# Supporting Information

## Haraguchi et al. 10.1073/pnas.1210804109

### SI Materials and Methods

**Animals.** Japanese quail, *Coturnix japonica*, at various ages were used in this study. Quail were incubated under daily photoperiods of 12-h light/12-h darkness cycles with the light provided by white fluorescent lamps. Pineal glands were isolated from the light-exposed animals at zeitgeber time 6. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals of Waseda University.

**PCR Protocol for Sex Determination.** Sexes of juvenile quail were determined by PCR to detect genomic size differences of the chromo-helicase–DNA-binding protein (CHD) between W (male) and Z (female) chromosomes (1). To isolate genomic DNA, brain tissue was lysed in 100 μL of digestion buffer [10 mM Tris·HCl (pH 8.0)/150 mM NaCl/10 mM EDTA/0.1% SDS] with 2 μg of Proteinase K at 65 °C overnight, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated in ethanol. The primers (Table S1) bind both *CHD-W* and *CHD-Z*. PCR was performed at 94 °C for 5 min for 1 cycle, followed by 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min for 32 cycles. Males had one band at ∼1,000 bp from *CHD-W*, and females had two bands, one at ∼1,000 bp from *CHD-W* and the other at 267 bp from *CHD-Z*.

**RT-PCR Analyses of Neurosteroidogenic Enzyme mRNAs.** Total RNA was extracted from the quail pineal gland with Sepazol-RNA I Super (Nacalai Tesque) and reverse-transcribed (2–4). All PCR amplifications (for *StAR*, *Cyp11a*, *Cyp7b*, *Hsd3b*, *Srd5a*, *Cyp17*, *Hsd17b*, and *Cyp19*) were performed in a reaction mixture containing Bio Taq polymerase (Bioline), 0.2 mM dNTP, and 0.5% dimethyl sulfoxide using a thermal cycler (3, 4). Forward primers and reverse primers (Table S1) were designed according to the nucleotide sequence of quail and chicken steroidogenic enzyme mRNAs. *β-Actin*, a housekeeping gene, was used as control. The PCR condition was the following: 1 cycle of 5 min at 94 °C, 30 cycles of 15 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C, and, finally, 1 cycle of 10 min at 72 °C.

**Biochemical Analysis of Pregnenolone Formation from Cholesterol.** To assess pregnenolone (PREG) formation from cholesterol in the quail pineal gland, conversion of [$^3$H]cholesterol (specific activity, 53.0 Ci/mmol; PerkinElmer) to [$^3$H]PREG was measured biochemically using organ-cultured pineal glands. Biochemical analysis in this study was performed as described previously (3, 5, 6). In brief, five pineal glands were cultured in medium 199 (medium 199 supplemented with 10 mM Hepes-NaOH at pH 7.4, 100 U/mL penicillin, and 100 μg/mL streptomycin; Invitrogen) containing 210 nM [$^3$H]cholesterol, 2% (vol/vol) propylene glycol, 1% protease inhibitor mixture (Nacalai Tesque) for 0, 12, or 24 h at 37 °C. The homogenates were incubated in a water-saturated atmosphere (80% $O_2$, 5% $CO_2$) to maintain the pH at 7.4. After incubation, steroids were extracted by ethyl acetate and subjected to HPLC analysis by using reversed-phase column, Capcell Pak C18 MG (Shiseido). The HPLC was performed with an isocratic condition of acetonitrile/isopropanol (60:40, vol/vol) at a flow rate of 0.3 mL/min. The eluate was counted in a flow scintillation analyzer (Radiomatic 525TR; PerkinElmer). Reference standards of tritiated cholesterol and PREG were chromatographed to detect their elution positions. To confirm the involvement of P450scc in the formation of PREG, five pineal glands and [$^3$H]cholesterol were cultured with aminoglutethimide (Sigma), an inhibitor of P450scc, at a final concentration of 50 μM (3).

**Biochemical Analysis of Neurosteroids Formed from PREG.** To assess neurosteroid formation from PREG in the quail pineal gland, conversions of substrate steroids [PREG, progesterone (PROG), androstenedione (AD), or testosterone (T)] were measured biochemically using pineal gland homogenates. Biochemical analysis in this study was performed as described previously (4, 7–13). In brief, 10 mg of pineal gland homogenates were incubated in PBS containing 70 nM [$^3$H]PREG (specific activity, 22.9 Ci/mmol; PerkinElmer) and NADPH or $NAD^+$, 70 nM [$^3$H]PROG (specific activity, 96.6 Ci/mmol; PerkinElmer) and NADPH, 70 nM [$^3$H]AD (specific activity, 98.2 Ci/mmol; PerkinElmer) and NADPH, or 70 nM [$^3$H]T (specific activity, 70 Ci/mmol; PerkinElmer) and NADPH for 0, 30, or 60 min at 37 °C. After incubation, neurosteroids were extracted by ethyl acetate and subjected to HPLC analysis using a reversed-phase column, LiChrospher 100 RP-18 (Kanto). Tritiated steroids [7α-hydroxypregnenolone (7α-OH PREG), PROG, 5α-dihydroprogesterone (5α-DHP), allopregnanolone (ALLO), AD, T, 5α-dihydrotestosterone (5α-DHT), and estradiol-17β (E2)] were chromatographed as standards to detect their elution positions. The isoforms 7α- and 7β-OH PREG; ALLO and EPI; and 5α- and 5β-DHT had the same retention times in HPLC. To confirm the involvement of steroidogenic enzymes in the formation of neurosteroids, tritiated steroids were cultured with 50 μM ketoconazole, an inhibitor of cytochrome P450s (Cyps) (12) (Sigma); 50 μM trilostane, an inhibitor of 3β-HSD (9) (Sigma); or 50 μM finasteride, an inhibitor of 5α-reductase (14) (Sigma). All of the tritiated steroids were purchased from PerkinElmer.

**Identification of Neurosteroids by Gas Chromatography/Mass Spectrometry.** Neurosteroids, produced in the quail pineal gland, were identified by gas chromatography/mass spectrometry (GC-MS) (GCMS-QP5000; Shimadzu) using a CP-Sil 5CB capillary column (Varian) as described previously (8, 9, 12, 13, 15, 16). In brief, 400 mg of pineal gland homogenates were incubated in PBS containing nonradioactive steroids for 60 min. The obtained samples were homogenized in an aliquot of methanol/$H_2O$ [75:25 (vol/vol); 1 mL] on ice. The samples were passed through the C-18 cartridge column, and the neurosteroid fractions were eluted with methanol and dried. For the identification of neurosteroids in the pineal gland, heptafluorobutyrate (for PREG, PROG, ALLO, EPI, AD, T, 5α- and 5β-DHT, and E2) or trimethylsilyl ether (for 7α- and 7β-OH PREG) derivatives of the neurosteroids were prepared before GC-MS by treating the dried samples with heptafluorobutyric anhydride (Wako Pure Chemical) or bis(trimethylsilyl)trifluoroacetamide (Wako Pure Chemical) for 30 min at room temperature. For the identification of neurosteroids, GC-SIM analysis was conducted at *m/z* 386 for 7α- and 7β-OH PREG, *m/z* 510 for PROG, *m/z* 514 for ALLO and EPI, *m/z* 482 for AD, *m/z* 680 for T, *m/z* 486 for 5α- and 5β-DHT, and *m/z* 664 for E2. Importantly, the isoforms 7α- and 7β-OH PREG; ALLO and EPI; and 5α- and 5β-DHT had different retention times in GC-MS (7α-OH PREG at 18.3 min, 7β-OH PREG at 19.1 min; ALLO at 15.3 min; EPI at 15.8 min; 5α-DHT at 14.8 min, and 5β-DHT at 15.9 min), respectively, unlike HPLC.

**Western Blot Analysis with P450scc Antibody.** To confirm that the mouse anti-human P450scc antibody (Abcam) recognizes galliform P450scc, Western blot analysis was performed as described previously (8). Quail and chicken are both galliforms with predicted high interspecific DNA sequence conservation (17). Chicken P450scc and quail P450scc have almost identical structure (96.3% identity in the amino acid sequences). The full-length ORF of the

chicken *Cyp11a* was amplified from chicken pineal gland cDNA using the chicken Cyp11a primers (Table S1) and subcloned into the mammalian expression vector pcDNA3.1/V5-His-TOPO (Invitrogen). Positive colonies were selected and subcultured, and the plasmid DNAs were purified by the Wizard plus SV minipreps DNA purification system (Promega). COS-7 cells were supplied from the Riken Cell Bank and maintained in DMEM (Sigma) supplemented with 10% (vol/vol) FBS, penicillin (50 U/mL), streptomycin (50 μg/mL), and Hepes (10 mM, pH 7.4). Transfection was performed with the TransFast transfection reagent (Promega) as described previously (8, 13). After transfection, the cells were harvested, centrifuged (10,000 × *g* for 5 min at 4 °C), and stored at –80 °C. Electrophoresis of proteins (3 μg each) derived from the quail pineal gland and extracts of COS-7 cells transfected with chicken *Cyp11a* cDNA was performed in 12.5% polyacrylamide gels. After transferring to a polyvinylidene fluoride membrane, the membrane was incubated with anti-human P450scc antibody at 1:1,000 dilution at 4 °C overnight and then with goat anti-mouse IgG-horseradish-peroxidase conjugate diluted at 1:1,000 for 1 h. The immunoreactive band was detected in the pineal gland or extracts of COS-7 cells transfected with chicken *Cyp11a* cDNA by using ECL prime Western blotting detection system (GE Healthcare). To confirm the specificity of the immunoreaction, the primary antibody was preadsorbed with chicken P450scc protein (10 μg/mL).

**Immunohistochemistry of P450scc.** Immunohistochemical localization of P450scc was performed as described previously (8, 9). In brief, quail chicks at P7 were terminated by decapitation. The brains were fixed in 4% (vol/vol) paraformaldehyde solution overnight, and after formic acid decalcification they were soaked in a refrigerated sucrose solution [30% (vol/vol) sucrose in 0.1 M phosphate buffer]. Whole brains were frozen in optimal cutting temperature (OCT) compound (Miles) and sectioned transversely at a 20-μm thickness on a cryostat at –20 °C. After blocking nonspecific binding with 5% (vol/vol) normal goat serum and 1% BSA in PBS containing 0.5% Triton X-100, the sections were immersed overnight at 4 °C in 1:100 dilution of mouse anti-human P450scc antibody (Abcam). The sections were then incubated for 60 min with Alexa Fluor 555 anti-mouse IgG (Invitrogen) at a dilution of 1:1,000 and examined with a fluorescence microscope (Leica).

**Quantification of Steroidogenic Enzyme mRNA Expressions.** To compare the expressions of *Cyp7b* and *Srd5a* mRNAs in the quail pineal gland, cerebellum, and diencephalon of both sexes at posthatch day 7 (P7) and P90, real-time PCR was conducted by using the StepOnePlus system (Applied Biosystems) as described previously (8). The oligonucleotide primers used in real-time PCR are listed in Table S1. *β-Actin* was used as the internal standard. The reaction mixture contained SYBR Green Real-Time PCR Mix (Toyobo), 400 nM each of forward and reverse primers, and 30 ng of cDNA in a final volume of 20 μL. PCR was run with a standard cycling program of 95 °C for 3 min, 40 cycles of 95 °C, 15 s; 60 °C, 15 s; and 72 °C, 15 s. An external standard curve was generated by a serial 10-fold dilution of cDNA obtained from the diencephalon, which had been purified, and its concentration was measured. To confirm the specificity of the amplification, the PCR products were subjected to a melting curve analysis and gel electrophoresis. The results were normalized to the expression of *β-actin* using the StepOnePlus 2.0 software (Applied Biosystems).

**Quantification of Neurosteroid Syntheses.** To compare neurosteroid syntheses in the pineal gland, cerebellum, and diencephalon of both sexes at P7 and P90, quail were terminated and tissues were dissected. Each homogenate containing 20 mg of the tissue was incubated separately with tritiated PREG or PROG for 30 min at 37 °C. After incubation, the extracted steroids were subjected to HPLC analysis as described previously (4, 7–13).

**Quantification of Neurosteroid Release.** The quail pineal gland, cerebellum, and diencephalon were isolated from the light-exposed animals and cultured in 500 μL of medium 199 in 12-well plates (each well containing 5 mg of tissue) at 37 °C under 5% $CO_2$/80% $O_2$. After 6 h of culture in the light, neurosteroids secreted into the medium were extracted by ethyl acetate and subjected to GC-SIM analysis to measure the concentrations of 7α-OH PREG, 7β-OH PREG, ALLO, and EPI as described previously (2, 8, 9, 12, 13, 15, 16).

**Quantification of the Number and the Dendritic Length of Purkinje Cells.** To investigate whether neurosteroids are involved in Purkinje cell survival in quail, pinealectomy (Px) at P2 and daily injection of ALLO or 7α-OH PREG were conducted using posthatched male quail chicks. Px and sham operation for P2 quail chicks were performed under nembutal anesthesia (40 mg/kg) as described previously (18). After the surgery, ALLO (30 ng/5 μL) or 7α-OH PREG (30 ng/5 μL) dissolved in sesame oil was injected into the pineal gland region of the quail chick brain once per day, for 6 d, during P2–P7. For daily injection of neurosteroids, a 5-μL volume of each reagent was injected at coordinates 1.0 mm lateral to the pineal gland region and 1 mm deep vertically to the surface of the skull as described in our previous study (19–21). The injection sites were confirmed at autopsy, and the samples were discarded if the injection site was not at the correct position. We injected 30 ng of 7α-OH PREG or ALLO once a day because ALLO and 7α-OH PREG released from the pineal gland were estimated to be around 4–6 ng/6 h of incubation. Control animals were treated with an equal volume of vehicle (sesame oil). Px quail chicks were s.c. implanted with a silastic (silicone type) plate containing melatonin (10 mg per plate) or vehicle as described in our previous study (18). The injection site was determined by visually inspecting the brains of the quail injected with 5 μL of 0.15% methylene blue dissolved in saline. After decapitation under deep anesthesia, cerebella of control, Px, ALLO, 7α-OH PREG, or melatonin-treated quail chicks at P3, P5, P7, and P21 were dissected. Cerebellar sections from 4% (vol/vol) paraformaldehyde-fixed brains were processed and stained with anticalbindin D-28k (Swant Swiss antibodies) as described previously (19–21). The number of calbindin-immunoreactive cell bodies of Purkinje cells was counted in the cerebellar lobes I–X at P21. The length of the molecular layer in the parasagittal section was evaluated as the maximal Purkinje dendritic length as described previously (19–21).

**Quantification of ALLO Concentration in the Cerebellum.** The concentration of ALLO in the whole cerebellum, rostral cerebellum, or caudal cerebellum of quail chicks was measured by GC-SIM analysis. To measure the concentration of ALLO, cerebella of control, Px, and ALLO-treated quail chicks were dissected after decapitation under deep anesthesia. Cerebella were subdivided into two regions: rostral cerebellum or caudal cerebellum. Each cerebellar tissue was combined to the total weight of ~500 mg and applied to GC-SIM analysis as described previously (2, 8, 9, 12, 13, 15, 16).

**Quantification of ALLO and/or EPI Synthesis.** To investigate whether melatonin influences the synthesis of cerebellar ALLO and/or EPI in the male juvenile quail, Px was performed at P2, and the quail chicks were s.c. implanted with a silastic plate containing melatonin (10 mg per plate) or vehicle (18). After decapitation under deep anesthesia, cerebella of control, Px, or melatonin-treated quail chicks at P3 were dissected and applied to HPLC analysis as described above.

**Quantification of ALLO Concentration in Serum.** Trunk blood was collected into heparinized glass tubes and centrifuged at 1,800 × *g* for 20 min at 4 °C. Individual plasma of quail chicks at P3 was

stored at –20 °C. Plasma samples (1 mL each) were homogenized in an aliquot of methanol/$H_2O$ [75:25 (vol/vol); 9 mL] on ice. The samples were passed through the C-18 cartridge column, and the neurosteroid fractions were eluted with methanol, dried, and applied to GC-SIM analysis as described above.

**Quantification of [$^3$H]ALLO Content in the Rostral and Caudal Cerebellum.** To better understand the mechanism of how pineal ALLO affects the adjacent cerebellar Purkinje cells, 20 pmol [$^3$H] ALLO (specific activity, 52.4 Ci/mmol; PerkinElmer) was injected close to the pineal lumen at P2 quail chicks. The content of [$^3$H] ALLO in the rostral and caudal cerebellum of quail chicks was counted in a liquid scintillation counter.

**Assessment of Neuronal Cell Death.** Parasagittal cerebellar sections of quail chicks at P3, P5, and P7 were examined by immunostaining with an antibody against cleaved caspase-3 (Asp175) (Cell Signaling Technology) to detect active-type caspase-3 (22) and by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (Roche) to detect apoptotic cells as described previously (23). The usefulness of the antibody against cleaved caspase-3 was validated in chickens as described previously (24).

**Statistical Analysis.** Results are expressed as the mean ± SEM. The significance of differences between the groups was evaluated by one-way ANOVA followed by the Tukey–Kramer test. The differences were considered significant if $P < 0.05$.


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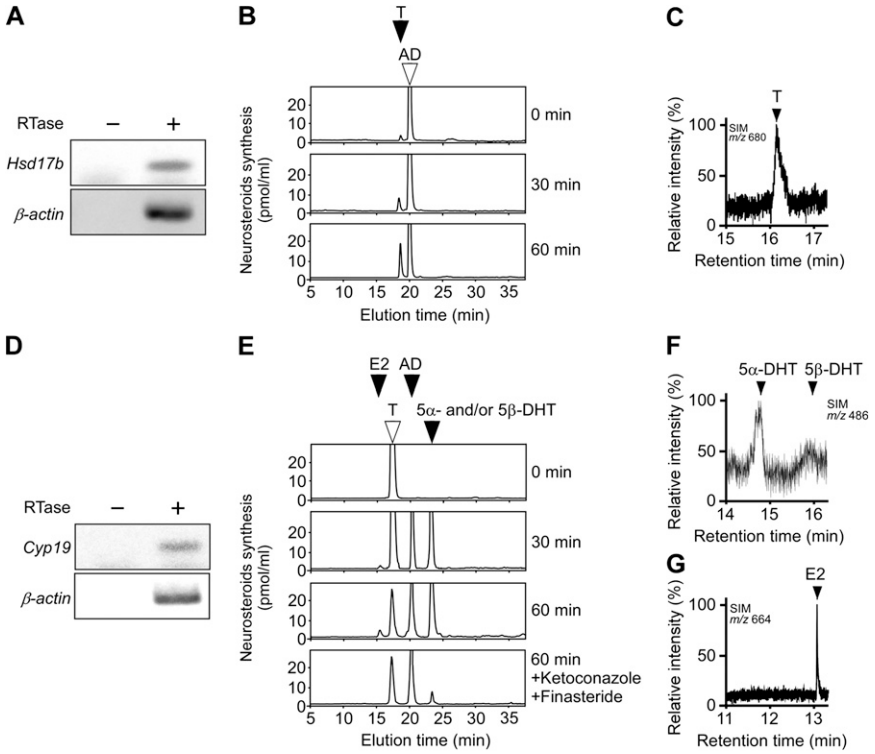


**Fig. S1.** Neurosteroid formation from AD or T in the pineal gland. (*A* and *D*) RT-PCR analyses of steroidogenic enzyme *Hsd17b* (*A*) and *Cyp19* (*D*) mRNAs in the pineal gland of male quail chicks. Total RNA was reverse-transcribed with (+) or without (–) RTase followed by PCR amplification. (*B* and *E*) HPLC analyses of neurosteroid formation in the pineal gland of male quail chicks. (*B*) The pineal gland homogenates were incubated with [$^3$H]AD + NADPH, and the extracts were subjected to HPLC. The arrowheads indicate elution positions of the substrate AD (open arrowhead) and its metabolite T (solid arrowhead). (*E*) The pineal gland homogenates were incubated with [$^3$H]T + NADPH, and the extracts were subjected to HPLC. The pineal gland homogenates with [$^3$H]T were also incubated with ketoconazole and finasteride. The arrowheads indicate elution positions of the substrate T (open arrowhead) and its metabolite AD, 5α- and/or 5β-DHT, and E2 (solid arrowheads). (*C*, *F*, and *G*) GC-SIM analyses of the metabolites of nonradioactive AD or T in the pineal gland of male quail chicks. GC-SIM was traced at *m/z* 680 for T (*C*), metabolite of nonradioactive AD, or at *m/z* 486 for 5α- and 5β-DHT (*F*) and at *m/z* 664 for E2 (*G*), metabolites of nonradioactive T. The arrowheads show the peaks corresponding to authentic T (*C*), 5α- and 5β-DHT (*F*), and E2 (*G*). Similar results were obtained in repeated experiments using three different samples.

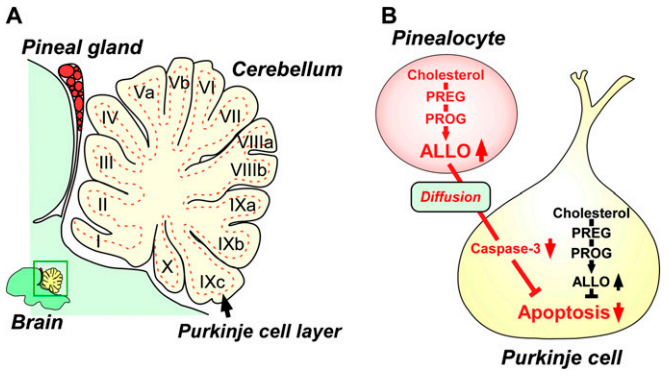


**Fig. S2.** A schematic model depicting the possible action of pineal ALLO on Purkinje cell survival in the developing cerebellum. (*A*) The location of the pineal gland in the quail chick brain. The pineal gland exists adjacent to the cerebellum. The square in the left bottom picture is magnified. (*B*) A schematic model of the neuroprotective action of pineal ALLO on Purkinje cells during development. ALLO is exceedingly produced in the pineal gland compared with the brain, may affect the adjacent cerebellar Purkinje cells by diffusion, and saves Purkinje cells from apoptosis in the juvenile quail. Secreted pineal ALLO inhibits the expression of active caspase-3 that may facilitate apoptosis of Purkinje cells in the cerebellum. Cerebellar ALLO may also act on Purkinje cells together with pineal ALLO to prevent Purkinje cell death (see text for details).

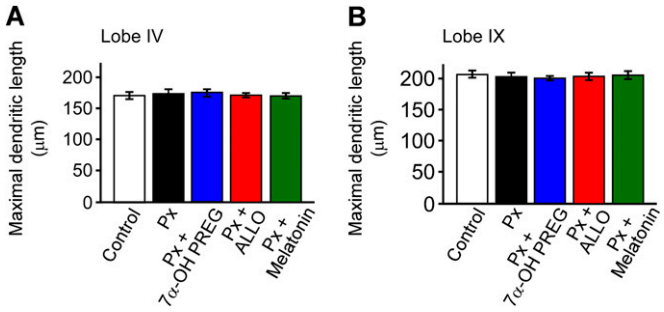


**Fig. S3.** Effect of pineal ALLO and 7α-OH PREG or melatonin on the maximal dendritic length of Purkinje cells. Cerebella of control male quail chicks, Px at P2 male quail chicks, and Px at P2 male quail chicks treated with daily injection of 7α-OH PREG (30 ng/5 μL) or ALLO (30 ng/5 μL), or Px at P2 male quail chicks s.c. implanted with a silastic plate containing melatonin (10 mg/plate) from P2 to P7 were analyzed. (*A* and *B*) Dendritic length of Purkinje cells in cerebellar lobes IV (*A*) and IX (*B*) ($n$ = 12). There was no statistical difference ($P > 0.05$). Each column and vertical line in *A* and *B* represent the mean ± SEM.

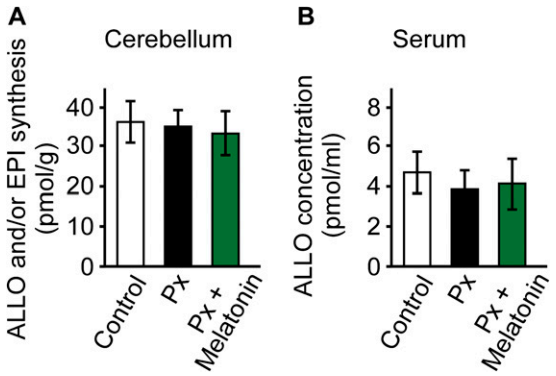


**Fig. S4.** Effect of pineal melatonin on the synthesis of ALLO and/or EPI synthesis and the level of circulating ALLO. Cerebella of control male quail chicks, Px at P2 male quail chicks, and Px at P2 male quail chicks s.c. implanted with a silastic plate containing melatonin from P2 to P3 were analyzed. (*A*) ALLO and/or EPI synthesis in the cerebellum at P3 ($n$ = 7). (*B*) Circulating ALLO concentration of serum at P3 ($n$ = 7). There was no statistical difference ($P > 0.05$). Each column and vertical line in *A* and *B* represent the mean ± SEM.

**Table S1. Primers for PCR analyses**

| Primer | Forward primer sequence (5′→3′) | Reverse primer sequence (5′→3′) |
|---|---|---|
| CHD | TTGCCAAGGATGAGAAACTG | TCTTCTCCTCCTACTGTGTT |
| chicken Cyp11a | ATGCTCTCCAGGGCTGCACC | TCACTCCTGGGGCTGGAGGG |
| StAR | AATCACTCAGCATCCTCGG | GGACCTGGTTGATGATGGTC |
| Cyp11a | TGCAGGTTGGTCTCTACGC | CTCCAGGATGTGCATGAGG |
| Cyp7b | ATGAACATTCGCATCAGCC | TCATCTCATTCATTGCGAGG |
| Hsd3b | AAGGCAGATGGCCAGATGTT | TGATGCGTCTGGCTTTCTGT |
| Srd5a | GTGCACCGACATCCGATTTA | CGATGGCAAAACCAAACCAT |
| Cyp17 | TGAGTACTCCATCCCCAAGG | CTCCTCGGGTTTATCCCACT |
| Hsd17b | GCTGTTTACCCACCTTTGGA | TTTTGAAATCCTCCCAGTGC |
| Cyp19 | CATTCTCATCTGCGTTCTGTTTCT | AGGCGTTACCTACTCCCATCC |
| β-Actin | TTGTGATGGACTCTGGTGATG | TTCTCTCTCGGCTGTGGTG |