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

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Short title: Melatonin synthesis in *Cry*-deficient mice

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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 wild-type mice, during both the day and night. Whereas, 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.

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 β1-adrenergic receptor in pinealocytes is known to be a critical step to increase intracellular cyclic AMP (cAMP). Increasing intracellular cAMP leads to a rapid activation of arylalkylamine N-acetyltransferase (AANAT), the rate-limiting enzyme in melatonin synthesis (Klein *et al.* 2007, Sugden *et al.* 1985) through inhibiting proteasomal proteolysis (Ganguly *et al.* 2001) as well as 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*, *Per2*, *Cryptochrome* (*Cry*) *1*, *Cry2*, *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

74 melatonin synthesis is also under the control of the core loop. Mutation or loss of these
75 canonical clock genes alters not only circadian behavioral rhythms but also pineal melatonin
76 synthesis in melatonin-proficient laboratory mice (Christ *et al.* 2010, Kennaway *et al.* 2003,
77 Yamanaka *et al.* 2010). In *Cry1* and *Cry2* double-deficient (*Cry1*$^{-/-}$/*Cry2*$^{-/-}$) mice, expression
78 of the clock genes *Per1* and *Per2* in the SCN and peripheral tissues are elevated and lose
79 circadian rhythmicity under a light–dark (LD) cycle or constant darkness (DD) (Okamura *et*
80 *al.* 1999, Van der Horst *et al.* 1999). These results are consistent with the core molecular loop
81 model, since a lack of CRY, a repressor of *Per* transcription, results in elevation of
82 transcription (Reppert & Weaver 2002). In addition, a circadian rhythm has been observed in
83 *Cry1* and *Cry2* expression in the rat pineal gland under a normal LD cycle and constant
84 darkness (Nakamura *et al.* 2001; Simonneaux *et al.* 2004), suggesting the functioning of
85 pineal peripheral clock. We previously examined the circadian profile of pineal melatonin
86 content in *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice on a melatonin-proficient C3H background and found that the
87 content was constantly low, without nocturnal increase (Yamanaka *et al.* 2010). The
88 mechanism underlying a lack of nocturnal melatonin synthesis in *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice
89 remains unexplored. Furthermore, this result seems to contradict the core molecular loop
90 model wherein *Aanat* mRNA expression and melatonin levels are expected to be high
91 throughout the day. Thus, *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice provide an ideal model for identifying the site
92 of circadian regulation by the master clock in the SCN and the mechanism of transcriptional

93 regulation of *Aanat* in the peripheral clock in the pineal gland. In the current study, we aim to
94 identify the site at which CRY is critically involved in the circadian melatonin synthesis
95 pathway and to determine whether the core molecular loop regulates *Aanat* expression.
96
97 **Materials and methods**
98 **Animals and housing**
99 *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice of C3H background (Masuki *et al.* 2005, Tanida *et al.* 2007) were
100 the kind gift of Dr. Todo. The mice were maintained at the Hokkaido University Graduate
101 School of Medicine as described previously (Yamanaka *et al.* 2010). Adult *Cry1*$^{-/-}$/*Cry2*$^{-/-}$
102 (*n*=324) and wild-type (C3H) (*n*=283) control mice of both sexes were used for the present
103 experiments. Mice were born and reared in animal quarters, where environmental conditions
104 were controlled (lights on from 0600 to 1800 hr; light intensity at the bottom of cage,
105 approximately 100 lux; humidity, 50–60%). Food and water were available ad libitum. The
106 present study was performed in compliance with the rules and regulations established by the
107 Animal Care and Use Committee of Hokkaido University with the permission of the Animal
108 Research Committee of Hokkaido University (approval no.13-0064).
109
110 **Sampling of pineal glands**
111 Pineal glands were sampled from 3- to 6-month-old wild-type (*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 euthanized 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 sec).

Melatonin radioimmunoassay (RIA)

Pineal glands were quickly removed at the indicated time and placed in 200 μL 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 15,000 rpm, 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 μL 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 *Period1* 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 wild-type (n = 30) and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ (n = 30) mice were quickly put in ice-cold tissue storage regent (RNA later, R0901, Sigma-Aldrich, St. Louis, MO, USA) 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, Hilden, Germany), and the concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). 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, Foster City, CA, USA) using the following primers: *mGAPDH* forward: 5'TGCGACTTCAACAGCAACTC3'; *mGAPDH* reverse: 5'ATGTAGGCCATGAGGTCCAC3'; *mAanat* forward: 5'CTCCAGCCCATCCAACTAG3'; *mAanat* reverse: 5'TGCACAGTTCAGAAGGCAAG3'; *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 wild-type (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 0.05M phosphate buffer (50 µL, pH. 6.5) and stored at −30°C until the assay. On the day of assay, 10µL each of 0.01M Tryptamine-HCL and 3.4 nM [1-^{14}C] acetyl coenzyme A (PerkinElmer, Walthan MA, USA) was added to 50µL 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 3,500 rpm 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 (wild-type, n = 8; *Cry1*$^{-/-}$/*Cry2*$^{-/-}$, n = 12) in tissue protein extraction regent (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

169 at −80°C until assay. After centrifuging at 15,000 rpm for 15 min at 4°C, the supernatant
170 protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher
171 Scientific). The protein extracts (50 μg/lane) were separated using SDS-polyacrylamide gel
172 electrophoresis (PAGE). A sample of rat pineal gland (2.5 μg/lane) collected at ZT22
173 (expected peak of pineal AANAT protein) was used as an inter-assay standard. The sample
174 was electrophoresed in NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA,
175 USA) according to the manufacturer's protocol and transferred onto a Hybond-P PVDF
176 Membrane (GE Healthcare, Little Chalfont, UK). The membrane was incubated with a
177 blocking reagent (Block Ace, DS Pharma, Osaka, Japan) overnight at 4°C, followed by
178 incubation with goat anti-AANAT (P-20) antibody (Santa Cruz Biotechnology, Santa Cruz,
179 CA) diluted 1:500 with immunoreaction enhancer solution (Can Get Signals, Toyobo, Osaka,
180 Japan) for 1 h at room temperature, and then with horseradish peroxidase (HRP)-conjugated
181 donkey anti-goat IgG (Promega, Madison, WI, USA) diluted 1:500,000 in Block Ace buffer
182 (Pharma Biomedical, Tokyo, Japan) for 1 h at room temperature. Immunoreactive bands were
183 detected using Super Signal, the enhanced chemiluminescent substrate for HRP (Thermo
184 Fisher Scientific) and Hyperfilm ECL (GE Healthcare, Little Chalfont, UK). The membrane
185 was stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and
186 reacted with mouse anti β-ACTIN IgG (Sigma-Aldrich) and subsequently with
187 HRP-conjugated goat anti-mouse IgG (Thermo Fisher Scientific). The bands of interest were

188 quantified using ImageJ software (National Institutes of Health). The band intensities were
189 calculated after subtracting the lane background from the raw data. The immunoblotting was
190 repeated three times.
191
192 **Pineal gland organ culture and isoproterenol stimulation**
193 Pineal glands were collected from mice housed in LD at project ZT3 (3 h after lights-on)
194 (wild-type, $n = 24$; *Cry1*$^{-/-}$/*Cry2*$^{-/-}$, $n = 23$) mice and project ZT15 (3 h after lights-off)
195 (wild-type, $n = 24$; *Cry1*$^{-/-}$/*Cry2*$^{-/-}$, $n = 20$). Each pineal gland was placed on a culture
196 membrane (Millicell PICM01250, Merck Millipore, Billerica, MA, USA) placed into a well
197 of a 24-well plate, and incubated with 200μL of Dulbecco's Modified Eagle's Medium
198 (DMEM; GIBCO/Thermo Fischer Scientific) at 37°C in a humidified atmosphere with 5%
199 CO_2 for 1 h. After this pre-incubation, the culture medium was replaced with DMEM
200 containing 200 μL of the β-adrenergic agonist isoproterenol (ISO; final concentration, 2 μM)
201 or vehicle (water). The dose was reported to increase *Aanat* mRNA and melatonin levels in
202 cultured pineal gland most efficiently (Barbosa *et al.* 2008, Wongchitrat *et al.* 2011). After
203 incubation for 2 h, each pineal gland was rinsed three times with 0.1M Tris-HCl buffer (pH
204 7.2) and homogenized in 200 μL of Tris-HCl buffer by four cycles of freezing in liquid
205 nitrogen and thawing. Two hours of incubation time was selected according to our
206 preliminary experiment and previous reports (Santana *et al.* 1988, Fukuhara *et al.* 2005,

207 Wongchitrat *et al.* 2011). The pineal homogenates were centrifuged at 15,000 rpm for 15 min
208 at 4°C and the supernatants were stored at −80 °C until measurement. The melatonin content
209 of each sample was determined by the RIA.
210
211 **AANAT enzyme activity after stimulation with isoproterenol and norepinephrine**
212 Pineal glands of the wild-type (n = 18; male n = 9, female n = 9) and $Cry1^{-/-}/Cry2^{-/-}$ (n =
213 19; male n = 9, female n = 10) mice were collected from mice housed in LD at project ZT3.
214 After preincubating for 1 h with DMEM, they were transferred to fresh medium containing
215 either 2μM of ISO or 10μM of norepinephrine (NE) and ascorbic acid (0.01mg/ml). After the
216 treatment for 2 h, the pineal glands were rinsed three times with 0.05M of phosphate buffer
217 (PB, pH6.5) and homogenized in 50 μL of PB by four cycles of freezing in liquid nitrogen
218 and thawing. The pineal homogenates were stored at −80 °C until measurement. The AANAT
219 activity was determined by the RIA.
220
221 **Cyclic AMP content in the pineal gland after treatment with forskolin**
222 Pineal glands of the wild-type (n = 21; male n = 9, female n = 12) and $Cry1^{-/-}/Cry2^{-/-}$ (n
223 = 21; male n = 10, female n = 11) mice were sampled from mice kept in LD at project ZT3
224 and individually incubated with 200 μL of DMEM at 37°C and 5% CO_2 for 30 min. After this
225 pre-incubation, 200 μL of forskolin (FSK, final concentration, 50 μM) or vehicle (DMEM)

was added to the culture medium and incubated at 37°C and 5% CO_2 for 1 h. The dose was reported to increase cAMP level and melatonin synthesis in cultured pineal gland (Santana *et al.* 1988, Santana *et al.* 2001). The cultured pineal glands were rinsed by transferring them three times to new culture wells containing 200 μL of cold 0.1M Tris-HCl buffer (pH 7.2). The pineal gland was placed in a tube containing 200 μL of cold 0.1M 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 assays. 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 ± SEM. 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 wild-type 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, wild-type 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 wild-type 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) 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 wild-type mice at ZT18 ($P < 0.05$) and ZT22 ($P < 0.001$).

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

Aanat is reported to have an E-box enhancer in the 1st 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 wild-type 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 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 wild type, 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 wild-type 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 wild-type mice, the AANAT protein concentration in the pineal 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 wild-type 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 wild-type 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 wild-type and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice were sampled at two time points, ZT3 and ZT15, and stimulated with isoproterenol (ISO, 2μM) 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 [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 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 were measured after the stimulation with 2μM of ISO or 10μM of NE for 2 h. 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 wild-type 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 two genotypes at similar extent (Fig. 5C). Two-way ANOVA test revealed significant effect of genotype (wild-type 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 μM and 50 μM) on cAMP levels were examined in cultured pineal glands. In both genotypes, FSK stimulation significantly increased the cAMP level in the pineal gland as compared to vehicle-treated controls (vehicle, 5 μM FSK and 50 μM FSK, 205 ± 10 nM [n = 7], 441 ± 48 nM [n = 7, P < 0.01] and 540 ± 33 nM [n = 7, P < 0.01] for wild type; 125 ± 20

nM [n = 7], 380 ± 45 nM [n = 7, $P < 0.01$] 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 wild type. 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 (wild-type 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 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

359 (Ganguly *et al.* 2001). In addition, the consistently high *Aanat* mRNA levels observed in the
360 *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ pineal glands indicate that the E-box enhancer plays a functional role in the
361 regulation of *Aanat* expression.

362 *Cry* genes are core components of the autoregulatory transcription/translation feedback
363 loop (core loop) in mammalian circadian clocks. *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice demonstrated circadian
364 rhythm in behavior under LD but immediately became arrhythmic after the transfer to DD
365 (Van der Horst *et al.* 1999). Furthermore, expression of the clock genes *Per1* and *Per2* and
366 multiunit neural activity in the SCN did not show circadian rhythms under DD (Albus *et al.*
367 2002, Okamura *et al.* 1999, Vitaterna *et al.* 1999). Nevertheless, we recently observed that
368 individual SCN cells of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice exhibit circadian rhythms in clock gene
369 expression and that the arrhythmicity in clock gene expression at the SCN tissue level in
370 adults is caused by desynchronization of individual cellular rhythms in the course of postnatal
371 development (Ono *et al.* 2013). Whereas in the neonatal period, circadian rhythms in single
372 SCN cells synchronize mainly via neuropeptides released in the SCN to exhibit robust
373 circadian rhythms at the tissue level (Ono *et al.* 2016). If the low level of melatonin
374 production in *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice is due to the absence of melatonin synthesis via rhythmic
375 neuronal signals from the SCN, the pineal glands of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice should exhibit
376 increased melatonin production in response to ISO. However, we observed here that
377 ISO-induced melatonin production occurred both during subjective day (ZT3) and night

(ZT15), suggesting that the pineal glands of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice can synthesize melatonin. Since the wild-type 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 (Wongchitrat *et al.* 2011, Fukuhara *et al.* 2005). 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 α1 receptors via Ca^{2+} and phosphatidyl inositol activation of protein kinase C (Klein *et al.* 1983b, Ho & Klein 1987, 1989, Sugden & Klein 1988), thereby activating *Aanat* transcription via cAMP-responsive elements in the promoter and 1st intron of *Aanat* (Humphries *et al.* 2007). Stimulation of α1- and β-adrenergic receptors results in a marked increase in both cAMP and cGMP accumulation in pinealocytes (Sugden & Klein 1987, Vanecek *et al.* 1986). 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

397 melatonin synthesis of the pineal gland of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice. The cGMP accumulation in
398 the pineal gland in *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice might be associated with the AANAT activity and
399 melatonin synthesis.

400 Another possible mechanism underlying the observed circadian rhythms in *Aanat*
401 expression is the E-box–mediated increase in a clock-controlled gene in the pineal peripheral
402 clock (Chen & Baler 2000). The *Aanat* gene also has a canonical E-box (CACGTG) and
403 E-box–like (CACATG) sequences in the 1st intron (Humphries *et al.* 2007), however, the role
404 of the peripheral clock in the pineal gland has still been unclear. Previously, transcription of
405 retinal but not pineal *Aanat* was shown to be increased by CLOCK and BMAL1 (Chen &
406 Baler 2000), and the intronic E-box was reported to be involved not in the circadian
407 regulation of transcription but in the tissue-specific silencing of *Aanat* expression (Humphries
408 *et al.* 2007). With respect to the rhythmic pattern of AANAT transcription, pinealocyte clock
409 of zebrafish and chicken is reported to drive AANAT directly via E-box mediated
410 transcription increase (Chong *et al.* 2000; Appelbaum *et al.* 2004). In the present study, a lack
411 of CRY, the negative element of the core molecular clock, resulted in a continual increase in
412 *Aanat* expression, similarly to *Per1* expression, indicating for the first time that the intronic
413 E-box of the mouse *Aanat* gene has a functional role *in vivo*. Although no reports are
414 available examining double labeling of CRY and AANAT so far, a continual increase of
415 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 occur via post-translational modifications of AANAT (Klein *et al.* 2007). The AANAT protein is phosphorylated in a cAMP-dependent phosphorylation of AANAT leads formation of a complex with the 14-3-3 protein (pAANAT/14-3-3 complex), which protects AANAT from degradation (Ganguly *et al.* 2001) and elevates 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 wild-type mice. Nevertheless, AANAT activity of *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ pineal increased by NE stimulation to the similar extent to the wild type, suggesting significant role of α1 adrenergic receptor in melatonin synthesis. The same amount of ISO significantly increased melatonin content but the increase AANAT activity was not statistically significant. The

435 reason of the discrepancy is not known, but the difference in assay system might be involved.
436 The possible mechanisms underlying this observation include the desynchronization of
437 cellular rhythms in the pineal caused by a lack of entraining signals from the SCN and,
438 alternatively, a decreased number and/or sensitivity of β-receptors on the pinealocytes.
439 Nevertheless, the pathways for circadian melatonin synthesis downstream of β-receptors are
440 intact in *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ pineal glands, indicating that CRYs are not involved in intracellular
441 melatonin synthesis pathways, since NE and FSK increased AANAT activity and cAMP
442 levels, respectively, in cultured pineal glands of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ similar to those in wild-type
443 mice (Fig. 6). Together, these results indicate that the pineal gland of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice
444 can induce melatonin synthesis in response to increased cAMP levels following NE release
445 from the sympathetic nerve terminals. Previously, Deguchi and Axelrod (1972) reported the
446 super-sensitivity *in vitro* to ISO stimulation in the denervated pineal gland and pineals from
447 rats exposed to continuous lighting. Whereas in the present study, AANAT activity in
448 *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ pineal was continuously low throughout the day but increased by the
449 stimulation of NE to the similar level of the wild type. Therefore, upregulation of adrenergic
450 receptors was prevented, somehow, in the pineals lacking CRYs. CRY deficiency may affect
451 the availability of receptor site and/or number of α1- and β-adrenergic receptor.
452 The limitation of the present study is that we used conventional *Cry* knockout mice. CRY
453 deficiency may have long-term effects on the development of adrenergic innervation of the

454 pineal gland, which needs to be studied using time-dependent *Cry* gene targeting in future.
455 Furthermore, it has been reported that adrenergic-stimulation turn on transcription of over
456 600 genes are induced by same signaling pathway that induces *Aanat* gene (Bailey *et al.* 2009,
457 Ho & Chik 2010). Genome-scale analysis might reveal a novel role of CRY in the pineal
458 physiology.

459 In conclusion, the present data demonstrate that continuously low pineal melatonin in
460 *Cry1*$^{-/-}$/*Cry2*$^{-/}$ mice is associated with continuously high *Aanat* mRNA levels and low
461 AANAT protein and enzyme activity. E-box elements of the *Aanat* gene are involved in
462 transcriptional regulation in the pineal gland *in vivo*. Finally, *Cry* genes are a necessary
463 requirement for generating the circadian rhythm in pineal melatonin synthesis. CRYs are
464 critically involved in the intracellular cAMP increase in response to nocturnal noradrenalin
465 stimulation, which is disrupted in the *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ SCN due to desynchronization of
466 cellular rhythms (Ono *et al.* 2013). CRYs are not necessary for melatonin synthesis via
467 intracellular pathways.

468

469 **Declaration of interest**

470 The authors declare that research was conducted with no commercial or financial relationship
471 that could be interpreted as a potential conflict of interest.

472

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Author contributions 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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References

Albus H, Bonnefont X, Chaves I, Yasui A, Doczy J, van der Horst GT, Meijer JH 2002 Cryptochrome-deficient mice lack circadian electrical activity in the suprachiasmatic

492 nuclei. *Curr Biol* **12** 1130-1133.

493 Appelbaum L, Toyama R, Dawid IB, Klein DC, Baler R & Gothilf Y 2004 Zebrafish

494 serotonin-N-acetyltransferase-2 gene regulation: pineal-restrictive downstream module

495 contains a functional E-box and three photoreceptor conserved elements. *Molecular*

496 *Endocrinology* 18 1210–1221.

497 Bailey MJ, Coon SL, Carter DA, Humphries A, Kim JS, Shi Q, Gaildrat P, Morin F, Ganguly

498 S, Hogenesch JB, *et al.* 2009 Night/day changes in pineal expression of >600 genes:

499 central role of adrenergic/cAMP signaling. *J Biol Chem* **284** 7606–7622.

500 Baler R, Covington S, Klein DC 1999 Rat arylalkylamine N-acetyltransferase gene: upstream

501 and intronic components of a bipartite promoter. *Biol Cell* **91** 699-705.

502 Barbosa R, Scialfa JH, Terra IM, Cipolla-Neto J, Simonneaux V, Afeche SC 2008 Tryptophan

503 hydroxylase is modulated by L-type calcium channels in the rat pineal gland. *Life Sci* **82**

504 529-535.

505 Chen W & Baler R 2000 The rat arylalkylamine N-acetyltransferase E-box: differential use in

506 a master vs. a slave oscillator. *Brain Res Mol Brain Res* **81** 43-50.

507 Chong NW, Bernard M & Klein DC 2000 Characterization of the chicken serotonin

508 N-acetyltransferase gene. Activation via clock gene heterodimer/E box interaction.

509 Journal of Biological Chemistry 275 32991–83299.

510 Christ E, Pfeffer M, Korf HW, von Gall C 2010 Pineal melatonin synthesis is altered in

511 Period1 deficient mice. *Neuroscience* **171** 398-406.

512 Deguchi T & Axelrod J 1972 Induction and super induction of serotonin N-acetyltransferase
513 by adrenergic drugs and denervation in rat pineal organ. *Proc Natl Acad Sci U S A* **69**
514 2208-2211.

515 Fukuhara C, Yamazaki S, Liang J 2005 Pineal circadian clocks gate arylalkylamine
516 N-acetyltransferase gene expression in the mouse pineal gland. *J Neurochem* **93**
517 156-162.

518 Ganguly S, Gastel JA, Weller JL, Schwartz C, Jaffe H, Namboodiri MA, Coon SL, Hickman
519 AB, Rollag M, Obsil T, et al. 2001 Role of a pineal cAMP-operated arylalkylamine
520 N-acetyltransferase/14-3-3-binding switch in melatonin synthesis. *Proc Natl Acad Sci U*
521 *S A* **98** 8083-8088.

522 Ganguly S, Weller JL, Ho A, Chemineau P, Malpaux B, Klein DC 2005 Melatonin synthesis:
523 14-3-3-dependent activation and inhibition of arylalkylamine N-acetyltransferase
524 mediated by phosphoserine-205. *Proc Natl Acad Sci USA* **102** 1222-1227.

525 Hartley SW, Coon SL, Savastano LE, Mullikin JC; NISC Comparative Sequencing Program,
526 Fu C, and Klein DC 2015 Neurotranscriptomics: The Effects of Neonatal Stimulus
527 Deprivation on the Rat Pineal Transcriptome. *PLoS One* **10(9)** e0137548.

528 Ho AK, and Klein DC 1987 Activation of alpha 1-adrenoceptors, protein kinase C, or
529 treatment with intracellular free Ca2+ elevating agents increases pineal phospholipase
530 A2 activity. Evidence that protein kinase C may participate in Ca2+-dependent alpha
531 1-adrenergic stimulation of pineal phospholipase A2 activity. *J Biol Chem* **262(24)**

11764-11770.

Ho AK, Chik CL, and Klein DC 1988 Permissive role of calcium in alpha 1-adrenergic stimulation of pineal phosphatidylinositol phosphodiesterase (phospholipase C) activity. *J Pineal Res* **5(6)** 553-564.

Ho AK, Chik CL, and Klein DC 1989 Forskolin stimulates pinealocyte cGMP accumulation. Dramatic potentiation by an alpha 1-adrenergic----[Ca2+]i mechanism involving protein kinase C. *FEBS Lett* **249(2)** 207-212.

Ho AK, Chik CL 2010 Modulation of Aanat gene transcription in the rat pineal gland. *J Neurochem* **112** 321–331

Humphries A, Wells T, Baler R, Klein DC, Carter DA 2007 Rodent Aanat: intronic E-box sequences control tissue specificity but not rhythmic expression in the pineal gland. *Mol Cell Endocrinol* **270** 43-49.

Kennaway DJ, Voultsios A, Varcoe TJ, Moyer RW 2003 Melatonin and activity rhythm responses to light pulses in mice with the Clock mutation. *Am J Physiol Regul Integr Comp Physiol* **284** R1231–R1240.

Klein DC & Moore RY 1979 Pineal N-acetyltransferase and hydroxyindole-O-methyltransferase: control by the retinohypothalamic tract and the suprachiasmatic nucleus. *Brain Res* **174** 245-262.

Klein DC 2007 Arylalkylamine N-acetyltransferase: “the Timezyme.” *J Biol Chem* **282** 4233-4237.

Klein DC, Smoot R, Weller JL, Higa S, Markey SP, Creed GJ, Jacobowitz DM 1983a Lesions

of the paraventricular nucleus area of the hypothalamus disrupt the suprachiasmatic leads to spinal cord circuit in the melatonin rhythm generating system. *Brain Res Bull* **10** 647-652.

Klein DC, Sugden D, Weller JL 1983b Postsynaptic alpha-adrenergic receptors potentiate the beta-adrenergic stimulation of pineal serotonin N-acetyltransferase. *Proc Natl Acad Sci U S A* **80** 599-603.

Larsen PJ, Enquist LW, Card JP 1998 Characterization of the multisynaptic neuronal control of the rat pineal gland using viral transneuronal tracing. *Eur J Neurosci* **10** 128-145.

Masuki S, Todo T, Nakano Y, Okamura H, Nose H 2005 Reduced alpha-adrenoceptor responsiveness and enhanced baroreflex sensitivity in Cry-deficient mice lacking a biological clock. *J Physiol* **566** 213-224.

Nakamura TJ, Shinohara K, Funabashi T, Mitsushima D, Kimura F 2001 Circadian and photic regulation of cryptochrome mRNAs in the rat pineal gland. *Neurosci Res* **41**(1) 25-32.

Nishide SY, Ono D, Yamada Y, Honma S, Honma K 2012 De novo synthesis of PERIOD initiates circadian oscillation in cultured mouse suprachiasmatic nucleus after prolonged inhibition of protein synthesis by cycloheximide. *Eur J Neurosci* **35** 291-299.

Okamura H, Miyake S, Sumi Y, Yamaguchi S, Yasui A, Muijtjens M, Hoeijmakers JH, van der Horst GT 1999 Photic induction of mPer1 and mPer2 in cry deficient mice lacking a

572 biological clock. *Science* **286** 2531-2534.

573 Ono D, Honma S, Honma K 2013 Cryptochromes are critical for the development of coherent
574 circadian rhythms in the mouse suprachiasmatic nucleus. *Nat Commun* **4** 1666.doi:
575 10.1038/ncomms2670.

576 Ono D, Honma S, Honma K 2016 Differential roles of AVP and VIP signaling in the postnatal
577 changes of neural networks for coherent circadian rhythms in the SCN. *Sci Adv* **2**
578 e1600960.doi: 10.1126/sciadv.1600960.

579 Perreau-Lenz S, Kalsbeek A, Garidou ML, Wortel J, van der Vliet J, van Heijningen C,
580 Simonneaux V, Pévet P, Buijs RM 2003 Suprachiasmatic control of melatonin synthesis
581 in rats: inhibitory and stimulatory mechanisms. *Eur J Neurosci* **17** 221-228.

582 Reppert SM, & Weaver DR 2002 Coordination of circadian timing in mammals. *Nature* **418**
583 935-941.

584 Rusak B, & Zucker I 1979 Neural regulation of circadian rhythms. *Physiol Rev* **59** 449–526.

585 Santana C, Guerrero JM, Reiter RJ, Troiani ME 1988 The in vitro activation of cyclic AMP
586 production by either forskolin or isoproterenol in the Syrian hamster pineal during the
587 day is not accompanied by an increase in melatonin production. *Biochem Biophys Res*
588 *Commun* **157** 930–936.

589 Santana C, Moujir F, Sanchez J, Reiter RJ, Abreu P 2001 Role of extracellular calcium on the
590 regulation of melatonin synthesis in the Syrian hamster pineal gland. *J Pineal Res* **31**

289-293.

Simonneaux V, Poirel VJ, Garidou ML, Nguyen D, Diaz-Rodriguez E, Pévet P 2004 Daily rhythm and regulation of clock gene expression in the rat pineal gland. *Brain Res Mol Brain Res* **20**(2):164-172.

Sugden D, Vanecek J, Klein DC, Thomas TP, Anderson WB 1985 Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. *Nature* **314** 359-361.

Sugden D, and Klein DC 1987 A cholera toxin substrate regulates cyclic GMP content of rat pinealocytes. *J Biol Chem* **262** 7447-7450.

Sugden D, and Klein DC 1988 Activators of protein kinase C act at a postreceptor site to amplify cyclic AMP production in rat pinealocytes. *J Neurochem* **50(1)** 149-155.

Tanida M, Yamatodani A, Niijima A, Shen J, Todo T, Nagai K 2007 Autonomic and cardiovascular responses to scent stimulation are altered in cry KO mice. *Neurosci Lett* **413** 177–182.

Van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, et al. 1999 Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* **398** 627-630.

Vanecek J, Sugden D, Weller J, and Klein DC 1986 See-saw signal processing in pinealocytes involves reciprocal changes in the α1-adrenergic component of the cyclic GMP response and the β-adrenergic component of cyclic AMP response. *J Neurochem* **47** 678-686.

611 Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, Hitomi K, Thresher
612 RJ, Ishikawa T, Miyazaki J, et al. 1999 Differential regulation of mammalian Period
613 genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc Natl Acad Sci U S A* **96**
614 12114-12119.
615 Wongchitrat P, Felder-Schmittbuhl MP, Govitrapong P, Phansuwan-Pujito P, Simonneaux V
616 2011 A noradrenergic sensitive endogenous clock is present in the rat pineal gland.
617 *Neuroendocrinology* **94** 75-83.
618 Yamanaka Y, Suzuki Y, Todo T, Honma K, Honma S 2010 Loss of circadian rhythm and
619 light-induced suppression of pineal melatonin levels in Cry1 and Cry2 double-deficient
620 mice. *Genes Cells* **15** 1063-1071.
621

622 **Figure legends**

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

630

631 **Figure 2** Circadian profiles of pineal *Per1* and *Aanat* mRNA levels.

632 Twenty-four–h patterns of mRNA levels of pineal *Per1* (A) and *Aanat* (B) in wild-type (open

633 circles) and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ (closed circles) mice on the 1st day in DD. Shadowed areas

634 indicate the dark phase. Dark and light gray areas indicate a subjective night and day,

635 respectively. Data are presented as the mean ± SEM (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$ vs.

636 *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ (post-hoc unpaired t-test followed by two-way ANOVA).

637

638 **Figure 3** Circadian rhythm of AANAT protein levels in wild-type and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice.

639 Representative western blot of pineal AANAT in wild-type and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice shown

640 together with that of β-actin (A). Relative level of AANAT in the pooled pineal glands (B).

641 Data are presented as the mean ± SEM of three independent experiments. White and black

642 columns indicate the relative AANAT protein level of wild-type and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice.

643 The values are normalized to the amount of β-actin in each sample.

644

645 **Figure 4** Circadian profile of pineal AANAT enzyme activity

646 Twenty-four–h profile of AANAT activity in wild-type (open circles) and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$

647 (closed circles) mice in DD. Dark and light gray areas indicate subjective night and day,

648 respectively. Data are presented as the mean ± SEM (n = 5–6). $^{**}P < 0.01$ vs. *Cry1*$^{-/-}$/*Cry2*$^{-/-}$

649 (post-hoc unpaired *t*-test followed by two-way ANOVA).

650

651 **Figure 5** Adrenergic-stimulant induced melatonin synthesis and AANAT activity in cultured

652 pineal glands.

653 Cultured pineal glands from wild-type (open columns) and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ (closed columns)

654 mice show a significant increase in melatonin content after stimulation with isoproterenol

655 (ISO) compared with vehicle controls at both two circadian phases (project ZT3 and ZT15).

656 Responses differed significantly between genotypes but not time of day. Data are presented as

657 the mean ± SEM, (n = 10–13). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle; $^{\dagger}P < 0.05$ vs. *Cry1*$^{-/-}$/*Cry2*$^{-/-}$

658 mice (post-hoc unpaired *t*-test followed by two-way ANOVA) (A). The AANAT activity in the

659 pineal glands of wild-type (open columns) and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ (closed columns) mice after

660 stimulation with 2μM of ISO (B) or 10μM NE (C) for 2 h. Data are presented as the mean ±

661 SEM, (n = 4–5). $^{*}P < 0.05$ vs. vehicle in each genotype (post-hoc unpaired *t*-test followed by

662 two-way ANOVA).

663

664 **Figure 6** Intracellular cAMP after forskolin stimulation in cultured pineal glands.

665 Cultured pineal glands from wild-type (open column) and *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ (closed columns)

666 mice demonstrate similar increases in cAMP content after the treatment with forskolin (FSK)

667 at project ZT3. Data are presented as the mean ± SEM (n = 7). $^{**}P < 0.01$ vs. vehicle

668 (post-hoc unpaired *t*-test followed by two-way ANOVA).

669

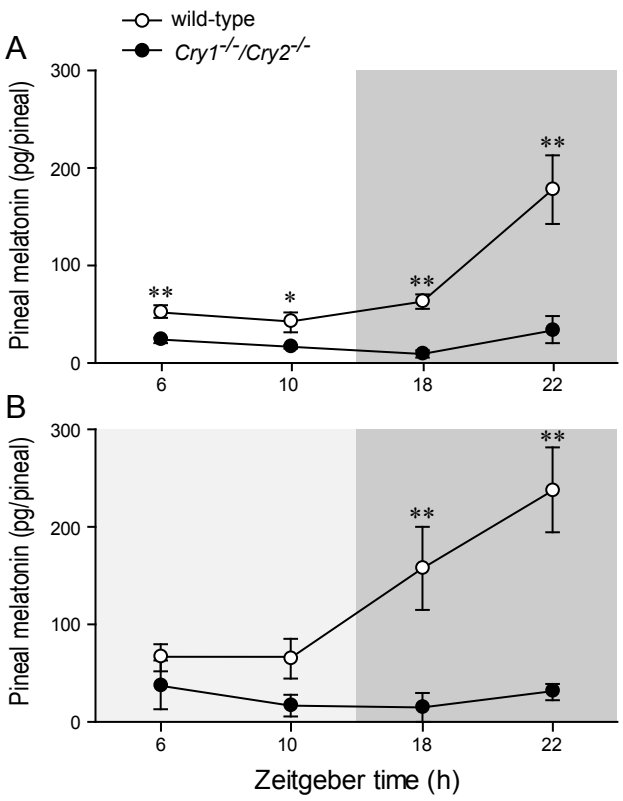

wild-type
Cry1-/-/Cry2-/-
A
B
Pineal melatonin (pg/pineal)
300
200
100
0
6
10
18
22
**
*
**
**
**
**
Zeitgeber time (h)

Figure 1

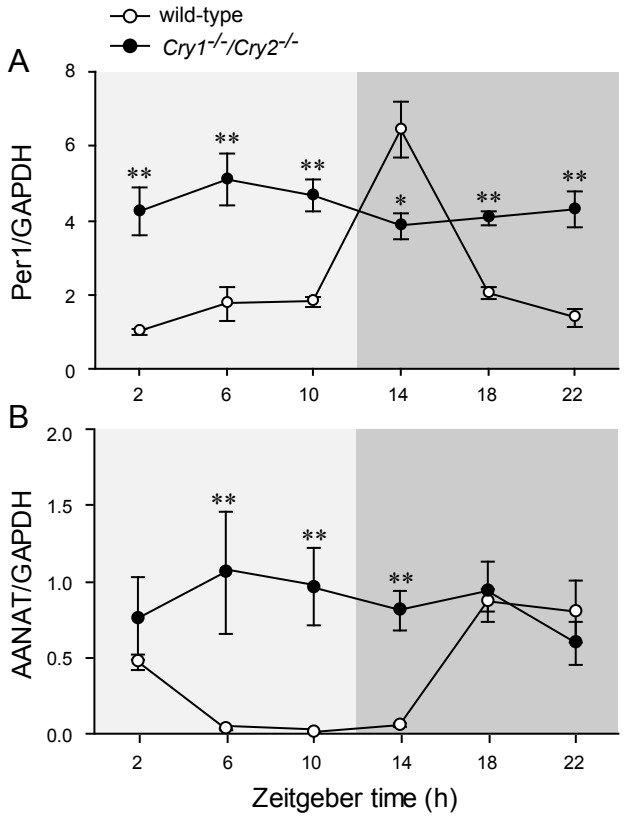

wild-type
Cry1-/-/Cry2-/-
A
Per1/GAPDH
8
6
4
2
0
**
**
**
*
**
**
2
6
10
14
18
22
B
AANAT/GAPDH
2.0
1.5
1.0
0.5
0.0
**
**
**
2
6
10
14
18
22
Zeitgeber time (h)

Figure 2

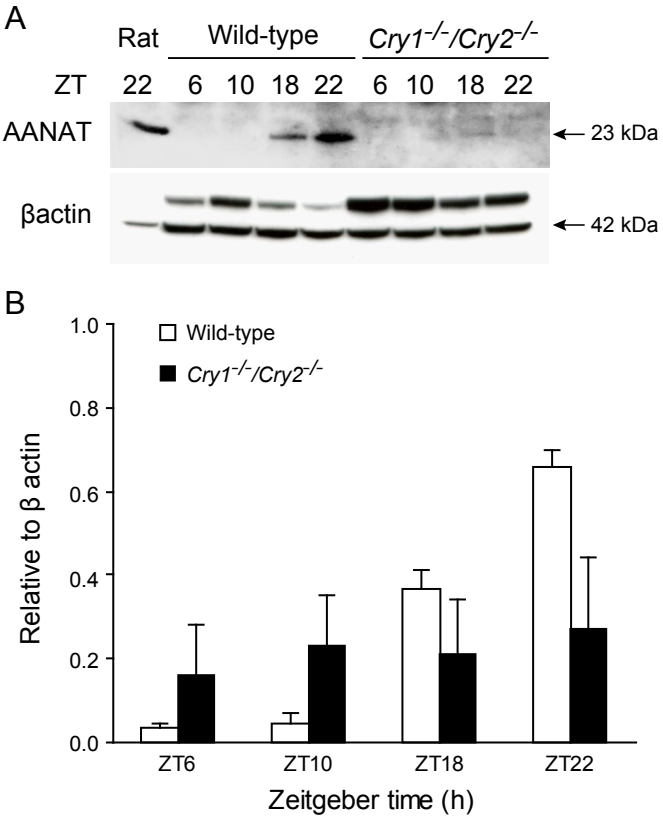

A
Rat
Wild-type
Cry1-/-/Cry2-/-
ZT 22 6 10 18 22 6 10 18 22
AANAT
23 kDa
βactin
42 kDa
B
Wild-type
Cry1-/-/Cry2-/-
Relative to β actin
1.0
0.8
0.6
0.4
0.2
0.0
ZT6
ZT10
ZT18
ZT22
Zeitgeber time (h)

Figure 3

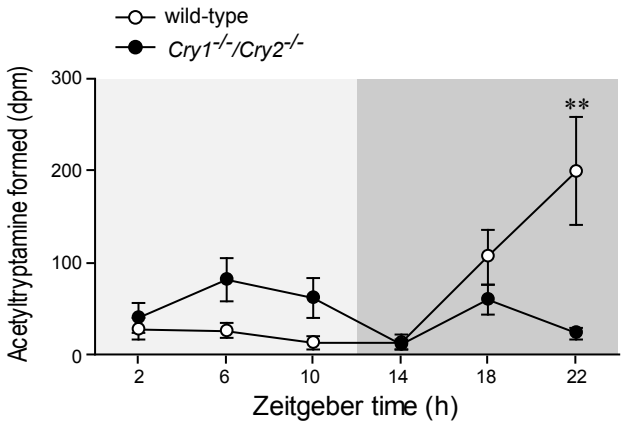

wild-type
Cry1-/-/Cry2-/-
Acetyltryptamine formed (dpm)
300
200
100
0
**
2
6
10
14
18
22
Zeitgeber time (h)

Figure 4

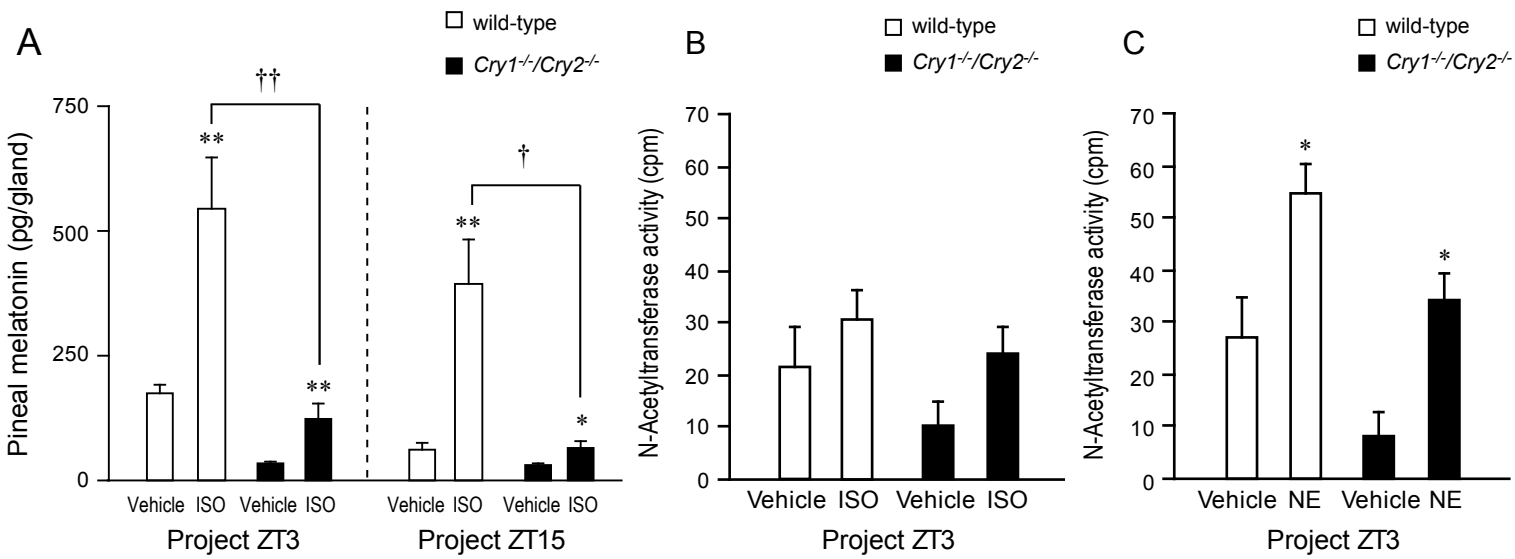

A
wild-type
Cry1-/-/Cry2-/-
Pineal melatonin (pg/gland)
750
500
250
0
Vehicle ISO Vehicle ISO
Project ZT3
Vehicle ISO Vehicle ISO
Project ZT15
B
wild-type
Cry1-/-/Cry2-/-
N-Acetyltransferase activity (cpm)
70
60
50
40
30
20
10
0
Vehicle ISO Vehicle ISO
Project ZT3
C
wild-type
Cry1-/-/Cry2-/-
N-Acetyltransferase activity (cpm)
Vehicle NE Vehicle NE
Project ZT3

Figure 5

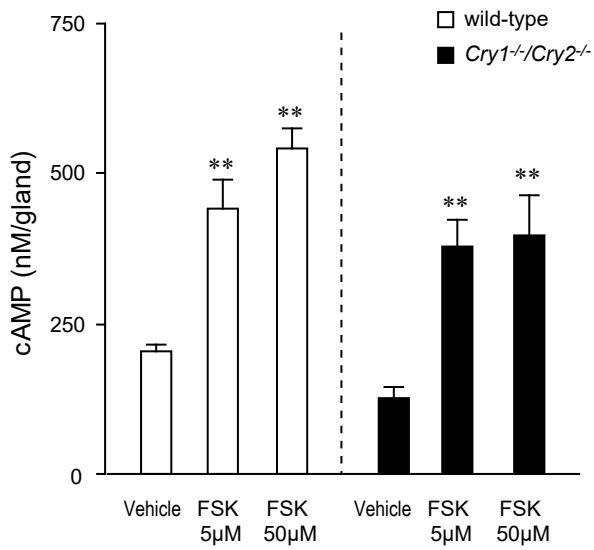

wild-type
Cry1-/-/Cry2-/-
cAMP (nM/gland)
750
500
250
0
**
**
**
**
Vehicle
FSK 5μM
FSK 50μM
Vehicle
FSK 5μM
FSK 50μM

Figure 6