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Cryptochrome deficiency enhances transcription but reduces protein levels of pineal *Aanat*

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Abstract

Cryptochrome (*Cry*) 1 and 2 are essential for circadian rhythm generation, not only in the suprachiasmatic nucleus, the site of the mammalian master circadian clock, but also in peripheral organs throughout the body. CRY is also known as a repressor of arylalkylamine-N-acetyltransferase (*Aanat*) transcription; therefore, *Cry* deficiency is expected to induce constantly high pineal melatonin content. Nevertheless, we previously found that the content was consistently low in melatonin-proficient *Cry1* and *Cry2* double-deficient mice (*Cry1*^{-/-}/*Cry2*^{-/-}) on C3H background. This study aims to clarify the mechanism underlying this discrepancy. In the *Cry1*^{-/-}/*Cry2*^{-/-} pineal, expression levels of *Aanat* and clock gene *Per1* were consistently high with no circadian fluctuation on the first day in constant darkness, demonstrating that CRY acts *in vivo* as a repressor of the pineal circadian clock and AANAT. In contrast, the enzyme activity and protein levels of AANAT remained low throughout the day, supporting our previous observation of continuously low melatonin. Thus, effects of *Cry* deficiency on the responses of β -adrenergic receptors were examined in cultured pineal glands. Isoproterenol, a β -adrenergic stimulant, significantly increased melatonin content, although the increase was smaller in *Cry1*^{-/-}/*Cry2*^{-/-} than in WT mice, during both the day and night. However, the increase in cAMP in response to forskolin was similar in both genotypes, indicating that CRY deficiency does not affect the pathway downstream of the β -adrenergic receptor. These results suggest that a lack of circadian adrenergic input due to CRY deficiency decreases β -receptor activity and cAMP levels, resulting in consistently low AANAT levels despite abundant *Aanat* mRNA.

Key Words

- ▶ melatonin
- ▶ pineal gland
- ▶ cryptochrome
- ▶ arylalkylamine-N-acetyltransferase
- ▶ C3H mice

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Introduction

Pineal melatonin synthesis in mammals is regulated by the circadian pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus. The circadian pacemaker is entrained to the environmental light–dark cycle by photic signals via the retinohypothalamic tract (Rusak & Zucker 1979) and controls melatonin synthesis in the pineal

gland through the sympathetic nerve, which releases noradrenalin at night (Larsen *et al.* 1998). Elimination of neural input to the pineal gland demonstrated an importance of this neural pathway not only for the nocturnal melatonin synthesis (Deguchi & Axelrod 1972) but also for day/night changes in transcription

of thousands of genes (Hartley *et al.* 2015). Activation of the β 1-adrenergic receptor in pinealocytes is known to be a critical step to increase intracellular cAMP. Increasing intracellular cAMP leads to a rapid activation of arylalkylamine N-acetyltransferase (AANAT), the rate-limiting enzyme in melatonin synthesis (Sugden *et al.* 1985, Klein 2007) by inhibiting proteasomal proteolysis (Ganguly *et al.* 2001) as well as by activating transcription level (Ganguly *et al.* 2005).

The molecular machinery generating the mammalian intracellular circadian rhythm is a transcription/translation feedback loop (core loop) involving the clock genes *Period* (*Per*) 1, *Per*2, Cryptochrome (*Cry*) 1, *Cry*2, *Clock* and *Bmal1* and their protein products (Reppert & Weaver 2002). The promoter region of the *Aanat* gene contains an E-box element that is activated by CLOCK and BMAL1 (Chen & Baler 2000). Therefore, it is likely that pineal melatonin synthesis is also under the control of the core loop. Mutation or loss of these canonical clock genes alters not only circadian behavioral rhythms but also pineal melatonin synthesis in melatonin-proficient laboratory mice (Kennaway *et al.* 2003, Christ *et al.* 2010, Yamanaka *et al.* 2010). In *Cry1* and *Cry2* double-deficient (*Cry1*^{-/-}/*Cry2*^{-/-}) mice, expression of the clock genes *Per1* and *Per2* in the SCN and peripheral tissues are elevated and lose circadian rhythmicity under a light–dark (LD) cycle or constant darkness (DD) (Okamura *et al.* 1999, Van der Horst *et al.* 1999). These results are consistent with the core molecular loop model, since a lack of CRY, a repressor of *Per* transcription, results in the elevation of transcription (Reppert & Weaver 2002). In addition, a circadian rhythm has been observed in *Cry1* and *Cry2* expression in the rat pineal gland under a normal LD cycle and constant darkness (Nakamura *et al.* 2001, Simonneaux *et al.* 2004), suggesting the functioning of pineal peripheral clock. We previously examined the circadian profile of pineal melatonin content in *Cry1*^{-/-}/*Cry2*^{-/-} mice on a melatonin-proficient C3H background and found that the content was constantly low, without nocturnal increase (Yamanaka *et al.* 2010). The mechanism underlying a lack of nocturnal melatonin synthesis in *Cry1*^{-/-}/*Cry2*^{-/-} mice remains unexplored. Furthermore, this result seems to contradict the core molecular loop model wherein *Aanat* mRNA expression and melatonin levels are expected to be high throughout the day. Thus, *Cry1*^{-/-}/*Cry2*^{-/-} mice provide an ideal model for identifying the site of circadian regulation by the master clock in the SCN and the mechanism of transcriptional regulation of *Aanat* in the peripheral clock in the pineal gland. In the current study, we aim to identify

the site at which CRY is critically involved in the circadian melatonin synthesis pathway and to determine whether the core molecular loop regulates *Aanat* expression.

Materials and methods

Animals and housing

Cry1^{-/-}/*Cry2*^{-/-} mice of C3H background (Masuki *et al.* 2005, Tanida *et al.* 2007) were the kind gift of Dr. Todo. The mice were maintained at the Hokkaido University Graduate School of Medicine as described previously (Yamanaka *et al.* 2010). Adult *Cry1*^{-/-}/*Cry2*^{-/-} ($n=324$) and WT (C3H) ($n=283$) control mice of both sexes were used for the present experiments. Mice were born and reared in animal quarters, where environmental conditions were controlled (lights on from 0600 to 1800h; light intensity at the bottom of cage, approximately 100lux; humidity, 50–60%). Food and water were available *ad libitum*. The present study was performed in compliance with the rules and regulations established by the Animal Care and Use Committee of Hokkaido University with the permission of the Animal Research Committee of Hokkaido University (approval no.13-0064).

Sampling of pineal glands

Pineal glands were sampled from 3- to 6-month-old WT ($n=40$) and *Cry1*^{-/-}/*Cry2*^{-/-} ($n=36$) mice under either LD or the first cycle after releasing to DD. Time of lights on was defined as Zeitgeber time 0 (ZT0) and that of lights-off as ZT12. The mice were killed by cervical dislocation without anesthesia at four time points (ZT 6, 10, 18, and 22; $n=4$ –5 per group) under LD or DD, and the pineal gland was collected under a dissecting microscope. Collection of the pineal gland during the dark phase was performed after cervical dislocation and eye enucleation under dim red light (<0.1 lux, <30 s).

Melatonin radioimmunoassay

Pineal glands were quickly removed at the indicated time and placed in 200 μ L of ice-cold assay buffer (0.1 M Tris–HCl, pH 7.2). The tissue was homogenized by repeated freezing and thawing in liquid nitrogen for three cycles. The pineal homogenates were centrifuged for 15 min at 21,500 g , and the supernatant was stored at -30°C . The melatonin content of each sample was determined by radioimmunoassay (RIA) as previously described (Yamanaka *et al.* 2010). Each sample was assayed using

200 μ L of supernatant. The minimum detection level for the melatonin concentration was 1.56 pg/tube. Intra- and inter-assay coefficient of variances were 5.6 and 6.2%, respectively.

Quantitative real-time PCR analysis of pineal *Aanat* and *Per1* mRNA

Period 1 (*Per 1*) and *Aanat* mRNAs in the pineal gland were measured by quantitative real-time reverse transcription-PCR (qRT-PCR) as previously reported (Nishide et al. 2012) with some modifications. The pineal glands of the WT ($n=30$) and *Cry1*^{-/-}/*Cry2*^{-/-} ($n=30$) mice were quickly put in ice-cold tissue storage reagent (RNA later, R0901, Sigma-Aldrich) at six time points (ZT 2, 6, 10, 14, 18, and 22; $n=5$ per group) under LD or DD and stored at -80°C until mRNA extraction. Total RNA was extracted using RNeasy Micro Kit (Qiagen), and the concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized using SuperScriptIII First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's instruction. qRT-PCR was performed with DyNAmo HS SYBR Green qPCR Kit (New England BioLabs, Ipswich, MA, USA) and a real-time thermal cycler (ABI PRISM 7700, Applied Biosystems) using the following primers: *mGAPDH* forward: 5'TGCGACTTCAACAGCAACTC3'; *mGAPDH* reverse: 5'ATGTAGGCCATGAGGTCCAC3'; *mAanat* forward: 5'CTCCAGCCCATCCAAGTAG3'; *mAanat* reverse: 5'TGCACAGTTCAGAAAGGCAAG3'; *mPer1* forward: 5'CGTCCTACCTCCTTTATCCAGA3'; *mPer1* reverse: 5'TGTTTGCATCAGTGTCATCAGC3'.

Measurement of pineal AANAT enzyme activity

The AANAT enzyme activity was assayed according to the method reported by Deguchi & Axelrod (1972) with slight modification. Pineal glands of the WT ($n=30$) and *Cry1*^{-/-}/*Cry2*^{-/-} ($n=31$) were quickly removed at one of six times of the day in DD (ZT2, 6, 10, 14, 18, and 22; $n=5-6$ per group). Each pineal gland was homogenized in chilled 0.05 M phosphate buffer (50 μ L, pH. 6.5) and stored at -30°C until the assay. On the day of assay, 10 μ L each of 0.01 M Tryptamine-HCL and 3.4 nM ($1-^{14}\text{C}$) acetyl coenzyme A (PerkinElmer) was added to 50 μ L of homogenate in a small glass tube. After incubating at 37°C for 10 min, the reaction was stopped by the addition of 0.5 mL of 0.5 M borate buffer (pH 10.0). The homogenate was transferred into a glass tube containing 6 mL of toluene-isoamyl alcohol (97:3)

and stirred using a vortex mixer. After centrifugation at 3000 g for 10 min, 5 mL of the organic phase was transferred into a scintillation vial containing 5 mL of scintillation cocktail (Aquazol2, PerkinElmer), and radioactivity was measured.

Immunoblotting of pineal AANAT protein

The AANAT protein concentration was determined by immunoblotting using the method reported elsewhere (Nishide et al. 2012) with some modifications. Briefly, the pineal glands were collected at four time points (ZT6, 10, 18 and 22) in DD. The pineal glands were pooled (WT, $n=8$; *Cry1*^{-/-}/*Cry2*^{-/-}, $n=12$) in tissue protein extraction reagent (T-PER, Thermo Scientific) and homogenized by sonication followed by freezing and thawing four times in liquid nitrogen. The homogenized samples were frozen in liquid nitrogen and stored at -80°C until assay. After centrifuging at 21,500 g for 15 min at 4°C , the supernatant protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). The protein extracts (50 μ g/lane) were separated using SDS- PAGE. A sample of rat pineal gland (2.5 μ g/lane) collected at ZT22 (expected peak of pineal AANAT protein) was used as an inter-assay standard. The sample was electrophoresed in NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen) according to the manufacturer's protocol and transferred onto a Hybond-P PVDF Membrane (GE Healthcare). The membrane was incubated with a blocking reagent (Block Ace, DS Pharma, Osaka, Japan) overnight at 4°C , followed by incubation with goat anti-AANAT (P-20) antibody (Santa Cruz Biotechnology) diluted 1:500 with immunoreaction enhancer solution (Can Get Signals, Toyobo, Osaka, Japan) for 1 h at room temperature, and then with HRP-conjugated donkey anti-goat IgG (Promega) diluted 1:500,000 in Block Ace buffer (Pharma Biomedical, Tokyo, Japan) for 1 h at room temperature. Immunoreactive bands were detected using Super Signal, the enhanced chemiluminescent substrate for HRP (Thermo Fisher Scientific) and Hyperfilm ECL (GE Healthcare). The membrane was stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and reacted with mouse anti β -ACTIN IgG (Sigma-Aldrich) and subsequently with HRP-conjugated goat anti-mouse IgG (Thermo Fisher Scientific). The bands of interest were quantified using ImageJ software (National Institutes of Health). The band intensities were calculated after subtracting the lane background from the raw data. The immunoblotting was repeated three times.

Pineal gland organ culture and isoproterenol stimulation

Pineal glands were collected from mice housed in LD at ZT3 (3 h after lights on) (WT, $n=24$; *Cry1*^{-/-}/*Cry2*^{-/-}, $n=23$) mice and ZT15 (3 h after lights-off) (WT, $n=24$; *Cry1*^{-/-}/*Cry2*^{-/-}, $n=20$). Each pineal gland was placed on a culture membrane (Millicell PICM01250, Merck Millipore) placed into a well of a 24-well plate and incubated with 200 μ L of Dulbecco's Modified Eagle's Medium (DMEM; GIBCO/Thermo Fischer Scientific) at 37°C in a humidified atmosphere with 5% CO₂ for 1 h. After this pre-incubation, the culture medium was replaced with DMEM containing 200 μ L of the β -adrenergic agonist isoproterenol (ISO; final concentration, 2 μ M) or vehicle (water) at projected ZT4 and 16. The dose was reported to increase *Aanat* mRNA and melatonin levels in cultured pineal gland most efficiently (Barbosa et al. 2008, Wongchitrat et al. 2011). After incubation for 2 h, each pineal gland was rinsed three times with 0.1 M Tris-HCl buffer (pH 7.2) and homogenized in 200 μ L of Tris-HCl buffer by four cycles of freezing in liquid nitrogen and thawing. Two hours of incubation time was selected according to our preliminary experiment and previous reports (Santana et al. 1988, Fukuhara et al. 2005, Wongchitrat et al. 2011). The pineal homogenates were centrifuged at 21,500 g for 15 min at 4°C and the supernatants were stored at -80°C until measurement. The melatonin content of each sample was determined by the RIA.

AANAT enzyme activity after stimulation with isoproterenol and norepinephrine

Pineal glands of the WT ($n=18$; male $n=9$, female $n=9$) and *Cry1*^{-/-}/*Cry2*^{-/-} ($n=19$; male $n=9$, female $n=10$) mice were collected from mice housed in LD at ZT3. After preincubating for 1 h with DMEM, they were transferred to fresh medium containing either 2 μ M of ISO or 10 μ M of norepinephrine (NE) and ascorbic acid (0.01 mg/mL) at projected ZT4. After the treatment for 2 h, the pineal glands were rinsed three times with 0.05 M of phosphate buffer (PB, pH 6.5) and homogenized in 50 μ L of PB by four cycles of freezing in liquid nitrogen and thawing. The pineal homogenates were stored at -80°C until measurement. The AANAT activity was determined by the RIA.

Cyclic AMP content in the pineal gland after treatment with forskolin

Pineal glands of the WT ($n=21$; male $n=9$, female $n=12$) and *Cry1*^{-/-}/*Cry2*^{-/-} ($n=21$; male $n=10$, female $n=11$) mice

were sampled from mice kept in LD at ZT3 and individually incubated with 200 μ L of DMEM at 37°C and 5% CO₂ for 30 min. After this pre-incubation, 200 μ L of forskolin (FSK, final concentration, 50 μ M) or vehicle (DMEM) was added to the culture medium at projected ZT3.5 and incubated at 37°C and 5% CO₂ for 1 h. The dose was reported to increase cAMP level and melatonin synthesis in cultured pineal gland (Santana et al. 1988, 2001). The cultured pineal glands were rinsed by transferring them three times to new culture wells containing 200 μ L of cold 0.1 M Tris-HCl buffer (pH 7.2). The pineal gland was placed in a tube containing 200 μ L of cold 0.1 M Tris-HCl buffer (pH 7.2) and homogenized by four cycles of freezing in liquid nitrogen and thawing. The pineal homogenates were stored at -80°C until assayed. The cAMP level in the pineal homogenate was measured using the cAMP-Glo Max assay kit (Promega) by monitoring bioluminescence with luminometer.

Statistical analysis

Data are reported as the mean $\pm$ S.E.M. A one-way ANOVA (main effect of time) followed by a *post hoc* Tukey-Kramer test was used to determine the time-dependent difference for each genotype. A two-way ANOVA (main effect of genotype and interaction between genotype and time) followed by a *post hoc* unpaired *t*-test was used to compare the data between the two genotypes. $P < 0.05$ was considered statistically significant.

Results

Circadian rhythms in pineal melatonin content in *Cry1*^{-/-}/*Cry2*^{-/-} mice

The circadian profile of pineal melatonin content under LD and DD differed significantly between WT and *Cry1*^{-/-}/*Cry2*^{-/-} mice ($P < 0.001$), with a significant interaction between genotype and time (two-way ANOVA test) (Fig. 1). Under both LD and DD conditions, WT mice exhibited a robust circadian variation, with a significant difference found between time points ($P < 0.001$; main effect time, one-way ANOVA), with the peak level at ZT22 (178 ± 35 pg/pineal in LD, 238 ± 44 pg/pineal in DD, $n=5$). In contrast, *Cry1*^{-/-}/*Cry2*^{-/-} mice exhibited no significant circadian variation in pineal melatonin content ($P=0.12$ in LD; $P=0.23$ in DD; main effect time, one-way ANOVA test, $n=4-5$). Under LD, the pineal melatonin content was significantly lower in *Cry1*^{-/-}/*Cry2*^{-/-} mice than in WT mice in the light phase ($P < 0.01$ at ZT6; $P < 0.05$ at ZT10; two-way ANOVA test with *post hoc* unpaired *t*-test)

Figure 1

Circadian profiles of pineal melatonin content in LD and DD. Pineal melatonin content was examined every 6 h as shown in WT (open circles) and *Cry1*^{-/-}/*Cry2*^{-/-} (closed circles) mice. Mice were either maintained under a 12:12 h LD cycle (A) or exposed to constant darkness (DD) for 1 day (B). Dark gray area indicates either dark phase of LD cycle or the subjective night phase under DD. Light gray area indicates the subjective day in DD. Data are presented as the mean $\pm$ s.e.m. ($n=4-5$). * $P<0.05$, ** $P<0.01$ vs *Cry1*^{-/-}/*Cry2*^{-/-} (post-hoc unpaired t-test followed by two-way ANOVA).

as well as in the dark phase ($P<0.01$ at ZT18 and ZT22). Under DD, the melatonin content was significantly lower in *Cry1*^{-/-}/*Cry2*^{-/-} mice than in WT mice at ZT18 ($P<0.05$) and ZT22 ($P<0.001$).

Circadian rhythms in *Per 1* and *Aanat* mRNA expression in the pineal lacking CRYs

Aanat is reported to have an E-box enhancer in the first intron, the function of which in the circadian regulation of *Aanat* expression is still unknown (Baler et al. 1999, Klein 2007). To examine the functional role of this E-box in *Aanat* expression, we determined the circadian profile of *Per1* and *Aanat* mRNA in *Cry1*^{-/-}/*Cry2*^{-/-} mice. In the WT mice, both *Per1* (Fig. 2A) and *Aanat* (Fig. 2B) mRNA levels in the pineal gland showed a significant rhythmicity (*Per1*, $P<0.05$; *Aanat*, $P<0.05$; main effect

Figure 2

Circadian profiles of pineal *Per1* and *Aanat* mRNA levels. Twenty-four-hour patterns of mRNA levels of pineal *Per1* (A) and *Aanat* (B) in wild-type (open circles) and *Cry1*^{-/-}/*Cry2*^{-/-} (closed circles) mice on the 1st day in DD. Shadowed areas indicate the dark phase. Dark and light gray areas indicate a subjective night and day, respectively. Data are presented as the mean $\pm$ s.e.m. ($n=5$). * $P<0.05$, ** $P<0.01$ vs *Cry1*^{-/-}/*Cry2*^{-/-} (post hoc unpaired t-test followed by two-way ANOVA).

time, one-way ANOVA test, $n=5$) and were significantly elevated in the subjective night as compared with those in the mid-subjective day ($P<0.05$, post-hoc Tukey–Kramer test). The peak of *Per1* expression was detected at ZT14, while that of *Aanat* was between ZT18 and ZT22. In *Cry1*^{-/-}/*Cry2*^{-/-} mice, the mRNA levels of both genes were constant throughout the day, exhibiting no significant rhythmicity ($P=0.23$; $P=0.81$; main effect time one-way ANOVA, $n=5$). Compared with WT, the *Per1* mRNA levels in *Cry1*^{-/-}/*Cry2*^{-/-} mice were significantly higher throughout the day except at ZT14, when the level was lower than that of wild type ($P<0.01$). Despite the findings of low pineal melatonin levels, the *Aanat* mRNA level in *Cry1*^{-/-}/*Cry2*^{-/-} mice was consistently high at the circadian peak level of WT mice with statistical significance during the mid-subjective day (ZT6 and 10) and early subjective night (ZT14) ($P<0.01$).

Circadian rhythm in pineal AANAT enzyme activity and protein concentration

To identify at which stage of melatonin synthesis the dissociation takes place between the consistently high *Aanat* mRNA and low melatonin levels, we examined the circadian rhythms in the levels of AANAT protein and enzyme activity. In WT mice, the AANAT protein concentration in the pineal gland exhibited a circadian rhythm with a peak level at ZT22 ($P<0.001$, one-way ANOVA test). Whereas in *Cry1^{-/-}/Cry2^{-/-}* mice, AANAT protein did not exhibit a significant rhythm across the four circadian phases examined (Fig. 3), instead remaining at intermediate levels of the circadian amplitude in WT throughout the day. To apply the same amount of total protein for electrophoresis, we pooled the extracts from 8 and 12 pineal glands from the WT and *Cry1^{-/-}/Cry2^{-/-}* mice, respectively. The results indicate that the total protein content is lower in *Cry1^{-/-}/Cry2^{-/-}* pineal glands. Similarly, the AANAT enzyme activity in WT mice showed a significant rhythmicity ($P<0.001$; main effect time, one-way ANOVA) (Fig. 4), with the peak reached at the late subjective night (ZT22), increased from the low daytime level ($P<0.05$, ZT2, ZT6, ZT10 vs ZT22; *post-hoc* Tukey–Kramer test). In contrast, the AANAT enzyme activity in *Cry1^{-/-}/Cry2^{-/-}* mice did not show significant rhythmicity across the circadian phases ($P=0.07$). Circadian patterns differed significantly between the two genotypes ($P<0.001$, two-way ANOVA), with a significant difference at ZT 22 ($P=0.002$, *post-hoc* unpaired *t*-test).

Effects of β -adrenergic stimulation on melatonin synthesis in the pineal lacking CRYs

We then sought to determine the mechanism underlying the discrepancy between *Aanat* mRNA and protein/enzyme activity levels in *Cry1^{-/-}/Cry2^{-/-}* pineal glands. The increase in intracellular cAMP in response to β -adrenergic stimulation via neural inputs from the SCN is known to be critical for stabilizing the AANAT protein and increasing melatonin synthesis (Ganguly et al. 2001). Therefore, we asked whether pinealocytes lacking CRYs can respond to β -adrenergic stimulation to increase melatonin synthesis. To determine the day–night difference, pineal glands of WT and *Cry1^{-/-}/Cry2^{-/-}* mice were sampled at two time points, ZT3 and ZT15, and stimulated with isoproterenol (ISO, 2 μ M) for 2 h after a pre-incubation of 1 h. In wild-type pineal glands, ISO treatment significantly increased melatonin content compared with those of vehicle-treated controls, both at ZT3 (vehicle vs ISO, 174 ± 18 pg/gland

Figure 3

Circadian rhythm of AANAT protein levels in WT and *Cry1^{-/-}/Cry2^{-/-}* mice. Representative western blot of pineal AANAT in WT and *Cry1^{-/-}/Cry2^{-/-}* mice shown together with that of β -actin (A). Relative level of AANAT in the pooled pineal glands (B). Data are presented as the mean $\pm$ S.E.M. of three independent experiments. White and black columns indicate the relative AANAT protein level of WT and *Cry1^{-/-}/Cry2^{-/-}* mice. The values are normalized to the amount of β -actin in each sample.

Figure 4

Circadian profile of pineal AANAT enzyme activity. Twenty-four-hour profile of AANAT activity in WT (open circles) and *Cry1^{-/-}/Cry2^{-/-}* (closed circles) mice in DD. Dark and light gray areas indicate subjective night and day, respectively. Data are presented as the mean $\pm$ S.E.M. ($n=5-6$). ** $P<0.01$ vs *Cry1^{-/-}/Cry2^{-/-}* (*post hoc* unpaired *t*-test followed by two-way ANOVA).

($n=12$) vs 544 ± 104 pg/gland ($n=12$); $P=0.002$, two-way ANOVA test with *post hoc* unpaired *t*-test) and ZT15 (vehicle vs ISO, 60 ± 14 pg/gland ($n=12$) vs 393 ± 91 pg/gland ($n=11$), $P=0.002$). In the *Cry1*^{-/-}/*Cry2*^{-/-} pineal gland, the melatonin content was also significantly elevated by ISO treatment, both at ZT3 (vehicle vs ISO, 34 ± 4 pg/gland ($n=13$) vs 124 ± 29 pg/gland ($n=10$); $P=0.004$) and at ZT15 (vehicle vs ISO, 32 ± 3 pg/gland ($n=10$) vs 65 ± 14 pg/gland ($n=10$); $P=0.045$) compared to the respective vehicle control. However, the response to ISO was markedly lower in the *Cry1*^{-/-}/*Cry2*^{-/-} than that in the wild type, both at ZT3 and ZT15 (ZT3, $P<0.001$; ZT15, $P=0.004$) (Fig. 5A). Three-way ANOVA revealed a significant interaction between the genotype (wild-type and *Cry1*^{-/-}/*Cry2*^{-/-}) and the stimulation (vehicle and ISO) ($P=0.003$), but no significant interaction the genotype and the stimulation time).

Effect of ISO and NE on the AANAT enzyme activity of the pineal lacking CRYs

To explore the mechanism of the different response to ISO in the wild-type and *Cry1*^{-/-}/*Cry2*^{-/-} mice, the pineal glands were sampled at ZT3, and the AANAT activity was measured 2 h after the stimulation with 2 μ M of ISO or 10 μ M of NE at projected ZT4. We examined only at ZT3 because no stimulation time effect was detected by ISO in either genotype. The AANAT activity after stimulation with ISO tended to increase the AANAT activity on average as compared with the vehicle control in WT and *Cry1*^{-/-}/*Cry2*^{-/-} mice, though it did not reach statistical significance (Fig. 5B). After the stimulation with NE, the significant increases of the AANAT activity were observed in both genotypes at similar extent (Fig. 5C). Two-way ANOVA test revealed significant effect of genotype (WT and *Cry1*^{-/-}/*Cry2*^{-/-}) ($P=0.011$) and the stimulation (Vehicle vs NE) ($P=0.002$), but no significant interaction between the genotype and stimulation.

Effect of FSK stimulation on the pineal cAMP level

To assess whether the decreased melatonin synthesis in the *Cry1*^{-/-}/*Cry2*^{-/-} mice is caused by a decrease in intracellular cAMP or defects in the downstreams, the effect of FSK (5 μ M and 50 μ M) on cAMP levels were examined in cultured pineal glands sampled at ZT3. In both genotypes, FSK stimulation significantly increased the cAMP level in the pineal gland as compared to vehicle-treated controls (vehicle, 5 μ M FSK and 50 μ M FSK, 205 ± 10 nM ($n=7$), 441 ± 48 nM ($n=7$, $P<0.01$) and 540 ± 33 nM ($n=7$, $P<0.01$) for WT; 125 ± 20 nM ($n=7$), 380 ± 45 nM ($n=7$, $P<0.01$)

Figure 5

Adrenergic stimulant induced melatonin synthesis and AANAT activity in cultured pineal glands. Cultured pineal glands from WT (open columns) and *Cry1*^{-/-}/*Cry2*^{-/-} (closed columns) mice show a significant increase in melatonin content after the stimulation with isoproterenol (ISO) compared with vehicle controls at the two circadian phases examined. Abscissa indicates the time of tissue sampling. Responses differed significantly between genotypes but not time of day. Data are presented as the mean $\pm$ s.e.m., ($n=10-13$). * $P<0.05$, ** $P<0.01$ vs vehicle; † $P<0.05$ vs *Cry1*^{-/-}/*Cry2*^{-/-} mice (*post hoc* unpaired *t*-test followed by two-way ANOVA) (A). The AANAT activity in the pineal glands of wild-type (open columns) and *Cry1*^{-/-}/*Cry2*^{-/-} (closed columns) mice after stimulation with 2 μ M of ISO (B) or 10 μ M NE (C) for 2 h. Data are presented as the mean $\pm$ s.e.m., ($n=4-5$). * $P<0.05$ vs vehicle in each genotype (*post hoc* unpaired *t*-test followed by two-way ANOVA).

and 397 ± 66 nM ($n=7$, $P<0.01$) for *Cry1*^{-/-}/*Cry2*^{-/-}, respectively) (Fig. 6). The *Cry1*^{-/-}/*Cry2*^{-/-} pineal exhibited FSK-induced increase in cAMP by FSK to a similar extent as WT. Thus, no genotype difference was detected in the response to FSK. Two-way ANOVA revealed a significant effect of stimulation (vehicle and FSK) on the cAMP level in the pineal gland ($P<0.001$), but there was no significant interaction between genotype (WT and *Cry1*^{-/-}/*Cry2*^{-/-}) and stimulation. While no significant dose-response was observed in the two doses examined, cAMP tended to increase with a higher dose in both genotypes.

Discussion

Our findings demonstrate that a loss of circadian melatonin rhythm in *Cry1*^{-/-}/*Cry2*^{-/-} mice is mainly caused by the loss of rhythmic noradrenergic signals from

Figure 6

Intracellular cAMP after forskolin stimulation in cultured pineal glands. Cultured pineal glands sampled at ZT3 from WT (open column) and *Cry1^{-/-}/Cry2^{-/-}* (closed columns) mice demonstrate similar increases in cAMP content after the treatment with forskolin (FSK). Data are presented as the mean $\pm$ S.E.M. ($n=7$). ** $P<0.01$ vs vehicle (post hoc unpaired t-test followed by two-way ANOVA).

the circadian pacemaker in the SCN and partly caused by a decreased response to β -adrenergic signals. The pineal gland of *Cry1^{-/-}/Cry2^{-/-}* mice showed consistently high *Aanat* mRNA expression and low AANAT protein and enzyme activity throughout the day. The discrepancy between these findings is explained by the lack of rhythmic β -adrenergic signals (Klein & Moore 1979, Klein *et al.* 1983a) by loss of neural input from the SCN, because cAMP-dependent phosphorylation of AANAT leads binding with 14-3-3 protein, which activates the AANAT activity (Ganguly *et al.* 2005) and protects from proteasomal degradation of AANAT (Ganguly *et al.* 2001). In addition, the consistently high *Aanat* mRNA levels observed in the *Cry1^{-/-}/Cry2^{-/-}* pineal glands indicate that the E-box enhancer plays a functional role in the regulation of *Aanat* expression.

Cry genes are core components of the autoregulatory transcription/translation feedback loop (core loop) in mammalian circadian clocks. *Cry1^{-/-}/Cry2^{-/-}* mice demonstrated circadian rhythm in behavior under LD but immediately became arrhythmic after the transfer to DD (Van der Horst *et al.* 1999). Furthermore, expression of the clock genes *Per1* and *Per2* and multiunit neural activity in the SCN did not show circadian rhythms under DD (Okamura *et al.* 1999, Vitaterna *et al.* 1999, Albus *et al.* 2002). Nevertheless, we recently observed that individual SCN cells of *Cry1^{-/-}/Cry2^{-/-}* mice exhibit circadian rhythms in clock gene expression and that the arrhythmicity in clock gene expression at the SCN

tissue level in adults is caused by desynchronization of individual cellular rhythms in the course of postnatal development (Ono *et al.* 2013). However, in the neonatal period, circadian rhythms in single SCN cells synchronize mainly via neuropeptides released in the SCN to exhibit robust circadian rhythms at the tissue level (Ono *et al.* 2016). If the low level of melatonin production in *Cry1^{-/-}/Cry2^{-/-}* mice is due to the absence of melatonin synthesis via rhythmic neuronal signals from the SCN, the pineal glands of *Cry1^{-/-}/Cry2^{-/-}* mice should exhibit increased melatonin production in response to ISO. However, we observed here that ISO-induced melatonin production occurred in both pineal glands sampled during subjective day (ZT3) and night (ZT15), suggesting that the *Cry1^{-/-}/Cry2^{-/-}* pineal glands can synthesize melatonin. Since the WT pineal glands also responded to ISO at both time points, this response to noradrenergic stimulation appears to be ungated. The ungated response has also been reported in the *Aanat* and *Per1* mRNA levels of cultured pineal gland after the treatment with ISO (Fukuhara *et al.* 2005, Wongchitrat *et al.* 2011). Taken together, these findings indicate that the loss of circadian rhythm of pineal melatonin expression in *Cry1^{-/-}/Cry2^{-/-}* mice is caused by a loss of rhythmic output signals from the SCN to the pineal gland.

In rodents, pineal AANAT enzyme activity dramatically increases at night (Deguchi & Axelrod 1972) in response to noradrenalin released under the control of the circadian clock in the SCN (Perreau-Lenz *et al.* 2003). Upon binding of noradrenalin to the pineal β -adrenergic receptor, intracellular cAMP content increases (Sugden *et al.* 1985, Ho *et al.* 1988, 1989), which leads to an activation of adenylate cyclase. This effect is enhanced by $\alpha 1$ receptors via Ca^{2+} and phosphatidyl inositol activation of protein kinase C (Klein *et al.* 1983b, Ho & Klein 1987, Sugden & Klein 1988, Ho *et al.* 1989), thereby activating *Aanat* transcription via cAMP-responsive elements in the promoter and 1st intron of *Aanat* (Humphries *et al.* 2007). Stimulation of $\alpha 1$ - and β -adrenergic receptors results in a marked increase in both cAMP and cGMP accumulation in pinealocytes (Vanecek *et al.* 1986, Sugden & Klein 1987). The effects of cGMP analogs on pineal physiology are usually weaker than the actions of cAMP analogs; however, it remains to be studied whether or not the cGMP accumulation is involved in the melatonin synthesis of the pineal gland of *Cry1^{-/-}/Cry2^{-/-}* mice.

Another possible mechanism underlying the observed circadian rhythms in *Aanat* expression is the E-box-mediated increase in a clock-controlled gene in the pineal peripheral clock (Chen & Baler 2000). The *Aanat*

gene also has a canonical E-box (CACGTG) and E-box-like (CACATG) sequences in the 1st intron (Humphries *et al.* 2007); however, the role of the peripheral clock in the pineal gland has still been unclear. Previously, transcription of retinal but not pineal *Aanat* was shown to be increased by CLOCK and BMAL1 (Chen & Baler 2000), and the intronic E-box was reported to be involved not in the circadian regulation of transcription but in the tissue-specific silencing of *Aanat* expression (Humphries *et al.* 2007). With respect to the rhythmic pattern of AANAT transcription, pinealocyte clock of zebrafish and chicken is reported to drive AANAT directly via E-box-mediated transcription increase (Chong *et al.* 2000, Appelbaum *et al.* 2004). In the present study, a lack of CRY, the negative element of the core molecular clock, resulted in a continual increase in *Aanat* expression, similar to *Per1* expression, indicating for the first time that the intronic E-box of the mouse *Aanat* gene has a functional role *in vivo*. Although no reports are available examining double labeling of CRY and AANAT so far, a continual increase of AANAT mRNA in the present results suggests that CRYs are expressed in the same cell as AANAT.

In addition to promoting *Aanat* transcription, an increase in cAMP also has a significant effect on nocturnal increases in melatonin synthesis that occurs via post-translational modifications of AANAT (Klein 2007). The AANAT protein is phosphorylated in a cAMP-dependent manner. The phosphorylation of AANAT leads to the formation of its complex with the 14-3-3 protein (pAANAT/14-3-3 complex), which protects AANAT from degradation (Ganguly *et al.* 2001) and elevates its enzymatic activity (Ganguly *et al.* 2005). Nocturnal melatonin synthesis critically depends on intracellular cAMP. Thus, melatonin synthesis is strictly influenced by the intracellular cAMP level, which is under the regulation of neural inputs from the circadian pacemaker in the SCN. According to our proposed mechanism, the decreased melatonin synthesis in *Cry1^{-/-}/Cry2^{-/-}* mice might be caused by the decrease in the intracellular cAMP level at night caused by a lack of coherent rhythm output from the SCN.

Although CRY deficiency resulted in a lack of circadian output from the SCN, we found that the pineal gland of *Cry1^{-/-}/Cry2^{-/-}* mice was capable of producing melatonin in response to noradrenergic signals. The response to ISO was reduced in *Cry1^{-/-}/Cry2^{-/-}* as compared to that of WT mice. Nevertheless, AANAT activity of *Cry1^{-/-}/Cry2^{-/-}* pineal increased by NE stimulation to the similar extent to the WT, suggesting significant role of $\alpha 1$ adrenergic receptor in melatonin synthesis. The same

amount of ISO significantly increased melatonin content but the increase in AANAT activity was not statistically significant. The reason of the discrepancy is not known, but the difference in assay system might be involved. The possible mechanisms underlying this observation include the desynchronization of cellular rhythms in the pineal caused by a lack of entraining signals from the SCN and, alternatively, a decreased number and/or sensitivity of β -receptors on the pinealocytes. Nevertheless, the pathways for circadian melatonin synthesis downstream of β -receptors are intact in *Cry1^{-/-}/Cry2^{-/-}* pineal glands, indicating that CRYs are not involved in intracellular melatonin synthesis pathways, since NE and FSK increased AANAT activity and cAMP levels, respectively, in cultured pineal glands of *Cry1^{-/-}/Cry2^{-/-}* similar to those in WT mice (Fig. 6). Together, these results indicate that the pineal gland of *Cry1^{-/-}/Cry2^{-/-}* mice can induce melatonin synthesis in response to increased cAMP levels following NE release from the sympathetic nerve terminals. Previously, Deguchi & Axelrod (1972) reported the super-sensitivity *in vitro* to ISO stimulation in the denervated pineal gland and pineals from rats exposed to continuous lighting. Whereas in the present study, AANAT activity in *Cry1^{-/-}/Cry2^{-/-}* pineal was continuously low throughout the day but increased by the stimulation of NE to the similar level of the WT. Therefore, upregulation of adrenergic receptors was prevented, somehow, in the pineals lacking CRYs. CRY deficiency may affect the availability of receptor site and/or number of $\alpha 1$ - and β -adrenergic receptor.

The limitation of the present study is that we used conventional *Cry*-knockout mice. CRY deficiency may have long-term effects on the development of adrenergic innervation of the pineal gland, which needs to be studied using time-dependent *Cry* gene targeting in future. Furthermore, it has been reported that adrenergic stimulation turns on transcription of nearly 600 genes via the same signaling pathway that induces *Aanat* gene (Bailey *et al.* 2009, Ho & Chik 2010). Genome-scale analysis might reveal a novel role of CRY in the pineal physiology.

In conclusion, the present data demonstrate that continuously low pineal melatonin in *Cry1^{-/-}/Cry2^{-/-}* mice is associated with continuously high *Aanat* mRNA levels and low AANAT protein and enzyme activity. E-box elements of the *Aanat* gene are involved in transcriptional regulation in the pineal gland *in vivo*. Finally, *Cry* genes are a necessary requirement for generating the circadian rhythm in pineal melatonin synthesis. CRYs are critically involved in the intracellular cAMP increase in response to nocturnal noradrenalin stimulation, which is disrupted

in the *Cry1^{-/-}/Cry2^{-/-}* SCN due to desynchronization of cellular rhythms (Ono et al. 2013). CRYs are not necessary for melatonin synthesis via intracellular pathways.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y Yamanaka and S Honma designed the experiments; Y Yamanaka, Y Yamada and S Honma performed the experiments; Y Yamanaka, Y Yamada and S Honma analyzed the data; and Y Yamanaka, K Honma and S Honma wrote the paper.

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