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Author(s)	Yamanaka, Yujiro; Yamada, Yoshiko; Honma, Ken-ichi; Honma, Sato
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1 **CRYPTOCHROME deficiency enhances transcription but reduces protein levels of**

2 **pineal *Aanat***

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4 Yujiro Yamanaka1, 2, 4 Yoshiko Yamada3, Ken-ichi Honma3, Sato Honma3, 4

5 1 Department of Physiology, Hokkaido University Graduate school of Medicine

6 2 Laboratory of Life and Health Science, Hokkaido University Graduate school of Education

7 3 Department of Chronomedicine, Hokkaido University Graduate school of Medicine

8 4 Research and Education Center for Brain Science, Hokkaido University

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

10 Address for correspondence to:

11 Prof. Sato Honma, Research and Education Center for Brain Science, Hokkaido University, Sapporo

12 060-8638, Japan

13 Email: sathonma@med.hokudai.ac.jp

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16

17   **Abstract**

18   *Cryptochrome* (*Cry*) *1* and *2* are essential for circadian rhythm generation, not only in the

19   suprachiasmatic nucleus, the site of the mammalian master circadian clock, but also in

20   peripheral organs throughout the body. CRY is also known as a repressor of

21   *arylalkylamine-N-acetyltransferase* (*Aanat*) transcription, therefore, *Cry*-deficiency is

22   expected to induce constantly high pineal melatonin content. Nevertheless, we previously

23   found that the content was consistently low in melatonin-proficient *Cry1* and *Cry2*

24   double-deficient mice ($Cry1^{-/-}/Cry2^{-/-}$) on C3H background. This study aims to clarify the

25   mechanism underlying this discrepancy.

26   In the $Cry1^{-/-}/Cry2^{-/-}$ pineal, expression levels of *Aanat* and clock gene *Per1* were

27   consistently high with no circadian fluctuation on the first day in constant darkness,

28   demonstrating that CRY acts *in vivo* as a repressor of the pineal circadian clock and AANAT.

29   In contrast, the enzyme activity and protein levels of AANAT remained low throughout the

30   day, supporting our previous observation of continuously low melatonin. Thus, effects of

31   *Cry*-deficiency on the responses of β-adrenergic receptors were examined in cultured pineal

32   glands. Isoproterenol, a β-adrenergic stimulant, significantly increased melatonin content,

33   although the increase was smaller in $Cry1^{-/-}/Cry2^{-/-}$ than in wild-type mice, during both the

34   day and night. Whereas, the increase in cAMP in response to forskolin was similar in both

35   genotypes, indicating that CRY deficiency does not affect the pathway downstream of the

36 β-adrenergic receptor. These results suggest that a lack of circadian adrenergic input due to

37 CRY deficiency decreases β-receptor activity and cAMP levels, resulting in consistently low

38 AANAT levels despite abundant *Aanat* mRNA.

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Introduction

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56   Pineal melatonin synthesis in mammals is regulated by the circadian pacemaker in the

57   suprachiasmatic nucleus (SCN) of the hypothalamus. The circadian pacemaker is entrained to

58   the environmental light–dark cycle by photic signals via the retinohypothalamic tract (Rusak

59   & Zucker 1979) and controls melatonin synthesis in the pineal gland through the sympathetic

60   nerve, which releases noradrenalin at night (Larsen *et al.* 1998). Elimination of neural input

61   to the pineal gland demonstrated an importance of this neural pathway not only for the

62   nocturnal melatonin synthesis (Deguchi & Axelrod 1972) but also for day/night changes in

63   transcription of thousands of genes (Hartley *et al.* 2015). Activation of the β1-adrenergic

64   receptor in pinealocytes is known to be a critical step to increase intracellular cyclic AMP

65   (cAMP). Increasing intracellular cAMP leads to a rapid activation of arylalkylamine

66   N-acetyltransferase (AANAT), the rate-limiting enzyme in melatonin synthesis (Klein *et al.*

67   2007, Sugden *et al.* 1985) through inhibiting proteasomal proteolysis (Ganguly *et al.* 2001) as

68   well as activating transcription level (Ganguly *et al.* 2005).

69   The molecular machinery generating the mammalian intracellular circadian rhythm is a

70   transcription/translation feedback loop (core loop) involving the clock genes *Period* (*Per*) *1*,

71   *Per2*, *Cryptochrome* (*Cry*) *1*, *Cry2*, *Clock*, and *Bmal1* and their protein products (Reppert &

72   Weaver 2002). The promoter region of the *Aanat* gene contains an E-box element that is

73   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.

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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 $Cry1^{-/-}/Cry2^{-/-}$

112   (*n*=36) mice under either LD or the first cycle after releasing to DD. Time of lights-on was

113   defined as Zeitgeber time 0 (ZT0), and that of lights-off as ZT12. The mice were euthanized

114   by cervical dislocation without anesthesia at four time points (ZT 6, 10, 18, and 22; $n = 4$–5

115   per group) under LD or DD, and the pineal gland was collected under a dissecting

116   microscope. Collection of the pineal gland during the dark phase was performed after cervical

117   dislocation and eye enucleation under dim red light (<0.1 lux, <30 sec).

118

119   **Melatonin radioimmunoassay (RIA)**

120   Pineal glands were quickly removed at the indicated time and placed in 200 μL of

121   ice-cold assay buffer (0.1 M Tris-HCl, pH 7.2). The tissue was homogenized by repeated

122   freezing and thawing in liquid nitrogen for three cycles. The pineal homogenates were

123   centrifuged for 15 min at 15,000 rpm, and the supernatant was stored at $-30°C$. The

124   melatonin content of each sample was determined by radioimmunoassay (RIA) as previously

125   described (Yamanaka *et al.* 2010). Each sample was assayed using 200 μL of supernatant.

126   The minimum detection level for the melatonin concentration was 1.56 pg/tube. Intra- and

127   inter-assay coefficient of variances were 5.6% and 6.2%, respectively.

128

129   **Quantitative real-time PCR analysis of pineal *Aanat* and *Period1* mRNA**

130   *Period 1* (*Per 1*) and *Aanat* mRNAs in the pineal gland were measured by quantitative

131   real-time reverse transcription-PCR (qRT-PCR) as previously reported (Nishide *et al.* 2012)

132   with some modifications. The pineal glands of the wild-type ($n = 30$) and $Cry1^{-/-}/Cry2^{-/-}$ ($n =$

133   30) mice were quickly put in ice-cold tissue storage regent (RNA later, R0901,

134   Sigma-Aldrich, St. Louis, MO, USA) at six time points (ZT 2, 6, 10, 14, 18, and 22; $n = 5$ per

135   group) under LD or DD and stored at -80 °C until mRNA extraction. Total RNA was

136   extracted using RNeasy Micro Kit (Qiagen, Hilden, Germany), and the concentration was

137   measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA,

138   USA). cDNA was synthesized using SuperScriptIII First-Strand Synthesis SuperMix

139   (Invitrogen) according to the manufacturer's instruction. qRT-PCR was performed with

140   DyNAmo HS SYBR Green qPCR Kit (New England BioLabs, Ipswich, MA, USA) and a

141   real-time thermal cycler (ABI PRISM 7700, Applied Biosystems, Foster City, CA, USA)

142   using the following primers: *mGAPDH* forward: 5'TGCGACTTCAACAGCAACTC3';

143   *mGAPDH* reverse: 5'ATGTAGGCCATGAGGTCCAC3'; *mAanat* forward:

144   5'CTCCAGCCCATCCAACTAG3'; *mAanat* reverse: 5'TGCACAGTTCAGAAGGCAAG3';

145   *mPer1* forward: 5'CGTCCTACCTCCTTTATCCAGA3'; *mPer1* reverse:

146   5'TGTTTGCATCAGTGTCATCAGC3'.

147

148   **Measurement of pineal AANAT enzyme activity**

149   The AANAT enzyme activity was assayed according to the method reported by Deguchi

150   & Axelrod (1972) with slight modification. Pineal glands of the wild-type ($n = 30$) and

151   *Cry1$^{-/-}$/Cry2$^{-/-}$* ($n = 31$) were quickly removed at one of six times of the day in DD (ZT2, 6,

152   10, 14, 18, and 22; $n = $ 5-6 per group). Each pineal gland was homogenized in chilled 0.05M

153   phosphate buffer (50 μL, pH. 6.5) and stored at −30°C until the assay. On the day of assay,

154   10μL each of 0.01M Tryptamine-HCL and 3.4 nM [1-^{14}C] acetyl coenzyme A (PerkinElmer,

155   Walthan MA, USA) was added to 50μL of homogenate in a small glass tube. After incubating

156   at 37°C for 10 min, the reaction was stopped by the addition of 0.5 mL of 0.5 M borate buffer

157   (pH 10.0). The homogenate was transferred into a glass tube containing 6 mL of

158   toluene-isoamyl alcohol (97:3) and stirred using a vortex mixer. After centrifugation at 3,500

159   rpm for 10 min, 5 mL of the organic phase was transferred into a scintillation vial containing

160   5 mL of scintillation cocktail (Aquazol2, PerkinElmer), and radioactivity was measured.

161

162   **Immunoblotting of pineal AANAT protein**

163   The AANAT protein concentration was determined by immunoblotting using the method

164   reported elsewhere (Nishide *et al.* 2012) with some modifications. Briefly, the pineal glands

165   were collected at four time points (ZT6, 10, 18, and 22) in DD. The pineal glands were

166   pooled (wild-type, n = 8; *Cry1$^{-/-}$/Cry2$^{-/-}$*, $n = 12$) in tissue protein extraction regent (T-PER,

167   Thermo Scientific) and homogenized by sonication followed by freezing and thawing four

168   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)

226   was added to the culture medium and incubated at 37°C and 5% CO_2 for 1 h. The dose was

227   reported to increase cAMP level and melatonin synthesis in cultured pineal gland (Santana *et*

228   *al.* 1988, Santana *et al.* 2001). The cultured pineal glands were rinsed by transferring them

229   three times to new culture wells containing 200 μL of cold 0.1M Tris-HCl buffer (pH 7.2).

230   The pineal gland was placed in a tube containing 200 μL of cold 0.1M Tris-HCl buffer (pH

231   7.2) and homogenized by four cycles of freezing in liquid nitrogen and thawing. The pineal

232   homogenates were stored at −80°C until assays. The cAMP level in the pineal homogenate

233   was measured using the cAMP-Glo Max assay kit (Promega) by monitoring bioluminescence

234   with luminometer.

235

236   **Statistical analysis**

237   Data are reported as the mean ± SEM. A one-way ANOVA (main effect of time) followed

238   by a post-hoc Tukey-Kramer test was used to determine the time-dependent difference for

239   each genotype. A two-way ANOVA (main effect of genotype and interaction between

240   genotype and time) followed by a post-hoc unpaired *t*-test was used to compare the data

241   between the two genotypes. $P < 0.05$ was considered statistically significant.

242

243   **Results**

244   **Circadian rhythms in pineal melatonin content in *Cry1^−/−^/Cry2^−/−* mice**

245   The circadian profile of pineal melatonin content under LD and DD differed significantly

246   between wild-type and $Cry1^{-/-}/Cry2^{-/-}$ mice ($P < 0.001$, with a significant interaction

247   between genotype and time (two-way ANOVA test) (Fig. 1). Under both LD and DD

248   conditions, wild-type mice exhibited a robust circadian variation, with a significant difference

249   found between time points ($P < 0.001$; main effect time, one-way ANOVA), with the peak

250   level at ZT22 (178 ± 35 pg/pineal in LD, 238 ± 44 pg/pineal in DD, $n = 5$). In contrast,

251    $Cry1^{-/-}/Cry2^{-/-}$ mice exhibited no significant circadian variation in pineal melatonin content

252   ($P = 0.12$ in LD; $P = 0.23$ in DD; main effect time, one-way ANOVA test, $n = 4$-5). Under LD,

253   the pineal melatonin content was significantly lower in $Cry1^{-/-}/Cry2^{-/-}$ mice than in wild-type

254   mice in the light phase ($P < 0.01$ at ZT6; $P < 0.05$ at ZT10; two-way ANOVA test with

255   post-hoc unpaired t-test) as well as in the dark phase ($P < 0.01$ at ZT18 and ZT22). Under DD,

256   the melatonin content was significantly lower in $Cry1^{-/-}/Cry2^{-/-}$ mice than in wild-type mice

257   at ZT18 ($P < 0.05$) and ZT22 ($P < 0.001$).

258

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

260   *Aanat* is reported to have an E-box enhancer in the 1st intron, the function of which in the

261   circadian regulation of *Aanat* expression is still unknown (Baler *et al.* 1999, Klein 2007). To

262   examine the functional role of this E-box in *Aanat* expression, we determined the circadian

263   profile of *Per1* and *Aanat* mRNA in $Cry1^{-/-}/Cry2^{-/-}$ mice. In the wild-type mice, both *Per1*

14

264   (Fig. 2A) and *Aanat* (Fig. 2B) mRNA levels in the pineal gland showed a significant

265   rhythmicity (*Per1*, $P < 0.05$; *Aanat*, $P < 0.05$; main effect time, one-way ANOVA test, $n = 5$)

266   and were significantly elevated in the subjective night as compared with those in the

267   mid-subjective day ($P < 0.05$, post-hoc Tukey–Kramer test). The peak of *Per1* expression was

268   detected at ZT14, while that of *Aanat* was between ZT18 and ZT22. In $Cry1^{-/-}/Cry2^{-/-}$ mice,

269   the mRNA levels of both genes were constant throughout the day, exhibiting no significant

270   rhythmicity ($P = 0.23$; $P = 0.81$; main effect time one-way ANOVA, $n = 5$). Compared with

271   wild type, the *Per1* mRNA levels in $Cry1^{-/-}/Cry2^{-/-}$ mice were significantly higher

272   throughout the day except at ZT14, when the level was lower than that of wild type ($P < 0.01$).

273   Despite the findings of low pineal melatonin levels, the *Aanat* mRNA level in $Cry1^{-/-}/Cry2^{-/-}$

274   mice was consistently high at the circadian peak level of wild-type mice with statistical

275   significance during the mid-subjective day (ZT6 and 10) and early subjective night (ZT14) (*P*

276   < 0.01).

277

278   **Circadian rhythm in pineal AANAT enzyme activity and protein concentration**

279   To identify at which stage of melatonin synthesis the dissociation takes place between the

280   consistently high *Aanat* mRNA and low melatonin levels, we examined the circadian rhythms

281   in the levels of AANAT protein and enzyme activity. In wild-type mice, the AANAT protein

282   concentration in the pineal exhibited a circadian rhythm with a peak level at ZT22 ($P < 0.001$,

283   one-way ANOVA test). Whereas in $Cry1^{-/-}/Cry2^{-/-}$ mice, AANAT protein did not exhibit a

284   significant rhythm across the four circadian phases examined (Fig. 3), instead remaining at

285   intermediate levels of the circadian amplitude in WT throughout the day. To apply the same

286   amount of total protein for electrophoresis, we pooled the extracts from 8 and 12 pineal

287   glands from the wild-type and $Cry1^{-/-}/Cry2^{-/-}$ mice, respectively. The results indicate that the

288   total protein content is lower in $Cry1^{-/-}/Cry2^{-/-}$ pineal glands. Similarly, the AANAT enzyme

289   activity in wild-type mice showed a significant rhythmicity ($P <0.001$; main effect time,

290   one-way ANOVA) (Fig. 4), with the peak reached at the late subjective night (ZT22),

291   increased from the low daytime level ($P < 0.05$, ZT2, ZT6, ZT10 vs. ZT22; post-hoc Tukey–

292   Kramer test). In contrast, the AANAT enzyme activity in $Cry1^{-/-}/Cry2^{-/-}$ mice did not show

293   significant rhythmicity across the circadian phases ($P = 0.07$). Circadian patterns differed

294   significantly between the two genotypes ($P < 0.001$, two-way ANOVA), with a significant

295   difference at ZT 22 ($P = 0.002$, post-hoc unpaired t-test).

296

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

298   We then sought to determine the mechanism underlying the discrepancy between *Aanat*

299   mRNA and protein/enzyme activity levels in $Cry1^{-/-}/Cry2^{-/-}$ pineal glands. The increase in

300   intracellular cAMP in response to β-adrenergic stimulation via neural inputs from the SCN is

301   known to be critical for stabilizing the AANAT protein and increasing melatonin synthesis

302 (Ganguly *et al.* 2001). Therefore, we asked whether pinealocytes lacking CRYs can respond

303 to β-adrenergic stimulation to increase melatonin synthesis. To determine the day–night

304 difference, pineal glands of wild-type and $Cry1^{-/-}/Cry2^{-/-}$ mice were sampled at two time

305 points, ZT3 and ZT15, and stimulated with isoproterenol (ISO, 2μM) for 2 h after a

306 pre-incubation of 1 h. In wild-type pineal glands, ISO treatment significantly increased

307 melatonin content compared with those of vehicle-treated controls, both at ZT3 (vehicle vs.

308 ISO, 174 ± 18 pg/gland [$n = 12$] vs. 544 ± 104 pg/gland [$n = 12$]; $P = 0.002$, two-way

309 ANOVA test with post-hoc unpaired *t*-test) and ZT15 (vehicle vs. ISO, 60 ± 14 pg/gland [$n =$

310 12] vs. 393 ± 91 pg/gland [$n = 11$], $P = 0.002$). In the $Cry1^{-/-}/Cry2^{-/-}$ pineal gland, the

311 melatonin content was also significantly elevated by ISO treatment, both at ZT3 (vehicle vs.

312 ISO, 34 ± 4 pg/gland [$n = 13$] vs. 124 ± 29 pg/gland [$n = 10$]; $P = 0.004$) and at ZT15

313 (vehicle vs. ISO, 32 ± 3 pg/gland [$n = 10$] vs. 65 ± 14 pg/gland [n = 10]; $P = 0.045$)

314 compared to the respective vehicle control. However, the response to ISO was markedly

315 lower in the $Cry1^{-/-}/Cry2^{-/-}$ than in the wild type, both at ZT3 and ZT15 (ZT3, $P < 0.001$;

316 ZT15, $P = 0.004$) (Fig. 5A). Three-way ANOVA revealed a significant interaction between

317 the genotype (wild-type and $Cry1^{-/-}/Cry2^{-/-}$) and the stimulation (vehicle and ISO) ($P =$

318 0.003), but no significant interaction the genotype and the stimulation time).

319

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

321   To explore the mechanism of the different response to ISO in the wild-type and

322    $Cry1^{-/-}/Cry2^{-/-}$ mice, the pineal glands were sampled at ZT3 and the AANAT activity were

323   measured after the stimulation with 2μM of ISO or 10μM of NE for 2 h. We examined only at

324   ZT3 because no stimulation time effect was detected by ISO in either genotype. The AANAT

325   activity after stimulation with ISO tended to increase the AANAT activity on average as

326   compared with the vehicle control in wild-type and $Cry1^{-/-}/Cry2^{-/-}$ mice, though it did not

327   reach statistical significance (Fig. 5B). After the stimulation with NE, the significant

328   increases of the AANAT activity were observed in both two genotypes at similar extent (Fig.

329   5C). Two-way ANOVA test revealed significant effect of genotype (wild-type and

330    $Cry1^{-/-}/Cry2^{-/-}$) (P=0.011) and the stimulation (Vehicle vs. NE) (P=0.002), but no significant

331   interaction between the genotype and stimulation.

332

333   **Effect of FSK stimulation on the pineal cAMP level**

334   To assess whether the decreased melatonin synthesis in the $Cry1^{-/-}/Cry2^{-/-}$ mice is

335   caused by a decrease in intracellular cAMP or defects in the downstreams, the effect of FSK

336   (5 μM and 50 μM) on cAMP levels were examined in cultured pineal glands. In both

337   genotypes, FSK stimulation significantly increased the cAMP level in the pineal gland as

338   compared to vehicle-treated controls (vehicle, 5 μM FSK and 50 μM FSK, 205 ± 10 nM [$n =$

339   7], 441 ± 48 nM [$n = 7$, $P < 0.01$] and 540 ± 33 nM [$n = 7$, $P < 0.01$] for wild type; 125 ± 20

18

340   nM [$n = 7$], 380 ± 45 nM [$n = 7$, $P < 0.01$] and 397 ± 66 nM [$n = 7$, $P < 0.01$] for

341     $Cry1^{-/-}/Cry2^{-/-}$, respectively) (Fig. 6). The $Cry1^{-/-}/Cry2^{-/-}$ pineal exhibited FSK-induced

342   increase in cAMP by FSK to a similar extent as wild type. Thus, no genotype difference was

343   detected in the response to FSK. Two-way ANOVA revealed a significant effect of

344   stimulation (vehicle and FSK) on the cAMP level in the pineal gland ($P < 0.001$), but there

345   was no significant interaction between genotype (wild-type and $Cry1^{-/-}/Cry2^{-/-}$) and

346   stimulation. While no significant dose response was observed in the two doses examined,

347   cAMP tended to increase with a higher dose in both genotypes.

348

349   **Discussion**

350   Our findings demonstrate that a loss of circadian melatonin rhythm in $Cry1^{-/-}/Cry2^{-/-}$

351   mice is mainly caused by the loss of rhythmic noradrenergic signals from the circadian

352   pacemaker in the SCN and partly caused by a decreased response to β-adrenergic signals. The

353   pineal gland of $Cry1^{-/-}/Cry2^{-/-}$ mice showed consistently high *Aanat* mRNA expression and

354   low AANAT protein and enzyme activity throughout the day. The discrepancy between these

355   findings is explained by the lack of rhythmic β-adrenergic signals (Klein & Moore 1979,

356   Klein *et al.* 1983a) by loss of neural input from the SCN, because cAMP-dependent

357   phosphorylation of AANAT leads binding with 14-3-3 protein, which activates the AANAT

358   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

378   (ZT15), suggesting that the pineal glands of $Cry1^{-/-}/Cry2^{-/-}$ mice can synthesize melatonin.

379   Since the wild-type pineal glands also responded to ISO at both time points, this response to

380   noradrenergic stimulation appears to be ungated. The ungated response has also been reported

381   in the *Aanat* and *Per1* mRNA levels of cultured pineal gland after the treatment with ISO

382   (Wongchitrat *et al.* 2011, Fukuhara *et al.* 2005). Taken together, these findings indicate that

383   the loss of circadian rhythm of pineal melatonin expression in $Cry1^{-/-}/Cry2^{-/-}$ mice is caused

384   by a loss of rhythmic output signals from the SCN to the pineal gland.

385   In rodents, pineal AANAT enzyme activity dramatically increases at night (Deguchi &

386   Axelrod 1972) in response to noradrenalin released under the control of the circadian clock in

387   the SCN (Perreau-Lenz *et al.* 2003). Upon binding of noradrenalin to the pineal β-adrenergic

388   receptor intracellular cAMP content increases (Sugden *et al.* 1985, Ho *et al.* 1988, 1989),

389   which leads to an activation of adenylate cyclase. This effect is enhanced by α1 receptors via

390   Ca^{2+} and phosphatidyl inositol activation of protein kinase C (Klein *et al.* 1983b, Ho & Klein

391   1987, 1989, Sugden & Klein 1988), thereby activating *Aanat* transcription via

392   cAMP-responsive elements in the promoter and 1st intron of *Aanat* (Humphries *et al.* 2007).

393   Stimulation of α1- and β-adrenergic receptors results in a marked increase in both cAMP and

394   cGMP accumulation in pinealocytes (Sugden & Klein 1987, Vanecek *et al.* 1986). The effects

395   of cGMP analogs on pineal physiology are usually weaker than the actions of cAMP analogs,

396   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

416   AANAT.

417   In addition to promoting *Aanat* transcription, an increase in cAMP also has a significant

418   effect on nocturnal increases in melatonin synthesis that occur via post-translational

419   modifications of AANAT (Klein *et al.* 2007). The AANAT protein is phosphorylated in a

420   cAMP-dependent phosphorylation of AANAT leads formation of a complex with the 14-3-3

421   protein (pAANAT/14-3-3 complex), which protects AANAT from degradation (Ganguly *et al.*

422   2001) and elevates enzymatic activity (Ganguly *et al.* 2005). Nocturnal melatonin synthesis

423   critically depends on intracellular cAMP. Thus, melatonin synthesis is strictly influenced by

424   the intracellular cAMP level, which is under the regulation of neural inputs from the circadian

425   pacemaker in the SCN. According to our proposed mechanism, the decreased melatonin

426   synthesis in $Cry1^{-/-}/Cry2^{-/-}$ mice might be caused by the decrease in the intracellular cAMP

427   level at night caused by a lack of coherent rhythm output from the SCN.

428   Although CRY deficiency resulted in a lack of circadian output from the SCN, we found

429   that the pineal gland of $Cry1^{-/-}/Cry2^{-/-}$ mice was capable of producing melatonin in response

430   to noradrenergic signals. The response to ISO was reduced in $Cry1^{-/-}/Cry2^{-/-}$ as compared to

431   that of wild-type mice. Nevertheless, AANAT activity of $Cry1^{-/-}/Cry2^{-/-}$ pineal increased by

432   NE stimulation to the similar extent to the wild type, suggesting significant role of α1

433   adrenergic receptor in melatonin synthesis. The same amount of ISO significantly increased

434   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

479   **Author contributions statement**

480   Y. Yamanaka and S. Honma designed the experiments; Y. Yamanaka, Y. Yamada, and S.

481   Honma performed the experiments; Y. Yamanaka., Y. Yamada, and S. Honma analyzed the

482   data; and Y. Yamanaka, K. Honma, and S. Honma wrote the paper.

483

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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).

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; †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

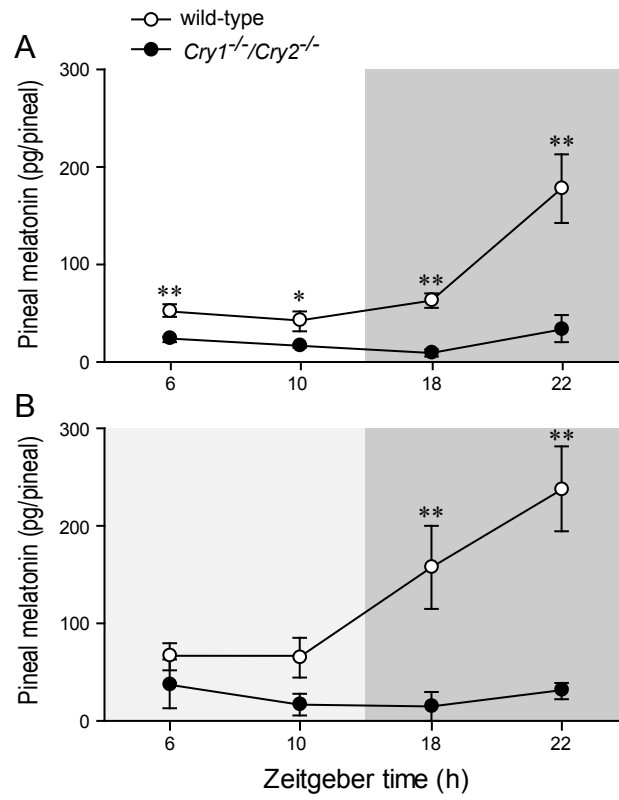668   (post-hoc unpaired t-test followed by two-way ANOVA).

669

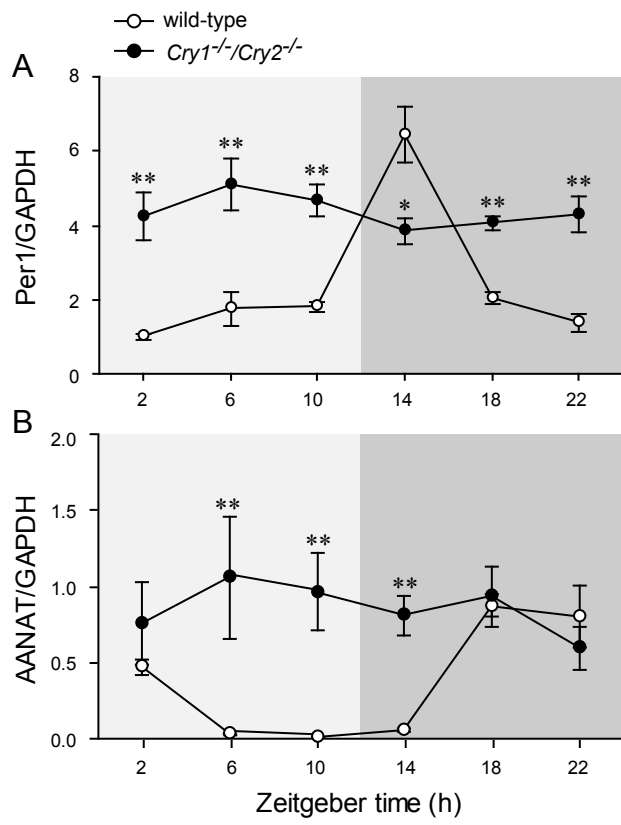Figure 1

Figure 2

A

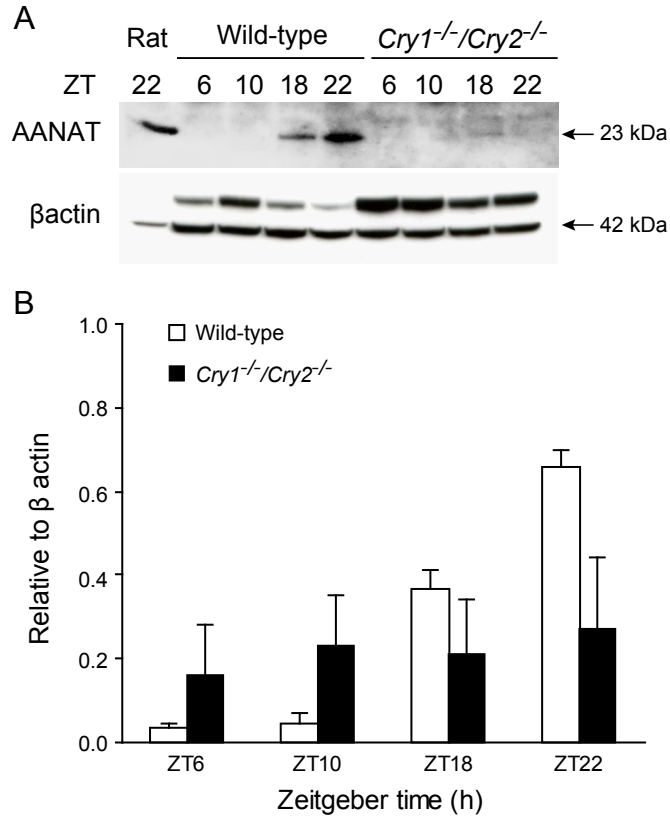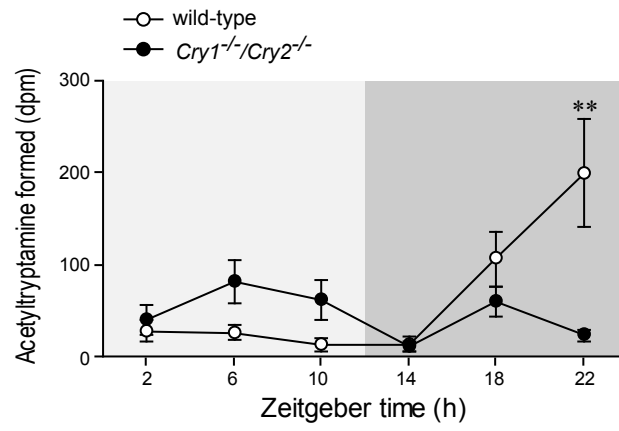| | Rat | Wild-type | | | | Cry1⁻ᐟ⁻/Cry2⁻ᐟ⁻ | | | |

ZT: 22, 6, 10, 18, 22, 6, 10, 18, 22

AANAT ← 23 kDa

βactin ← 42 kDa

B

Figure 3

Figure 4

Figure 5

Figure 6