounts of the samples and normalizing TSHβ values and related genes, because Six5 and Kcnq5 expression is specific to the PT and does not respond to melatonin (Dupre et al. 2008; Masumoto et al. 2010). Consistent with in vivo observations (Ono et al. 2008; Unfried et al. 2009; Dardente et al. 2010; Yasuo et al. 2010) and bioluminescence results, the expression levels of endogenous TSHβ, Eya3 and Qqa were significantly decreased under melatonin treatment, excluding the possibility that the decrease in the bioluminescence by melatonin might be an artifact (Fig. 3E,F and S2 in Supporting Information). In addition, there was a strong positive correlation between TSHβ mRNA levels by qPCR analysis and bioluminescence counts of the PT slices by PMT measurements (r2 = 0.71; Fig. 3F, right). Furthermore, the culture medium of neonatal PT slices in Fig. 3F and S2 in Supporting Information was collected, and secreted TSHβ amounts were measured by mass spectrometry. In the melatonin-treated culture medium, secreted TSHβ amounts were 50% lower than vehicle-treated samples (Fig. 3G). There was a strong correlation between secreted TSHβ amounts and the bioluminescence counts (r2 = 0.81; Fig. 3G, middle) as well as the TSHβ mRNA levels (r2 = 0.66; Fig. 3G, right). These results indicate that the responsiveness of the luc activity to an external signal reflects TSHβ mRNA expression and secreted TSHβ protein.

Recently, it was reported that glutamine and glutamic acid induce the TSHβ expression in the rat PT (Aizawa et al. 2012). To check whether the mouse PT also responds to glutamine and glutamic acid, the slices were treated with 1 mM glutamine or 1 mM glutamic acid after 2 h of glutamine and glutamic acid starvation. Although no response was observed by glutamine treatment, a transient increase in luc
Figure 3 Real-time monitoring of the TSHβ expression by bioluminescence. (A) Experimental schema. Neonatal PTs were collected at postnatal 6–8 (P6–8) under L12 : D12 conditions, and 5- to 6-week-old mice were collected after being maintained in short-day (SD) or long-day (LD) conditions for 2–3 weeks. (B and D) Real-time bioluminescence recording in adult or neonatal PT slices of indicated genotypes. White and black bars on the graphs indicate the subjective day and night on the following days after sampling, respectively [n = 2, wild-type (WT) mice or n = 6, heterozygous TSHβ Luc mice (+/L) in B; n = 5, WT and n = 17, +/L in D, average ± SEM]. (C and E) Real-time monitoring of the luciferase (luc) reporter activities in adult or neonatal PT slices after the addition of vehicle (Veh) or melatonin (Mel). The PT slices were prepared from animals kept under LD (adult, C) or L12 : D12 (neonate, E) conditions. Vehicle or melatonin was applied at the start of the culture. Normalized luc reporter activities of PT slices from +/L mice are shown (n = 4 in C and n = 3–4 in E, average ± SEM). (F) Confirmation of expression level changes in TSHβ, Eya3 and Cga in the cultured PT by qPCR analysis. Neonatal PT slices were treated with vehicle or 10 nm melatonin 48 h with PMT monitoring. Expression levels of TSHβ, Eya3 and Cga were normalized by the value of Six5, which was specifically expressed in the PT. *P < 0.05, **P < 0.01 (two-sample t-test, n = 9, average ± SEM). Correlation between TSHβ mRNA levels and bioluminescence counts (right). White circles (Veh) and gray circles (Mel) indicate data points. The line is regression: r² = 0.71. (G) Quantification of secreted amounts of TSHβ into the culture medium of the PT slices in 3F and S2 by mass spectrometry analysis. **P < 0.01 (two-sample t-test, n = 9, average ± SEM). Correlation between secreted TSHβ amounts (middle) and TSHβ mRNA levels or bioluminescence counts (right). White circles (Veh) and gray circles (Mel) indicate data points. The line is regression: r² = 0.81 (middle), r² = 0.66 (right).
activity was observed by glutamic acid treatment. These results suggest that the slices can also respond to an inducer of TSHβ (Fig. S3 in Supporting Information).

In summary, we visualized the TSHβ expression dynamics in the PT by using adult and neonatal TSHβLuc mice and observed responsiveness of the TSHβ expression to an external signal.

Discussion

In the present study, we established a TSHβ real-time monitoring system in which the TSHβ expression dynamics can be monitored as real-time bioluminescence activities. The difference in luc activity of PT slices derived from adult mice under short- or long-day conditions was consistent with in vivo TSHβ expression levels in response to each day-length condition (Figs 2 and 3B). Moreover, the luc activities of adult and neonatal PT slices were suppressed by melatonin treatment (Fig. 3C,E). We also observed dose-dependent suppression of TSHβ in neonatal PT slices by melatonin (Fig. 3E) at physiological levels (Kennaway et al. 2002), which is consistent with the results using ovine PT cell cultures (Morgan et al. 1989). Furthermore, the luc activities were transiently up-regulated by glutamic acid treatment as seen in the rat PT (Aizawa et al. 2012) (Fig. S3 in Supporting Information). These observations suggest that PT slices possess at least TSHβ responsiveness to melatonin and glutamic acid, and changes in TSHβ expression dynamics under other stimuli are also expected to be observed by using our monitoring system.

Chronic melatonin treatment in this monitoring system may not necessarily the same as physiological conditions, comparing with nocturnal melatonin secretion in vivo. However, because TSHβ was induced within several hours after long-day stimulation in vivo, this monitoring system is at least available for screening of candidate molecules upstream of TSHβ, by observing luc activity during the same period. Transiently increased luc activity by glutamic acid treatment supports this idea (Fig. S3 in Supporting Information).

Several studies show that melatonin implantation (constant release of melatonin) has no effect on testicular regression in hamsters (Goldman et al. 1979). Furthermore, melatonin injection under long-day conditions does not suppress TSHβ expression for at least 4 days in hamster (Yasuo et al. 2010), which is inconsistent with the immediate suppression presented in Fig. 3C. We speculate that difference between these in vivo observations and our in vitro results may be due to experimental conditions such as existence of TSHβ-inducible signals. Therefore, careful design of experiments will be necessary in each purpose.

One possible application of our experimental system is to screen upstream light-information signals of the TSHβ induction. Our system has several advantages for this purpose: (i) The PT slices can be used and easily treated with drugs compared with previous methods such as subcutaneous injection (Yasuo et al. 2007), intracerebroventricular injection (Ono et al. 2008) or dissociated PT cells (Morgan et al. 1989), (ii) availability of neonatal tissues increases the throughput of the experiment and viability of the slices. Previous reports support that neonatal PT has the similar photoperiodic properties to adult PT. First, high expression levels of TSHβ (Schulze-Bonhage & Wittkowski 1990; Japon et al. 1994), MT1 receptor (Williams et al. 1991; Johnston et al. 2003) and rhythmic expression of the clockwork genes (Ansari et al. 2009) were observed in both embryonic and neonatal PT. Second, fetal and neonatal lambs showed changes in prolactin concentration in response to day length (Ebling et al. 1988, 1989).

We consider that neurotransmitters or humoral factors are possible candidates involving the extracellular upstream signals of TSHβ. For example, there are several known neurotransmitters of deep brain photoreceptor–expressing cells in birds (Halford et al. 2009; Nakane et al. 2010). The afferent signals mediated by these neurotransmitters might transfer light stimuli to the PT. Additionally, receptors expressed in PT cells like Gs-coupled receptors and downstream cAMP signaling are possible candidates because these signals can antagonize decreases in TSHβ by melatonin and the Gi-coupled MT1 receptor (Morgan et al. 1995).

Our system is also applicable to investigate the mechanism of photoinducible phase, by which the response of the PT to a light signal including TSHβ induction is gated during the late night (Masumoto et al. 2010). Internal circadian time is probably involved in the determination of the gate timing, because photoperiodic responses can be induced by light stimuli at specific time (Kavault & Ortavant 1997). Our previous study showed that the brain slices of mPer2Luc mice containing PT exhibited circadian oscillations of luc (Masumoto et al. 2010). Also, it was reported that PER2::LUC rhythms in slices were detected in the median eminence/paras
tuberalis (Guilding et al. 2009). These reports support that the PT region contains its intrinsic circadian oscillator. Analyzing circadian time dependency of TSHβ expression after chemical/neurotransmitter stimulation in the PT may facilitate reconstitution of the in vivo phenomenon and help to show the details of gating.

The TSHβ<sup>Luc</sup> mice generated here are the first report of TSHβ null mice. TSHβ<sup>Luc</sup> mice enabled us to study the photoperiodic roles of TSHβ. Although TSHβ is also expressed in the pituitary and functions as a part of the hypothalamus–pituitary–thyroid (HPT) axis, it is believed that the molecule in the PT acts differently from the HPT axis. This idea is supported by the results that TSHβ expression in ovine PT did not change with thyroid-releasing hormone (TRH) and thyroid hormone (TH) treatment, because the lack of TRH and TH receptor or transcription factor Pit-1 induces TSHβ in the pituitary (Bockmann et al. 1997). Growth retardation observed in our homozygous TSHβ<sup>Luc</sup> mice (Fig. 1D) is consistent with previous observations in hypothryroid mice, such as TSHR knockout mice (Marians et al. 2002), the Snell dwarf mice (Cordier et al. 1976), the cug mice (Taylor & Rowe 1987) and the hyt mice (Beamer et al. 1981), and could be recovered by supplementation with thyroid powder (Fig. 1D). Thus, photoperiodic phenotypes in the TSHβ<sup>Luc</sup> mice, if any, can be discriminated from the effects of the impaired HPT axis by supplementation with thyroid powder. Because no obvious photoperiodic phenotype has not been identified in laboratory mice, such as testicular growth, which is a typical photoperiodic phenomenon (Ono et al. 2008), it will be intriguing to further explore photoperiodic phenotypes in laboratory mice with reference to known seasonal physiological changes such as gonad growth in female European hamsters (Hanon et al. 2010), metabolic changes such as adipose depot mass and serum leptin (Atcha et al. 2000) and immune system such as NK cell cytolytic activity and phagocytosis in Siberian hamsters (Yellon et al. 1999; Martin et al. 2008). Even if no obvious photoperiodic phenotype can be identified in laboratory mice, molecular mechanisms underlying the induction of TSHβ by long-day light exposure are evolutionary conserved (Hanon et al. 2008, 2010; Nakao et al. 2008; Ono et al. 2008; Dardente et al. 2010; Dupre et al. 2010; Masumoto et al. 2010; Yashuo et al. 2010). Therefore, TSHβ<sup>Luc</sup> mice, established in this study, will provide useful insights into the evolutionary conserved molecular mechanism of photoperiodism.

### Experimental procedures

#### Generation of TSHβ<sup>Luc</sup> mice

TSHβ<sup>Luc</sup> mice [Tshb(Luc); Acc. No. CDB0713K: http://www.cdb.riken.jp/arg/mutant%20mice%20list.html] were generated as follows: a 1.9 kbp of Luc cassette that contains Luciferase (Luc)–coding region and simian virus 40 polyadenylation signal was amplified by PCR from pGL4.10 (Promega, Madison, WI, USA) using Luc-F–EcoRI (5′-AGAATTCCTACTGTTGGTAAAGCCACCAGGAGAT-3′) and Luc-R–Norl (5′-CGGAGCGCCGCCGATTTTACACCATGGTACAGGTTTTTACTTGCG-3′) primers (Hokkaido System Science, Sapporo, Japan). The amplified product was ligated into the EcoRI/NorI site of pBluescript SK (−) (Stratagene, La Jolla, CA, USA). The 6.3-kb 5′-arm was PCR-amplified from a bacterial artificial chromosome (BAC) clone containing TSHβ (RP24-230F23; BACPAC Resources, Oakland, CA, USA) using 5′–F–SalI (5′-GCCAGGTCCAGCCATAGAGGAACAC-AAGGATCCAAAAGGCTCGTCTCTCTGATCATCAGGAGGAACATCCTTATGACAGGAGATC-3′) primers, and the amplicon was ligated into the SalI/NcoI site upstream of the Luc of pBluescript SK (−) to make the 5′-arm–Luc cassette, in which the Luc cassette was connected in frame to the first ATG of the TSHβ gene. The 5′-arm–Luc cassette was inserted into the SalI/NorI site of the PGK-Neo-pA/DT-A vector (detailed descriptions of the vector are available from http://www.cdb.riken.go.jp/arg/cassesette.html). The 2.8-kbp 3′-arm was also PCR-amplified from the BAC clone using 3′–F–AvrII (5′-GACCCCTAGGATGTGTTCATATGCTATTCTTCTTTAGCTGTAA-3′) and 5′-R–SalI (5′-ATTTAGTCCAGTATCCATTATGCGTTAACACGCGCAACCAC-3′) primers, and the amplicon was ligated into the Xbal/XhoI site of the PGK-Neo-pA/DT-A vector. The resultant targeting vector was linearized with the Ascl site and introduced into TT2 embryonic stem cells by electroporation (Yagi et al. 1993; Murata et al. 2004). Screening of homologous recombinant embryonic stem cells and production of chimera mice are described elsewhere (http://www.cdb.riken.jp/arg/Methods.html). PCR primers used to routinely identify the wild-type allele are P1 (5′-GCCAGGGCCCGAGGATGACTTA GCATGCTATTCTTCTTTAGCTGTAA-3′) and P2 (5′-GCCAGGTCCAGCCATAGAGGAACAC-AAGGATCCAAAAGGCTCGTCTCTCTGATCATCAGGAGGAACATCCTTATGACAGGAGATC-3′), and those to identify the mutant allele are P3 (5′-GCCAGGTCCAGCCATAGAGGAACAC-AAGGATCCAAAAGGCTCGTCTCTCTGATCATCAGGAGGAACATCCTTATGACAGGAGATC-3′) and P4 (5′-GCCAGGTCCAGCCATAGAGGAACAC-AAGGATCCAAAAGGCTCGTCTCTCTGATCATCAGGAGGAACATCCTTATGACAGGAGATC-3′) (Fig. 1A). PCR products of 305 and 446 bp were derived from wild-type and mutant alleles, respectively. For Southern blot analysis, genomic DNA extracted from ES cells or a mouse tail was digested with AvrII and extracted using phenol and chloroform. For blotting, blots were probed with 32P-labeled probes of a 14-kbp fragment of TSHβ cDNA (Accession No. AB159420) and a 6.8-kbp fragment of TSHβ cDNA (Accession No. AB159419).
limiting enzymes for melatonin synthesis. By contrast, CBA/N mice are melatonin proficient and they show significant TSHb induction after long-day stimulation (Ono et al. 2008; Masumoto et al. 2010). Thus, TSHb-Lac mice were backcrossed for more than 10 generations and maintained on a CBA/N background for all experiments except the measurement of growth in Fig. 1D (more than four generations).

**Animals**

Mice were carefully kept and handled according to the RIKEN Regulations for Animal Experiments. For in situ hybridization experiments, L12 : D12 conditioned male 3-week-old wild-type and heterozygous TSHb-Lac mice were housed under short-day conditions (L8 : D16, 400 lux) or under long-day conditions (L16 : D8, 400 lux) for 3 weeks.

For neonatal PT slice culture experiments, neonatal wild-type and heterozygous TSHb-Lac mice were generated by overnight breeding of heterozygous TSHb-Lac and CBA/N mice (Japan SLC, Hamamatsu, Japan). Pregnant mice and pups were housed under L12 : D12 conditions (400 lux) because it was reported that exposure to different light/dark conditions from birth affects circadian properties (Carleighlo et al. 2011). For adult PT slice culture experiments, L12 : D12 conditioned 3-week-old wild-type and heterozygous TSHb-Lac mice were housed under short- or long-day conditions for 2 to 3 weeks. For other experiments, TSHb-Lac mice were housed under L12 : D12 conditions. All mice were given commercial chow and water ad libitum except the supplementation of thyroid powder. Homozygous TSHb-Lac mice were given commercial chow with 100 ppm thyroid powder (Sigma, St. Louis, MO, USA) and water ad libitum (Fig. 1D).

**In situ hybridization**

Mice were deeply anesthetized with isoflurane (Mylan, Tokyo, Japan) and intracardially perfused with 10 mL saline and 20 mL of a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Mouse brain samples were post-fixed in the same fixative for 24 h at 4 °C, soaked in PB containing 20% sucrose for several days and stored frozen at −80 °C for further use. In situ hybridization was carried out as previously described (Shigeyoshi et al. 1997; Masumoto et al. 2010). Serial coronal sections (40 μm thick) of the mouse brain were prepared using a cryostat (CM 1850; Leica Microsystems, Wetzlar, Germany). To prepare probes, fragments of cDNA were obtained by PCR and subcloned into the pGEM-T easy vector (Promega). Radiolabeled probes were generated using [35S]-UTP (PerkinElmer, Norwalk, CT, USA) via a standard protocol for cRNA synthesis. The primers used in the construction of in situ hybridization cRNA probes were as follows:

- **TSHb cRNA probe:**
  - Forward primer: 5′-TGGGTTGGAAGAAGGTGACCG-3′
  - Reverse primer: 5′-ACACAGATTCGACTGCTATTG-3′

Cga cRNA probe:

- Forward primer: 5′-GCAGGCACTGAAAAATCCAGAGACATTTGTC-3′
- Reverse primer: 5′-ACACACAGCGCCATTGAATGGC-TC-3′

Luc cRNA probe:

- Forward primer: 5′-CACCCTGAAGACACTGGGTGTAACCCAC-3′
- Reverse primer: 5′-CAGGGCATCTTTGCCGCCTT-CTTGAC-3′

**PT slice culture**

TSHb-Lac mice were decapitated 1–4 h before light-off. PT slices were prepared from neonatal pups (6–8 days old) or adult mice (5 to 6 weeks old). For neonatal PT slice culture experiments, brains were rapidly removed and were cut by a tissue chopper (McIlwain Tissue Chopper; McIlwain Laboratory Engineering, Gomshall, Surrey, UK) to a thickness of 350 μm. For adult PT slice culture experiments, brains were rapidly removed and were cut by a vibratome type linerslicer (PRO7; Dosaka EM, Kyoto, Japan) to a thickness of 300 μm. The PT region slices were dissected using a surgical knife. The slices were then placed on a culture membrane (MilliCell-CM; Millipore, Bedford, MA, USA) and set on a dish with a 1.2 mL culture medium containing DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 300 mg/L NaHCO3 (Sigma), 20 mg/L L kanamycin (In-vitrogen), 100 mg/L apo-transferrin (Sigma), 100 μM putrescine (Sigma), 20 nM progesterone (Sigma), 30 nM sodium selenite (Sigma) and 1 nM luciferin (Biosynth, Staad, Switzerland). The dish was sealed with silicone grease (HVG; Dow Coming-Toray, Tokyo, Japan). Explants from different pups were cultured separately, and only those of appropriate genotypes were chosen for further experiments. Slices were treated with vehicle [ethanol (Sigma)] or melatonin (Sigma) (Fig. 3 and S2 in Supporting Information). For glutamine and glutamic acid starvation, culture medium with DMEM/F12 without glutamine and glutamic acid (Fig. S3 in Supporting Information). For glutamine treatment, the glutamine- and glutamic acid-starved culture medium was replaced with the above fresh culture medium or with added glutamine (Sigma) or glutamic acid (Sigma) (Fig. S3 in Supporting Information).

**Quantitative RT-PCR (qPCR)**

For qPCR experiments of the pituitary, TSHb-Lac mice were decapitated at ZT2 to 6 under L12 : D12 conditions to collect the part of the tissue embedded in the sella turcica (pituitary fossa) from 12-week-old male TSHb-Lac mice and temporarily stocked at −80 °C for further use. The total RNA was prepared from each pituitary, using RNeasy micro kit (Qiagen, Valencia, CA, USA). The cDNAs were synthe-
sized from 0.25 μg of the total RNA using Superscript III transcriptase (Invitrogen). qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA).

For qPCR experiments of the cultured PT samples, the cultured PT samples were collected 48 h after the start of the culture. The total RNA was prepared from each cultured PT, using NucleoSpin RNA XS kit (Takara, Otsu, Japan). The cDNAs were synthesized from 0.25 μg of the total RNA of the cultured PT using Superscript Vilo transcriptase (Invitrogen). qPCR was performed using Quantitect SYBR Green PCR mastermix (Qiagen).

qPCR was performed using the ABI PRISM 7900 (Applied Biosystems) at the following conditions: Samples contained 1 × SYBR Green PCR Master Mix or QuantiTect SYBR Green PCR Master Mix, 0.8 μl primers and 1/50 synthesized cDNA in a 10-μl volume. The PCR conditions were as follows: 10 min at 95 °C, then 45 cycles of 15 s at 94 °C, 1 min at 59 °C. The absolute cDNA abundance was calculated using a standard curve obtained from murine genomic DNAs. We used TATA box binding protein (Tβp) or actin, beta (Actb) as the internal controls. Primer information is as follows:

**TSHβ mRNA (exon 4):**

Forward primer: 5′-GTGGGCAACGACATCCCTTTTGT-3′
Reverse primer: 5′-GCACACTCTCTCTATCCACGTAC-3′

**Cδα mRNA:**

Forward primer: 5′-TGCTGACGGCCAGGCTTCAATG-3′
Reverse primer: 5′-GAAGTCTGGTAGGAGGAGGGTG-3′

**Luc mRNA:**

Forward primer: 5′-TCCCTCAACGTGCAAAAGACGCCACCT-3′
Reverse primer: 5′-GTCGGTTCTTCTATCCATGTATGCATGATC-3′

**Actb mRNA:**

Forward primer: 5′-TTGTCCCCTCAACTTGTG-3′
Reverse primer: 5′-CCTGGCTGCCTCAACACTCCT-3′

**TSHβ mRNA:**

Forward primer: 5′-CTGCATAACAGGCGTCTGCA-3′
Reverse primer: 5′-GCCAGATAGAAAGACTGCGG-3′

**Eya3 mRNA:**

Forward primer: 5′-TTCAGCTCAAAGTAGTGAATCTGAT-3′
Reverse primer: 5′-TATGGAAAGCCGCTATCC-3′

**Tβp mRNA:**

Forward primer: 5′-CCCCCTCTGACTGAAATC-3′
Reverse primer: 5′-GGTGCCAGCAGCAGGCAA-3′

### PMT bioluminescence measurements

The time-course bioluminescence results were obtained as reported previously (Isojima et al. 2009). PMT measurements with a high-sensitivity bioluminescence detection system (LM-2400; Hamamatsu Photonics, Hamamatsu, Japan) were started immediately after the start of PT slice culture at the time of light-off (ZT12 for neonatal PT slices, ZT8 or 16 for adult short- or long-day slices). For the data analysis in Fig. 3B,D, raw photon counts were plotted. For the data analysis in Figs 3C,E and S3 in Supporting Information, photon counts of the PT slice from heterozygous TSHβ<sup>−/−</sup> mice were calculated by subtracting background photon counts of PT slice from wild-type mice. The resultant values were further normalized by photon counts at ZT4.75, which is 12.75 h after the start of the culture in adult long-day slices (Fig. 3C), or at ZT23, which is 11 h after the start of the culture in neonatal PT slices (Fig. 3E), which were the median time for the lowest bioluminescence values from all vehicle-treated samples in adult and neonatal slices, respectively (Fig. S4 in Supporting Information). The resultant values were normalized by photon counts at ZT13.45, which is just before glutamine and glutamic acid treatment in neonatal PT slices (Fig. S3 in Supporting Information).

### Bioluminescence imaging

Samples were prepared as described above. Sealed 35-mm culture dishes were placed on the stage of a macrozoom microscope (MVX10; Olympus, Tokyo, Japan) in a dark hood. The culture dishes were kept at approximately 37 °C in a heated chamber (Tokai Hit, Shizuoka, Japan) on the microscope stage. Bioluminescence was imaged using a 1.6 × Plan Apo-chromat objective (NA 0.24; Olympus) with 6.3 × zoom and transmitted to an electron-multiplying charge-coupled device (EM-CCD) camera cooled to ~80 °C (ImagEM; Hamamatsu Photonics). The dimension of an image is 512 × 512 pixels, and each pixel corresponds to the size of 1.587 × 1.587 μm. Exposure time was 29 min with EM gain of 200 × for snapshot images, and exposure time was 14 min with EM gain of 300 × for time-lapse images. Images were transferred at 690 KHz to minimize readout noise, and analyzed using MetaMorph software (Molecular device, Sunnyvale, CA, USA). Single-cell tracking was performed as previously described (Ukai et al. 2007).

### Mass spectrometry

The YALSQDVCTYR peptide, which was the unique sequence for TSHβ, was synthesized on a peptide synthesizer (Syro Wave; Biotage, Uppsala, Sweden) using Fmoc solid-phase chemistry. The peptide was dimethyl-labeled with formaldehyde (CD<sub>2</sub>O) and desalted by C18 StageTips according to previously described method (Boersma et al. 2009). The dimethyl labeling of the peptide was checked by liquid chromatography coupled with tandem mass spectrometry (LC-MS/
MS) with an LTQ orbitrap velos mass spectrometer (Thermo scientific, Waltham, MA, USA) coupled to a nano-Advance UHPLC system (Bruker Daltonics, Leipzig, Germany).

The culture medium of the PT slices was collected and temporarily stocked at −80 °C for further use. Proteolytic digestion was performed using a phase-transfer surfactant protocol (Masuda et al. 2008; Narumi et al. 2012). The resultant digest was dimethyl-labeled with formaldehyde (CH2O).

For quantification by mass spectrometry, a triple quadrupole mass spectrometer (TSQ Vantage EMR; Thermo scientific) was used to design a selected reaction monitoring (SRM) method. LC-SRM/MS was performed by a TSQ Vantage EMR mass spectrometer equipped with a nanoLC interface (AMR), a nano-Advance UHPLC system (Bruker Daltonics) and an HTC-PAL autosampler (CTC Analytics, Basingstoke, UK). The parameters of the mass spectrometer were set as follows: 0.002 m/z scan width, 40 msec scan time, 0.7 fwhm Q1 and Q3 resolution and 1.8 mTorr gas pressure.

For data analysis of the peak area in the chromatogram of each SRM transition, the peak area was extracted by using Pinpoint software (Thermo scientific). To confirm whether the peaks of SRM chromatogram were derived from the endogenous YALSQDVCTYR peptide, we checked that ratios among the peak areas of 6 SRM transitions were comparable with those of the standard YALSQDVCTYR peptide. Three of six SRM transitions, which had a lower variability in the ratio between the 18 runs of LC-SRM/MS analysis, were used for the quantification of the endogenous YALSQDVCTYR peptide. Chromatograms of SRM transition are shown in Fig. S5 in Supporting Information. Interassay coefficient of variation was 7.9%. The lower limit of sensitivity was 13.27 attomole.

Statistical analyses

All data are shown as means ± SEM. Comparison of two groups was made using two-sample t-test. A value of P < 0.05 was considered as statistically significant. The analyses were performed by Microsoft Excel (Microsoft, Redmond, WA, USA) or R software (see The Foundation for Statistical Computing, http://www.R-project.org).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web site:

Figure S1 (A) Time-lapse images of neonatal PT slice from heterozygous TSHβ+ mice kept under L12:D12 conditions. Scale bar: 100 µm. (see also Video S1 in Supporting Information) (B) Representative bioluminescence patterns in individual neonatal PT cells in Fig. S1A. Scale bar: 100 µm.

Figure S2 Confirmation of expression level changes of TSHβ, Eya3, and Cga in the cultured PT by qPCR analysis.
**Figure S3** Real-time monitoring of the luciferase reporter activities in neonatal PT slices with glutamine or glutamic acid treatment.

**Figure S4** Histograms of the bottom time of bioluminescence of adult long-day PT samples ($n = 19$, upper) and neonatal PT samples ($n = 157$, bottom) that were treated with vehicle.

**Video S1** Real-time imaging of bioluminescence in a neonatal PT slice for 4 days.

**Figure S5** The chromatograms of each selected reaction monitoring (SRM) transition.