Behavioral/Systems/Cognitive

7α-Hydroxypregnenolone Mediates Melatonin Action Underlying Diurnal Locomotor Rhythms

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Melatonin regulates diurnal changes in locomotor activity in vertebrates, but the molecular mechanism for this neurohormonal regulation of behavior is poorly understood. Here we show that 7α-hydroxypregnenolone, a previously undescribed avian neurosteroid, mediates melatonin action on diurnal locomotor rhythms in quail. In this study, we first identified 7α-hydroxypregnenolone and its stereoisomer 7β-hydroxypregnenolone in quail brain. These neurosteroids have not been described previously in avian brain. We then demonstrated that 7α-hydroxypregnenolone acutely increased quail locomotor activity. To analyze the production of 7α-hydroxypregnenolone, cytochrome $P450_{7\alpha}$, a steroidogenic enzyme of this neurosteroid, was also identified. Subsequently, we demonstrated diurnal changes in 7α-hydroxypregnenolone synthesis in quail. 7α-Hydroxypregnenolone synthesis and locomotor activity in males were much higher than in females. This is the first demonstration in any vertebrate of a clear sex difference in neurosteroid synthesis. This sex difference in 7α-hydroxypregnenolone synthesis corresponded to the sex difference in locomotion. We show that only males exhibited marked diurnal changes in 7α-hydroxypregnenolone synthesis, and these changes occurred in parallel with changes in locomotor activity. Finally, we identified melatonin as a key component of the mechanism regulating 7α-hydroxypregnenolone synthesis. Increased synthesis of 7α-hydroxypregnenolone occurred in males *in vivo* after melatonin removal via pinealectomy and orbital enucleation (Px plus Ex). Conversely, decreased synthesis of this neurosteroid occurred after melatonin administration to Px plus Ex males. This study demonstrates that melatonin regulates synthesis of 7α-hydroxypregnenolone, a key factor for induction of locomotor activity, thus inducing diurnal locomotor changes in male birds. This is a previously undescribed role for melatonin.

Key words: **neurosteroids; 7α-hydroxypregnenolone; melatonin; locomotor activity; diurnal changes; quail brain**

Introduction

A ubiquitous property of vertebrates is fluctuation of locomotor activity over the 24 h circadian cycle (Saper et al., 2005). The endogenous diurnal rhythm of melatonin is known to control the diurnal locomotor rhythm in vertebrates, including birds (Binkley et al., 1971; John et al., 1978; Cassone and Menaker, 1984; Chabot and Menaker, 1992; Hau and Gwinner, 1994; Warren and Cassone, 1995). However, molecular mechanisms for this neurohormonal regulation of behavior are poorly understood.

Conversely, the brain has traditionally been considered to be a target site of peripheral steroid hormones. In contrast, new findings over the past decade have shown that the brain itself also has the capability of forming steroids *de novo*, the so-called "neurosteroids." Studies on mammals (for review, see Baulieu, 1997; Compagnone and Mellon, 2000) and nonmammals (for review, see Tsutsui et al., 1999, 2003; Mellon and Vaudry, 2001) indicate that *de novo* neurosteroidogenesis in the brain from cholesterol is a conserved property of vertebrates. Our studies using Japanese quail have demonstrated that the avian brain possesses the key steroidogenic enzyme, cytochrome P450 side-chain cleavage enzyme (P450scc) (CYP11A), and produces pregnenolone, a precursor of neurosteroids (Tsutsui and Yamazaki, 1995; Usui et al., 1995; Tsutsui et al., 1997). Other steroidogenic enzymes also are expressed in the avian brain and convert pregnenolone to progesterone, 3β,5β-tetrahydroprogesterone, androstenedione, testosterone, and estradiol (Ukena et al., 1999, 2001; Matsunaga et al., 2001, 2002, 2004a; Tsutsui and Schlinger, 2001). However, the biosynthetic pathway of neurosteroids in birds and in other vertebrates may be still incompletely mapped out (for review, see Tsutsui et al., 2006).

We recently found that the newt brain actively produces 7α-hydroxypregnenolone, a previously undescribed amphibian neurosteroid, from pregnenolone (Matsunaga et al., 2004b). Interestingly, 7α-hydroxypregnenolone acts as a novel neuronal activator to stimulate locomotor activity of newts (Matsunaga et al., 2004b). We therefore hypothesized that 7α-hydroxypregnenolone may be a key factor for the induction of diurnal changes in locomotor activity in vertebrates. To demonstrate this

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hypothesis, we conducted a series of experiments using Japanese quail, a species that displays a robust diurnal locomotor activity rhythm (Wilson, 1972; Wada, 1979). We first identified 7α- and 7β-hydroxypregnenolone in quail brain by using biochemical techniques. We then demonstrated that only 7α-hydroxypregnenolone acutely increased quail locomotor activity. Cytochrome $P450_{7\alpha}$ (CYP7B), a steroidogenic enzyme of 7α-hydroxypregnenolone, was also identified. Subsequently, we demonstrated diurnal changes in 7α-hydroxypregnenolone synthesis in quail. Only males exhibited marked diurnal changes in 7α-hydroxypregnenolone synthesis, which paralleled with locomotor activity. In contrast, females showed constantly lower levels of 7α-hydroxypregnenolone synthesis and locomotor activity. Finally, we investigated the mechanism that regulates diurnal changes in 7α-hydroxypregnenolone synthesis in males based on evidence that melatonin controls diurnal changes in locomotor activity in birds (Binkley et al., 1971; John et al., 1978; Cassone and Menaker, 1984; Chabot and Menaker, 1992; Hau and Gwinner, 1994; Warren and Cassone, 1995). Here, we show that melatonin regulates synthesis of 7α-hydroxypregnenolone, a key factor for induction of locomotor activity, thus inducing diurnal locomotor rhythms in male birds.

Materials and Methods

Animals. Adult Japanese quail *Coturnix japonica*, 3 months of age, were housed in a temperature-controlled room (25 ± 2°C) under daily photoperiods of 16/8 h light/dark (LD) (lights on at 7:00 A.M., off at 11:00 P.M.). All birds were isolated in individual cages, and the experimental protocol was approved in accordance with guidelines prepared by Waseda University (Tokyo, Japan) and Hiroshima University (Higashi-Hiroshima, Japan).

Identification of 7α- and 7β-hydroxypregnenolone by biochemical analyses combined with HPLC, TLC, and gas chromatography/mass spectrometry. To identify previously undescribed avian neurosteroids produced from pregnenolone in the quail brain, the radioactive metabolites of [7-^{3}H]pregnenolone [specific activity, 14 Ci/mmol (1 Ci = 37 GBq); distribution of ^{3}H, 7α, 47.6%; 7β, 31.5%; 4(α + β), 8.7%; 2α, 10%; PerkinElmer, Boston, MA] were analyzed by HPLC using brain homogenates as described by Matsunaga et al. (2001, 2002, 2004a,b). In brief, brain homogenates containing 40 mg of tissue from male quail were incubated in PBS (10 mM phosphate buffer/140 mM NaCl, pH 7.5) containing 1 million cpm [^{3}H]pregnenolone, 0.24 mM NADPH, and 4% propylene glycol for 30 min at 40°C. After incubation, steroids were extracted by ethyl acetate and subjected to HPLC analysis by using a reversed-phase column, LiChrospher 100 RP-18 (4.0 × 250 mm; Kanto, Tokyo, Japan). The column was eluted with a 30 min linear gradient of 40–70% acetonitrile at a flow rate of 0.7 ml/min, followed by an isocratic elution of 70% acetonitrile. The eluate was fractionated every minute from 5 to 35 min and counted in a liquid scintillation counter. To confirm the involvement of steroidogenic enzyme in the formation of the unknown avian neurosteroids, brain homogenates and [^{3}H]pregnenolone were incubated with ketoconazole (Sigma, St. Louis, MO), an inhibitor of cytochrome P450s (CYPs), at a final concentration of 10^{-4} M.

The radioactive metabolites of pregnenolone also were analyzed by TLC using a silica gel plate (Merck, Darmstadt, Germany). The HPLC peak corresponding to the unknown avian neurosteroids were collected, dried, and subjected to TLC analysis by using ethyl acetate/*n*-hexane/acetic acid (16:8:1 ratio) as the mobile phase. The unknown avian neurosteroids were further examined in gas chromatography/mass spectrometry (GC-MS) analysis. Trimethylsilyl ether derivatives of the metabolites obtained from native pregnenolone (Sigma) were prepared before GC-MS by reacting the dried sample with bis(trimethylsilyl)trifluoroacetamide (Wako Pure Chemical, Osaka, Japan) for 30 min at 60°C. For the identification of the unknown avian neurosteroids, a GC-MS system (GCMS-QP5000; Shimadzu, Kyoto, Japan) using a CP-Sil 5CB capillary column (0.25 mm × 30 m; Varian, Palo Alto, CA) was used as described by Matsunaga et al. (2004b). The column was maintained at 220°C for 5 min, and then the temperature was raised to 300°C at the rate of 5°C/min. These biochemical analyses were repeated independently a minimum of four times. Both 7α-hydroxypregnenolone and its stereoisomer 7β-hydroxypregnenolone, which were used as reference standards in these analyses, were purchased from Steraloids (Newport, RI).

Cloning and sequencing of a cDNA encoding quail CYP7B, a steroidogenic enzyme of 7α-hydroxypregnenolone. CYP7B is considered to catalyze the conversion of pregnenolone to 7α-hydroxypregnenolone in mammalian brains (Stapleton et al., 1995; Rose et al., 1997). Male quail were used for the identification of a putative cDNA encoding quail CYP7B. Total RNA of the diencephalon was extracted with Sepazol-RNA I Super (Nacalai Tesque, Kyoto, Japan). All PCR amplifications were performed in a reaction mixture containing *Taq* polymerase [Ex *Taq* polymerase; Takara, Shiga, Japan) or gene *Taq* polymerase (Nippon Gene, Tokyo, Japan)] and 0.2 mM dNTP on a thermal cycler (Program Temperature Control System PC-700; Astec, Fukuoka, Japan). To determine the 3′-end sequence of quail CYP7B, first-strand cDNA was synthesized with the oligo-dT anchor primer supplied in the 5′/3′ rapid amplification of cDNA ends (RACE) kit (Roche Diagnostics, Indianapolis, IN) and amplified with the anchor primer and chicken CYP7B primer 1 (chicken CYP7B, nucleotides 1900–1919, 5′-CTGCAGTCAACAGGTCAGAA-3′). First-round PCR products were reamplified with the anchor primer and chicken CYP7B primer 2 (chicken CYP7B, nucleotides 1946–1965, 5′-TCACCAGAGAACAATTGGAC-3′). Both primers were designed on the basis of the nucleotide sequence of chicken CYP7B (GenBank accession number XM_418276). The second-round PCR products were subcloned into a pGEM-T Easy vector (Promega, Madison, WI). The DNA inserts of the positive clones were amplified by PCR with universal M13 primers. The 3′-end sequence of quail CYP7B was determined and used for the determination of the 5′-end sequence of quail CYP7B. Template cDNA was synthesized with an oligonucleotide primer complementary to quail CYP7B (nucleotides 1411–1430, 5′-CATCTCATTCATTGC-GAGGA-3′); this synthesis was followed by dA-tailing of the cDNA with dATP and terminal transferase (Roche Diagnostics). The tailed cDNA was amplified with the oligo-dT anchor primer and quail CYP7B primer 1 (quail CYP7B, nucleotides 1300–1319, 5′-GCCATTCTCTATGTAAC-GAT-3′); this was followed by additional amplification of the first-round PCR products with the anchor primer and the quail CYP7B primer 2 (quail CYP7B, nucleotides 1258–1278, 5′-CTTCATAGACCTC-GGGGTCC-3′). The second-round PCR products were subcloned, and the inserts were amplified as described above.

All nucleotide sequences of a putative quail CYP7B were determined with a Thermo Sequenase cycle sequencing kit (GE Healthcare, Freiburg, Germany), IRDye 800 termination mixes version 2 (PerkinElmer Life Sciences, Boston, MA), and a model 4200-1 G DNA sequencing system and analysis system (LI-COR, Lincoln, NE) and then analyzed with DNASIS-MAC software (Hitachi Software Engineering, Kanagawa, Japan). Universal M13 primers or gene-specific primers were used to sequence both strands.

Enzymatic activity of quail CYP7B transfected in COS-7 cells. To assess its enzymatic activity, COS-7 cells were transfected with the quail putative CYP7B as described by Yin et al. (2005). The full-length open reading frame of a putative quail CYP7B was amplified from quail diencephalon cDNA using the forward primer 5′-GCCGCCACCATGGGCCCCG-AAGCGCTGCC-3′ and the reverse primer 5′-GCTCAATTTCTTAA-GGACCT-3′ and subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA), a mammalian expression vector. 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 Riken Cell Bank (Tsukuba, Japan) and maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum, 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 (Yin et al., 2005). After transfection, the cells were harvested, centrifuged (10,000 × *g* for 5 min at 4°C), and stored at −80°C. The cell pellets were homogenized and reacted with [^{3}H]pregnenolone for HPLC analysis or nonradioactive pregnenolone for GC-MS analysis, as described by Matsunaga et al. (2004b).

Measurements of the production and concentration of 7α- and 7β-hydroxypregnenolone by HPLC and GC-MS. To measure the production of 7α- and 7β-hydroxypregnenolone in different brain regions, quail exposed to LD (lights on at 7:00 A.M.) were terminated by decapitation between 10:00 A.M. and 11:00 A.M., and each brain was subdivided into the telencephalon, diencephalon, and mesencephalon. Samples from each brain region were pooled and homogenized. Each homogenate containing 40 mg of tissue was incubated separately with tritiated pregnenolone for 20 min at 40°C, and extracted steroids were subjected to HPLC analysis. To measure the concentration of endogenous 7α- and 7β-hydroxypregnenolone in different brain regions, GC-MS analysis was performed as described by Matsunaga et al. (2004b). Steroids in each brain region were extracted by solid-phase extraction using C18 columns. Each pooled brain region (200 mg) was homogenized in an aliquot of methanol/H_2O (75:25, v/v; 1 ml) on ice. After centrifugation (3000 × *g*, 5 min), each sample (supernatant of homogenate) was diluted to a final concentration of 5% methanol. Each sample then was extracted with C18 columns previously equilibrated with methanol and methanol/H_2O (5:95, v/v) successively. Samples were passed through the cartridge, and the steroid fraction was eluted with methanol and evaporated to dryness. Then, the sample was applied to a GC-MS system as described above. In addition, the internal standard [17,21,21,21-^{2}H]pregnenolone was prepared as described by Vallée et al. (2000) and Matsunaga et al. (2004b). In brief, [17,21,21,21-^{2}H]pregnenolone was prepared from the unlabeled parent steroid for the internal standard. The parent steroid pregnenolone (100 mg) was subjected to a reflux exchange reaction using a 35% solution of ^{2}HCl (99% ^{2}H, 50 μl) in $CH_3CH_2O^2H$ (99% ^{2}H, 3.3 ml) overnight. The reaction mixture was evaporated to dryness, the exchange reaction was repeated, and the product was crystallized twice from aqueous ethanol. The synthesized ^{2}H-labeled steroid then was isolated as a single peak from a preparative HPLC column before using this steroid as an internal standard. Because the production and concentration of 7α- and 7β-hydroxypregnenolone in the diencephalon were much higher than those in other brain regions (see Fig. 3), diurnal changes of these parameters were analyzed in the diencephalon.

Measurement of the expression of quail CYP7B mRNA by real-time quantitative PCR. To quantify the expression of quail CYP7B mRNA in the brain, real-time quantitative PCR was conducted by using the LineGene system (LineGene FQD-33A; BioFlux, Tokyo, Japan) according to the recommendations of the manufacturer. β-Actin, a housekeeping gene, was used for the internal standard. The PCR primers used for the amplification of quail CYP7B cDNA fragments were 5′-TAACATCCACCTCACCAGAG-3′ (identical with nucleotides 1061–1080; GenBank accession number AB329632) and 5′-TTTCCTCAAACTGACTTCCTG-3′ (complementary to nucleotides 1200–1220; GenBank accession number AB329632). The PCR primers for β-actin were 5′-TTGTGATGGACTCTGGTGATG-3′ (identical with nucleotides 389–409; GenBank accession number AF199488) and 5′-TTCTCTCTCGGCTGTGGTG-3′ (complementary to nucleotides 540–558; GenBank accession numbers AF199488). PCR was performed using a SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan). An external standard curve was generated by dilutions of the target PCR product, which had been purified and its concentration measured previously. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and gel electrophoresis. The quail CYP7B gene expression in each reaction was normalized by the expression of β-actin. The relative mRNA expression levels were calculated according to the comparative C_T ($\Delta\Delta C_T$) method described previously (Ohnishi et al., 2007).

In situ *hybridization of CYP7B mRNA.* To examine the cellular localization of CYP7B mRNA, *in situ* hybridization of CYP7B mRNA was conducted. Because the production and concentration of 7α- and 7β-hydroxypregnenolone in the male diencephalon were much higher than those in other brain regions (see Fig. 3), the site of CYP7B mRNA expression in the male diencephalon was localized by *in situ* hybridization. Partial quail CYP7B cDNA (corresponding to nucleotides 1212–1693; GenBank accession number AB329632) was obtained by reverse transcription (RT)-PCR using the following specific primers: sense primer, 5′-AAGGTGGCCCCTAAGATTCC-3′ (identical with nucleotides 1212–1233; GenBank accession number AB329632) and antisense primer 5′-AGAAATTCCGTATGTCAGCA-3′ (complementary to nucleotides 1672–1693; GenBank accession number AB329632). The RT-PCR product was subcloned into pGEM-T Easy vector (Promega). Quail CYP7B probes were labeled with digoxigenin (DIG) RNA labeling kit according to the instructions of the manufacturer (Roche Diagnostics). Quail were killed by decapitation; brains were rapidly dissected, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), and frozen. Sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, rinsed in PBS, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 5 min, and then washed in 2× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0). Subsequently, the sections were prehybridized for 1 h at 50°C in prehybridization buffer consisting of 50% formamide, 2× SSC, 1× Denhardt's solution, 0.5 mg/ml yeast transfer RNA, 0.5 mg/ml heparin sodium, and 0.1% sodium pyrophosphate. Hybridization was performed at 50°C for 16 h with DIG-labeled CYP7B RNA probe diluted with hybridization buffer (prehybridization buffer supplemented with 10% dextransulfate). After hybridization, the sections were sequentially washed in four bathes of 2× SSC at room temperature for 10 min each, washed in 2× SSC–50% formamide at 50°C for 1 h, treated with RNase A (20 μg/ml) in RNase buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 1 mM EDTA) at 37°C for 30 min, washed sequentially in RNase buffer, 2× SSC at room temperature, and then washed in 1× SSC–50% formamide at 50°C for 1 h. Slides were rinsed in buffer 1 (100 mM Tris-HCl, pH 7.5, and 150 mM NaCl) and then incubated with buffer 1 containing 2% blocking reagent (Roche Diagnostics). Then, sections were incubated with alkaline phosphatase-labeled sheep anti-DIG antibody (Roche Diagnostics; diluted 1:1000 for detection of CYP7B mRNA expression) at 4°C for overnight. Sections were washed in buffer 1, then immersed in buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$), and then incubated with the solution of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at room temperature for 48 h under dark condition. Control for specificity of *in situ* hybridization of CYP7B mRNA was performed by using the DIG-labeled sense RNA probe complementary to the antisense probe sequence.

Surgery, steroid administration, and behavioral analysis. All surgery was performed under Nembutal anesthesia (40 mg/kg). Using a stereotaxic instrument, male birds were chronically implanted with a 12 mm, 23 gauge steel guide cannula (Eicom, Kyoto, Japan) aimed at the lateral ventricle of the brain. Ten days after surgery, birds received an intracerebroventricular injection of vehicle or 7α-hydroxypregnenolone via a 13 mm, 30 gauge stainless steel injector. 7α-Hydroxypregnenolone dissolved in isotonic saline containing 0.2% DMSO was injected over a period of 30 s into the lateral ventricle at different doses (10 and 100 ng in a 5 μl solution). Control treatment consisted of an equal volume of vehicle alone. For behavioral testing, quail were placed individually in an empty soundproof chamber. For 30 min after administration of 7α-hydroxypregnenolone, locomotor activity was measured by using an implantable telemetry system (IMT-200; Star Medical, Tokyo, Japan). The obtained data were analyzed using Chart version 4.0 software (ADInstruments, Castle Hill, New South Wales, Australia). The effect of 7β-hydroxypregnenolone, a stereoisomer of 7α-hydroxypregnenolone, on locomotion was also examined. Behavioral experiments were performed during 6:00–10:00 P.M. when spontaneous locomotor activity of males was low (see Fig. 6*A*). To confirm the action of 7α- or 7β-hydroxypregnenolone on locomotor activity, male birds received an intracerebroventricular injection of ketoconazole (5 μg in a 5 μl solution), an inhibitor of CYPs, during 5:00–6:00 A.M., and behavioral experiments were performed during 10:00 A.M. to 12:00 P.M. when spontaneous locomotor activity of males was high (see Fig. 6*A*). To investigate the effect of 7α-hydroxypregnenolone on locomotor activity in the female, female birds received an intracerebroventricular injection of 7α-hydroxypregnenolone (100 ng in a 5 μl solution) and subsequently locomotion was examined during 10:00 A.M. to 12:00 P.M. Diurnal changes in locomotor activity of intact birds of both sexes were also measured by using an implantable telemetry system at intervals of 1 h during 1:00 A.M. to 12:00 A.M.

Pinealectomy combined with orbital enucleation and melatonin admin-

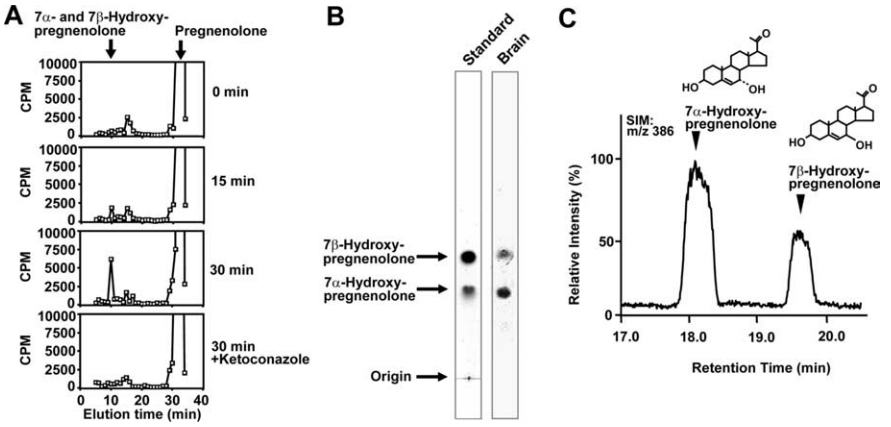

Figure 1. Identification of 7α- and 7β-hydroxypregnenolone in the quail brain. ***A***, HPLC profile of unknown metabolites of pregnenolone by using a reversed-phase column. The brain homogenates were incubated with [^{3}H]pregnenolone, and the extracts were subjected to HPLC. The ordinate indicates the radioactivity measured in each HPLC fraction, and the arrows indicate elution positions of standard steroids, pregnenolone, and 7α- and 7β-hydroxypregnenolone. ***B***, Autoradiography of the unknown pregnenolone metabolites (right column) and standard steroids 7α- and 7β-hydroxypregnenolone (left column) on TLC under the same condition as in ***A***. ***C***, GC-SIM mass trace of *m/z* 386 in the extract from male brain homogenates. The arrowheads indicate the retention times of 7α-hydroxypregnenolone and 7β-hydroxypregnenolone. The *m/z* 386 ion is a diagnostic ion of 7α- and 7β-hydroxypregnenolone (see Fig. 2*F*) (Matsunaga et al., 2004b).

istration. In this experiment, we investigated the effect of melatonin on the production of 7α- and 7β-hydroxypregnenolone in the diencephalon of males. The pineal gland and eyes are the major sources of melatonin in the quail (Underwood et al., 1984). Pinealectomy with orbital enucleation (Px plus Ex) and sham-operation (SH) were performed under Nembutal anesthesia as described previously (Oishi and Lauber, 1974; Ubuka et al., 2005). Px plus Ex birds were divided into two groups and subcutaneous implanted with a SILASTIC (silicone type; Dow Corning, Midland, MI) plate containing melatonin (Sigma) at a dose of 10 mg/plate or vehicle as described previously (Tsutsui et al., 1998; Ubuka et al., 2001, 2005). One week after surgery, SH, Px plus Ex, and Px plus Ex plus melatonin birds were terminated by decapitation at 3:00 A.M. for the collection of diencephalic samples. The production of 7α- and 7β-hydroxypregnenolone in the male diencephalon was measured by HPLC as described above. The completeness of Px and Ex was verified at autopsy and confirmed by measuring melatonin in the diencephalon by means of an RIA as described by Ubuka et al. (2005). In brief, melatonin was extracted from each diencephalic sample by using chloroform as described previously (Ubuka et al., 2005). The RIA was performed as described previously (Ubuka et al., 2005), by using rabbit anti-melatonin serum supplied by the Institute for Molecular and Cellular Regulation (Gunma University, Maebashi, Japan). For the measurement of melatonin, separate groups of identically treated male birds of SH, Px plus Ex, and Px plus Ex plus melatonin groups were terminated at 3:00 A.M.

Treatment with melatonin receptor antagonist. To demonstrate whether luzindole, an antagonist of melatonin receptor, influences the production of 7α- and 7β-hydroxypregnenolone in the diencephalon, male quail received an intracerebroventricular injection of luzindole (100 μg in a 5 μl solution; Sigma) during 11:00 P.M. to 12:00 A.M. when melatonin secretion started. Birds were terminated at 3:00 A.M. when endogenous melatonin was high. The production of 7α- and 7β-hydroxypregnenolone in the diencephalon was measured by HPLC as described above.

Results

Identification of 7α- and 7β-hydroxypregnenolone in the brain

First, we identified previously undescribed avian neurosteroids from the quail brain by using biochemical techniques combined with HPLC, TLC, and GC-MS analyses. The initial finding was that unknown avian neurosteroids were metabolized from pregnenolone in the quail brain. Quail brain homogenates were incubated with tritiated pregnenolone as a precursor, and radioactive metabolites were analyzed by reversed-phase HPLC. A major radioactive peak of the metabolites was detected ~20 min before the elution of pregnenolone, a precursor (Fig. 1*A*). Several nonradioactive steroids were used as reference standards for HPLC analysis, and 7α-hydroxypregnenolone and its stereoisomer 7β-hydroxypregnenolone exhibited the same retention time of the radioactive peak under a similar chromatographic condition (Fig. 1*A*). The detection of [^{3}H]7α- and 7β-hydroxypregnenolone was feasible by HPLC because the radioactive pregnenolone used in this study was labeled with ^{3}H at multiple positions, including the 7α or 7β position (see Materials and Methods). The radioactive metabolites corresponding to 7α- and 7β-hydroxypregnenolone increased in a time-dependent manner (Fig. 1*A*), and the inhibitor of CYPs, ketoconazole (10^{-4} M), reduced the metabolites (Fig. 1*A*). The HPLC peak was collected and subjected to TLC. Nonradioactive 7α- and 7β-hydroxypregnenolone were used as reference standards (visualized by iodine atmosphere), and the metabolites of tritiated pregnenolone were detected by autoradiography. As shown in Figure 1*B*, quail brain homogenates produced two radioactive metabolites from [^{3}H]pregnenolone corresponding to the positions of the 7α- and 7β-hydroxypregnenolone standards. The metabolites of pregnenolone were further analyzed by GC-MS. Trimethylsilyl ether derivatives of the authentic 7α- and 7β-hydroxypregnenolone and the metabolites obtained from nonradioactive pregnenolone were prepared and subsequently applied to GC-MS analysis. Based on GC-selected ion monitoring (SIM) analysis [mass/charge (*m/z*) 386], the metabolites had retention times that were identical to 7α-hydroxypregnenolone (18.1 min) and 7β-hydroxypregnenolone (19.7 min) (Fig. 1*C*). Thus, the unknown avian neurosteroids converted from pregnenolone in the quail brain were identified as 7α- and 7β-hydroxypregnenolone.

Identification of quail CYP7B, a steroidogenic enzyme of 7α-hydroxypregnenolone, and demonstration of its enzymatic activity

To determine the mode of 7α-hydroxypregnenolone synthesis, we identified a cDNA from brain tissue encoding a putative quail CYP7B, a steroidogenic enzyme producing 7α-hydroxypregnenolone from pregnenolone. A combination of 3′- and 5′-RACE produced a sequence that we then compared with chicken, mouse, and human CYP7B (Stapleton et al., 1995; Rose et al., 1997; Schwarz et al., 1998). The identified putative quail CYP7B cDNA revealed a full length of 2341 bp (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). The putative quail CYP7B open reading frame commences with a methionine at nucleotide 72 and terminates with a TGA codon at nucleotide 1581, encoding a protein of 503 amino acids (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). The deduced amino acid sequence of the open reading frame (503 amino acids) displayed 99, 49, and 52% identities with chicken, mouse, and human CYP7B, respectively. The putative

quail CYP7B contains a highly conserved motif, FXXGXXXCXG(XXXA) of CYPs (see double underlining in supplemental Fig. S1, available at www.jneurosci.org as supplemental material) (Nelson et al., 1993), which is thought to represent the heme binding site with the arrangement of amino acids around the cysteine residue postulated to preserve the three-dimensional structure of this region for ligand binding (Poulos, 1988). Another domain (see underlining in supplemental Fig. S1, available at www.jneurosci.org as supplemental material), which may be conserved in CYPs responsible for steroid interconversions (Chung et al., 1987; Noshiro and Okuda, 1990), also featured in quail CYP7B.

After its identification, we demonstrated the enzymatic activity of this putative quail CYP7B. The homogenate of COS-7 cells transfected with the putative quail CYP7B cDNA converted pregnenolone to 7α- and/or 7β-hydroxypregnenolone by HPLC analysis (Fig. 2*A*), whereas the inhibitor of CYPs, ketoconazole (10^{-4} M), reduced this metabolic process (Fig. 2*B*). COS-7 cells that were not transfected with the putative quail CYP7B cDNA did not convert pregnenolone to 7α- and/or 7β-hydroxypregnenolone (Fig. 2*C*). Subsequently, 7α-hydroxypregnenolone synthesis was confirmed by GC-MS analysis. The homogenate of COS-7 cells transfected with the putative quail CYP7B cDNA produced a metabolite that had the same retention time as 7α-hydroxypregnenolone characterized by GC-MS total ion current (TIC) trace (Fig. 2*D*). GC-MS TIC trace also showed that 7α-hydroxypregnenolone and the metabolite had the same diagnostically important ions (*m*/*z* 386 and 476 in Fig. 2*F*). However, we could not detect a metabolite corresponding 7β-hydroxypregnenolone using the same trace (Fig. 2*D*). COS-7 cells without transfection of the putative quail CYP7B cDNA did not convert pregnenolone to 7α-hydroxypregnenolone (Fig. 2*E*).

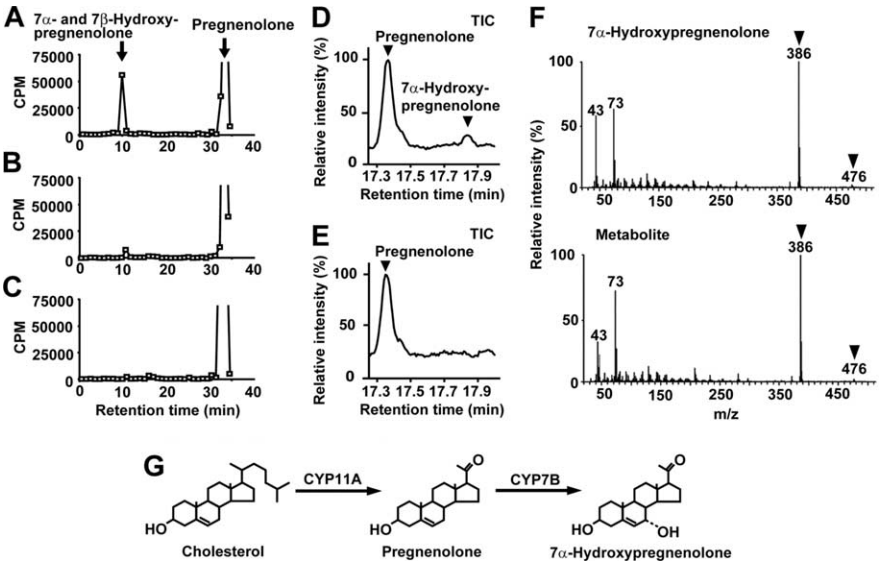

Figure 2. 7α-Hydroxypregnenolone synthesis in COS-7 cells expressing the putative quail CYP7B. ***A***, HPLC profile of 7α- and/or 7β-hydroxypregnenolone and pregnenolone extracted from the COS-7 cells that were transfected with the putative quail CYP7B cDNA by pcDNA3.1/V5-His-TOPO and incubated with [^{3}H]pregnenolone. The ordinate indicates the radioactivity measured in each HPLC fraction, and the arrows indicate elution positions of standard steroids pregnenolone and 7α- and/or 7β-hydroxypregnenolone. ***B***, HPLC profile of the extract from the COS-7 cells that were transfected with the putative quail CYP7B cDNA and incubated with [^{3}H]pregnenolone and ketoconazole, an inhibitor of CYPs. ***C***, HPLC profile of the extract from the COS-7 cells that were transfected with pcDNA3.1/V5-His-TOPO expression construct alone and incubated with [^{3}H]pregnenolone. ***D***, GC-MS analysis of 7α-hydroxypregnenolone. GC-MS TIC trace of the extract from the COS-7 cells that were transfected with the putative quail CYP7B cDNA and incubated with pregnenolone. The arrowheads show the peaks corresponding to 7α-hydroxypregnenolones and pregnenolone. ***E***, GC-MS TIC trace of the extract from the COS-7 cells without transfection of the putative quail CYP7B cDNA. ***F***, GC-MS of trimethylsilyl ether derivatives of an unknown pregnenolone metabolite and the authentic 7α-hydroxypregnenolone. The arrowheads indicate diagnostically important ions of 7α-hydroxypregnenolone (*m*/*z* 386 and 476). ***G***, The quail brain expresses CYP11A (P450scc) (Tsutsui and Yamazaki, 1995; Usui et al., 1995; Tsutsui et al., 1997) and CYP7B ($P450_{7\alpha}$) (present study) and produces pregnenolone (Tsutsui and Yamazaki, 1995; Usui et al., 1995; Tsutsui et al., 1997) and 7α-hydroxypregnenolone (present study).

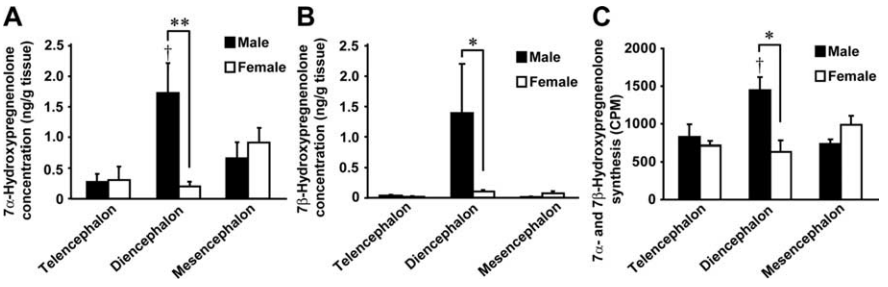

Figure 3. Sex differences in the concentration and production of 7α- and 7β-hydroxypregnenolone in different brain regions. Quail exposed to LD (lights on at 7:00 A.M.) were terminated between 10:00 and 11:00 A.M. Each column and vertical line represent the mean ± SEM ($n = 5$). *$p < 0.05$ and **$p < 0.01$, male vs female; †$p < 0.05$ vs telencephalon by one-way ANOVA, followed by Duncan's multiple range test.

Comparison of the production and concentrations of 7α- and 7β-hydroxypregnenolone among different brain regions

To understand the action of 7α- and 7β-hydroxypregnenolone, data on the regio-specific synthesis of these neurosteroids are needed. We therefore compared the concentrations of 7α- and 7β-hydroxypregnenolone among different brain regions of the quail brain in both sexes by GC-MS analysis. As shown in Figure 3, *A* and *B*, 7α- and 7β-hydroxypregnenolone concentrations in the diencephalon of males were higher than those of females ($p < 0.01$ or $p < 0.05$, male vs female). However, the concentrations of these neurosteroids in other brain regions were very low in both sexes (Fig. 3*A*,*B*). We then compared the production of 7α- and 7β-hydroxypregnenolone by HPLC analysis (Fig. 3*C*). As shown in Figure 3*C*, the production of 7α- and 7β-hydroxypregnenolone in the diencephalon of males was also higher than that of females ($p < 0.05$, male vs female).

Cellular localization of CYP7B mRNA in the diencephalon

In situ hybridization of CYP7B mRNA was examined in the male diencephalon. As shown in Figure 4, *A* and *C*, an intense expression of CYP7B mRNA was detected in several diencephalic regions. Clusters of the cells expressing CYP7B mRNA were localized in the following restricted regions: the nucleus preopticus medialis (POM) (Fig. 4*A*), nucleus paraventricularis magnocellularis (PVN) (Fig. 4*C*), nucleus ventromedialis hypothalami (VMN), nucleus dorsolateralis anterior thalami (DLA), and nu-

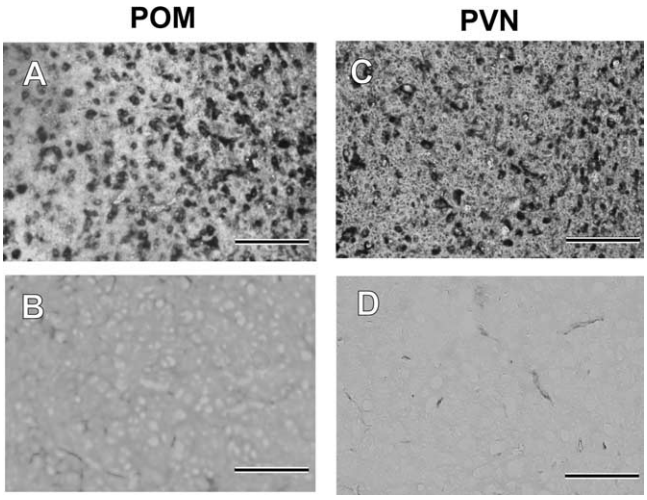

Figure 4. Expression of CYP7B mRNA in the male diencephalon. *In situ* hybridization using the antisense probe for CYP7B mRNA (***A***, ***C***) or the sense probe for CYP7B mRNA (***B***, ***D***) in the POM (***A***, ***B***) and PVN (***C***, ***D***). Scale bars, 100 μm.

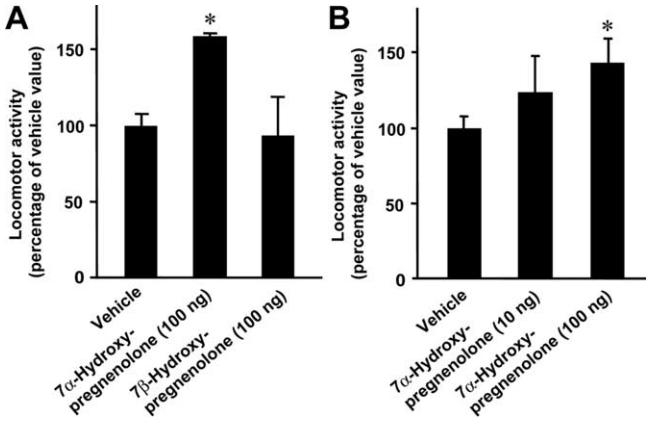

Figure 5. Effect of 7α- and 7β-hydroxypregnenolone on locomotor activity of the male quail. ***A***, Male quail received an intracerebroventricular injection of vehicle (saline alone, $n = 8$), 7α-hydroxypregnenolone (100 ng, $n = 8$), or 7β-hydroxypregnenolone (100 ng, $n = 8$). ***B***, Male quail received an intracerebroventricular injection of vehicle (saline alone, $n = 8$), 7α-hydroxypregnenolone (10 ng, $n = 8$), or 7α-hydroxypregnenolone (100 ng, $n = 8$). Locomotor activity of each group is expressed as the percentage of the vehicle value. Each column and vertical line represent the mean ± SEM. *$p < 0.05$ vs vehicle by one-way ANOVA, followed by Duncan's multiple range test.

cleus lateralis anterior thalami (LA). A complete absence of CYP7B mRNA expression in these positively stained cells was observed in controls in which the sense probe was used (Fig. 4*B*,*D*).

Stimulation of locomotor activity by 7α-hydroxypregnenolone administration

To demonstrate whether 7α- and 7β-hydroxypregnenolone are involved in the regulation of locomotor activity, we used male quail because the production and concentrations of these neurosteroids in the male diencephalon were much higher than those in the female (Fig. 3). Behavioral experiments were performed during 6:00–10:00 P.M. when spontaneous locomotor activity of males was low (see Fig. 6*A*). In the 30 min after intracerebroventricular injection, 7α-hydroxypregnenolone significantly increased locomotor activity of male quail ($p < 0.05$, 7α-hydroxypregnenolone vs vehicle) as shown in Figure 5*A*. This stimulatory effect tended to be dose dependent; the effective dose ranged between 10 and 100 ng per intracerebroventricular injection (Fig. 5*B*). In contrast, 7β-hydroxypregnenolone at the same high dose (100 ng) did not influence locomotor activity (Fig. 5*A*).

Diurnal changes in locomotor activity and 7α-hydroxypregnenolone production and concentration

To understand the functional significance of 7α-hydroxypregnenolone in the regulation of locomotor activity, diurnal changes in locomotor activity were examined alongside changes in the production and concentration of 7α-hydroxypregnenolone. Behavioral analysis indicated that locomotor activity of male birds was much higher ($p < 0.01$ or $p < 0.05$, male vs female) than that in females during 7:00 A.M. to 1:00 P.M. under LD (lights on at 7:00 A.M., off at 11:00 P.M.) (Fig. 6*A*). Locomotor activity of males decreased thereafter to that of females (Fig. 6*A*). The concentration of 7α-hydroxypregnenolone in the male diencephalon changed markedly, with a maximal level at 11:00 A.M. ($p < 0.05$, 11:00 A.M. vs 3:00 A.M. or 7:00 P.M.; $p < 0.05$, male vs female at 11:00 A.M.) when locomotor activity of males was high (Fig. 6*A*,*B*). Similar diurnal changes in the production of 7α- and 7β-hydroxypregnenolone were detected in the male diencephalon ($p < 0.01$, 11:00 A.M. vs 3:00 A.M. or 7:00 P.M.; $p < 0.05$, male vs female at 11:00 A.M.) (Fig. 6*D*). However, diurnal changes in the concentration of 7β-hydroxypregnenolone were less pronounced than those of 7α-hydroxypregnenolone (Fig. 6*B*,*C*). The production and concentrations of 7α- and 7β-hydroxypregnenolone in the diencephalon were constantly low in females (Fig. 6*B–D*), which correspondingly exhibited lower locomotor activity than males (Fig. 6*A*).

Changes in locomotor activity after 7α-hydroxypregnenolone manipulation

As shown in Figure 5, 7α-hydroxypregnenolone significantly increased locomotor activity of male quail. To demonstrate whether ketoconazole, an inhibitor of CYPs, inhibits locomotor activity of male quail, ketoconazole (5 μg) was treated during 5:00–6:00 A.M., and behavioral experiments were performed during 10:00 A.M. to 12:00 P.M. when spontaneous locomotor activity of males was high (Fig. 6*A*). Ketoconazole significantly decreased locomotor activity of male quail for a 30 min observation (67.0 ± 8.7% of vehicle value; $n = 7$ in each group; $p < 0.05$). Subsequently, we characterized the effect of 7α-hydroxypregnenolone administration on locomotor activity of female quail. Behavioral experiments were performed during 10:00 A.M. to 12:00 P.M. when spontaneous locomotor activity and 7α-hydroxypregnenolone concentration of females were lower than those of males (Fig. 6). In the 30 min after intracerebroventricular injection, 7α-hydroxypregnenolone did not significantly increase locomotor activity of female quail (101.4 ± 6.2% of vehicle value; $n = 7$ in each group; $p > 0.05$). These results indicate that 7α-hydroxypregnenolone is involved in the regulation of diurnal changes in locomotor activity only in males.

Reduction of 7α-hydroxypregnenolone production and concentration by melatonin administration

To investigate whether melatonin is involved in the regulation of 7α-hydroxypregnenolone synthesis, melatonin was manipulated in male birds. Px plus Ex significantly increased 7α-hydroxypregnenolone concentration ($p < 0.01$) (Fig. 7*A*) as well as the production of 7α- and 7β-hydroxypregnenolone ($p < 0.01$) (Fig. 7*B*) in the diencephalon compared with SH. Px plus Ex

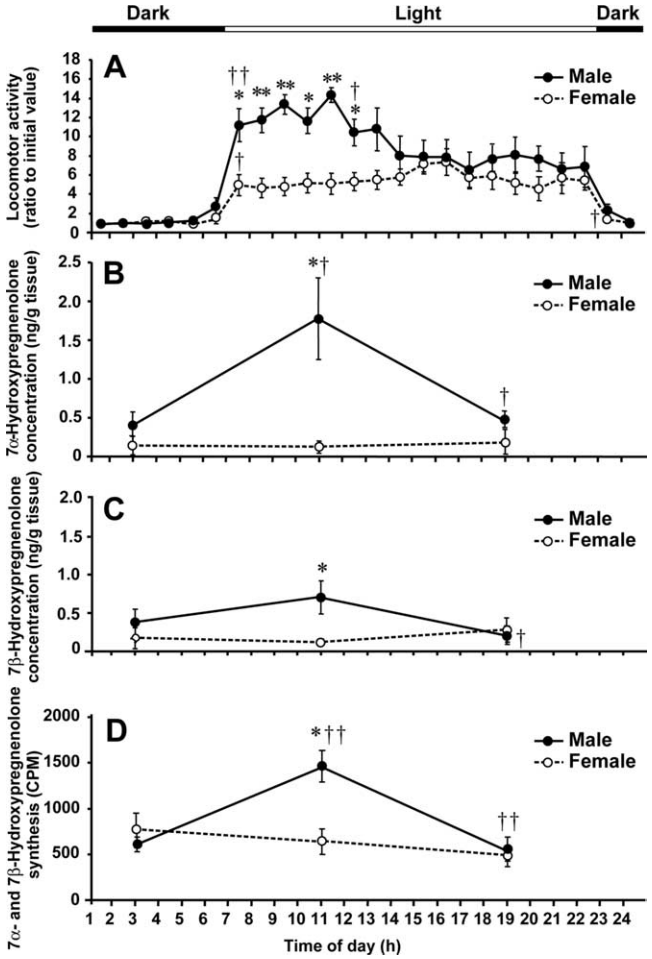

Figure 6. Diurnal changes in locomotor activity and 7α- and 7β-hydroxypregnenolone production and concentration. Quail were housed under LD photoperiods (lights on at 7:00 A.M.). Locomotor activities of males and females are expressed as the ratio to the initial value observed during 1:00–2:00 A.M. in each sex. Values represent the mean ± SEM ($n = 5$) for each time. *$p < 0.05$ and **$p < 0.01$ vs female; †$p < 0.05$ and ††$p < 0.01$ vs previous time by one-way ANOVA, followed by Duncan's multiple range test.

also induced a significant increase in the expression of quail CYP7B mRNA ($p < 0.01$, Px plus Ex vs SH) in the diencephalon (Fig. 7*C*). In contrast, melatonin administration for 1 week to Px plus Ex male birds was followed by significant decreases in the same three parameters ($p < 0.01$, Px plus Ex plus melatonin vs Px plus Ex), reducing them to SH levels (Fig. 7).

To investigate whether melatonin inhibits 7α-hydroxypregnenolone synthesis via its receptor, male birds were treated with luzindole, a melatonin receptor antagonist. An intracerebroventricular injection of luzindole was followed by a significant increase in the production of 7α- and 7β-hydroxypregnenolone at 3:00 A.M. when endogenous melatonin was high (vehicle, 637 ± 56 cpm; luzindole, 951 ± 93 cpm; $n = 8$ in each group; $p < 0.05$).

Discussion

We demonstrated previously that the quail brain expresses CYP11A and produces pregnenolone from cholesterol (Fig. 2*G*) (Tsutsui and Yamazaki, 1995; Usui et al., 1995; Tsutsui et al., 1997). In this study, we first identified previously unknown avian neurosteroids as 7α-hydroxypregnenolone and its stereoisomer 7β-hydroxypregnenolone in the quail brain. Subsequently, we found that quail brain expresses CYP7B, a steroidogenic enzyme of 7α-hydroxypregnenolone synthesis, and produces 7α-hydroxypregnenolone from pregnenolone (Fig. 2*G*). Although it is still unclear whether CYP7B also can produce 7β-hydroxypregnenolone, the presence of 7β-hydroxypregnenolone as well as 7α-hydroxypregnenolone was confirmed in quail brain. The production of 7α-hydroxypregnenolone in the brain may be a conserved property of vertebrates, because we also recently identified this neurosteroid as a novel neuronal activator in the newt brain (Matsunaga et al., 2004b). The formation of 7α-hydroxylated neurosteroids, such as 7α-hydroxypregnenolone and 7α-hydroxydehydroepiandrosterone, has also been observed in mammalian brains (Akwa et al., 1992; Doostzadeh and Morfin, 1997; Weill-Engerer et al., 2003; Yau et al., 2003). Behavioral analysis of male quail demonstrated that administration of 7α-hydroxypregnenolone, a previously undescribed avian neurosteroid, acutely increases locomotor activity. This stimulatory effect tended to be dose dependent; the effective dose ranged between 10 and 100 ng intracerebroventricular injection. In contrast, 7β-hydroxypregnenolone at the same high dose (100 ng) did not influence locomotor activity. We therefore consider that 7α-hydroxypregnenolone acts as a neuronal activator to stimulate locomotor activity of male quail.

The production and concentration of 7α-hydroxypregnenolone in the male diencephalon were much higher than those in the female. This is the first demonstration, to our knowledge, of a clear sex difference in the neurosteroid biosynthesis in any vertebrate class. Such a sex difference was evident only in the diencephalon. Because the male quail displays a robust locomotor activity rhythm when held under typical light/dark lighting schemes (Wilson, 1972; Wada, 1979), this bird may serve as an excellent animal model to demonstrate the physiological role of 7α-hydroxypregnenolone. Behavioral analysis using quail exposed to LD (lights on at 7:00 A.M., lights off at 11:00 P.M.) revealed that locomotor activity of males was much higher than that of females from the time of lights on until noon. Locomotor activity of males decreased thereafter and diminished to female levels. Interestingly, the production and concentration of 7α-hydroxypregnenolone in the male diencephalon changed markedly during the observed 24 h period, with a maximal level at 11:00 A.M. when locomotor activity of males was high. These parallel changes suggest that 7α-hydroxypregnenolone is involved in the regulation of diurnal changes in locomotor activity in males. In contrast to males, locomotor activity and 7α-hydroxypregnenolone synthesis and concentration were constantly low in females during the same observed period. Thus, 7α-hydroxypregnenolone may contribute to the higher locomotor activity observed in males. This hypothesis was confirmed by the present finding, indicating that administration of ketoconazole, an inhibitor of CYPs, decreased locomotor activity of males. Unlike males, 7α-hydroxypregnenolone administration did not increase locomotor activity of females. This finding suggests that the receptor for 7α-hydroxypregnenolone is not present or is otherwise inactivated in the female. Based on this finding and lower levels of 7α-hydroxypregnenolone synthesis and concentration in the female diencephalon, 7α-hydroxypregnenolone may not function in the female.

Identification of the cells producing 7α-hydroxypregnenolone in the brain must be taken into account when studying the stimulatory action of 7α-hydroxypregnenolone on locomotor activity of male quail. In this study, therefore, we further characterized the site showing the CYP7B expression in the male diencephalon by *in situ* hybridization, because 7α-hydroxypregnenolone synthesis in the diencephalon was much higher

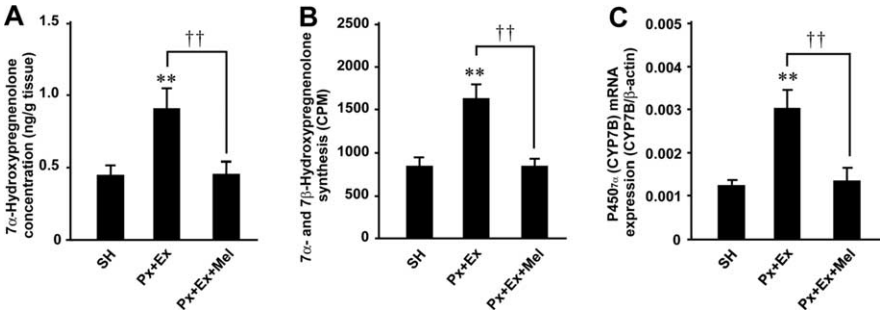

Figure 7. Effects of Px combined with Ex (Px+Ex) and melatonin administration to Px+Ex quail (Px+Ex+Mel) on 7α-hydroxypregnenolone concentration, 7α- and 7β-hydroxypregnenolone synthesis, and quail CYP7B mRNA expression in the male diencephalon. Each column and vertical line represent the mean ± SEM ($n = 5$). **$p < 0.01$ vs SH; ††$p < 0.01$ Px+Ex vs Px+Ex+Mel by one-way ANOVA, followed by Duncan's multiple range test.

than that in other brain regions. In the male diencephalon, the expression of CYP7B mRNA was localized in the POM, PVN, VMN, DLA, and LA. Based on our previous study with newts (Matsunaga et al., 2004b), 7α-hydroxypregnenolone increased the concentration of dopamine in the telencephalic region including the striatum, which is known to be involved in the regulation of locomotor behavior in vertebrates (Sanberg, 1983; Sharp et al., 1987; Bardo et al., 1990). It has been reported that, in birds, dopamine neurons are localized in the mesencephalic region, such as the area ventralis (AVT) and substantia nigra (SN), and that they project to the telencephalic region, such as the striatum (Mezey and Csillag, 2002; Hara et al., 2007). It also has been reported that, as in mammal brain, the avian brain possesses dopamine D_1- and D_2-like receptors in the telencephalon (Ball et al., 1995; Levens et al., 2000). The present and previous studies with birds and amphibians suggest that the stimulatory effect of 7α-hydroxypregnenolone on locomotor activity of male quail may be mediated by the dopaminergic system. 7α-Hydroxypregnenolone synthesized actively in the diencephalon may, by acting on dopamine neurons localized in the AVT and SN, induce dopamine release from their termini in the telencephalic region, such as the striatum, and consequently increase locomotor activity of male quail.

This study also provides new findings on the mechanism underlying regulation of 7α-hydroxypregnenolone synthesis and 7α-hydroxypregnenolone-dependent locomotor activity. Until now, no such mechanism for regulating had been determined. We hypothesized that melatonin may regulate 7α-hydroxypregnenolone synthesis, thus influencing locomotor activity. We based our hypothesis on evidence that melatonin is involved in the regulation of locomotor activity in birds (Binkley et al., 1971; John et al., 1978; Cassone and Menaker, 1984; Chabot and Menaker, 1992; Hau and Gwinner, 1994; Warren and Cassone, 1995; Murakami et al., 2001) and on our observations of parallel changes in locomotor activity and 7α-hydroxypregnenolone synthesis. Our hypothesis was confirmed by a combination of experiments involving melatonin manipulation in male quail. In this study, Px combined with Ex and melatonin replacement were performed. A combination of Px plus Ex increased the production and concentration of 7α-hydroxypregnenolone and the expression of quail CYP7B in the diencephalon, concomitant with a decrease in endogenous melatonin in the diencephalon (SH, 0.318 ± 0.081 pg/mg tissue; Px plus Ex, 0.059 ± 0.032 pg/mg tissue; $p < 0.05$). In an additional experiment, melatonin administration to Px plus Ex birds decreased the production and concentration of 7α-hydroxypregnenolone and the expression of quail CYP7B in the diencephalon. These changes after melatonin administration were also closely related to changes in melatonin in the diencephalon (Px plus Ex, 0.059 ± 0.032 pg/mg tissue; Px plus Ex plus melatonin, 0.720 ± 0.099 pg/mg tissue; $p < 0.01$). In addition, an inhibitory effect of melatonin on 7α-hydroxypregnenolone synthesis was abolished by luzindole, an antagonist of melatonin receptor. In summary, melatonin most likely acts to reduce CYP7B expression through melatonin receptor-mediated mechanisms. Together, melatonin derived from the pineal gland and eyes appears to act as a potent inhibitory factor of 7α-hydroxypregnenolone synthesis in birds. This hypothesis is in agreement with the previous finding showing that melatonin decreases locomotor activity of quail (Murakami et al., 2001; Nakahara et al., 2003) as well as other avian species (Murakami et al., 2001). To the best of our knowledge, this is the first report showing the action of melatonin on the regulation of neurosteroid synthesis in the vertebrate brain.

In quail, as in all vertebrates, the nocturnal secretion of melatonin is night-length dependent (Cockrem and Follett, 1985), and the onset of melatonin secretion occurs soon after the onset of darkness (Kumar and Follett, 1993). Therefore, the increase in 7α-hydroxypregnenolone synthesis during the light period is likely to be a result of the decrease in endogenous melatonin secretion in male quail. The increase in 7α-hydroxypregnenolone synthesis appears to enable the increase in locomotor activity. Thus, 7α-hydroxypregnenolone is considered to play a crucial role in the process of diurnal changes in locomotor activity of male quail by mediating melatonin action. A similar novel mechanism underlying regulation of diurnal locomotor rhythms may also be present in other vertebrates, because the formation of 7α-hydroxypregnenolone in the brain is evident in mammals (Akwa et al., 1992; Doostzadeh and Morfin, 1997; Weill-Engerer et al., 2003; Yau et al., 2003) and newts (Matsunaga et al., 2004b).

In this study, we found the sex difference in 7α-hydroxypregnenolone synthesis in the quail diencephalon. However, we could not detect such a clear sex difference in the diencephalon of castrated quail (K. Inoue, S. Haraguchi, and K. Tsutsui, unpublished observation). The sex difference of this neurosteroid might be dependent on gonadal sex steroids. Additional study is needed to clarify the mechanism(s) that induces the sex difference in 7α-hydroxypregnenolone synthesis.

In conclusion, we show that, in male birds, 7α-hydroxypregnenolone acts as a key factor for the induction of locomotor activity and mediates the melatonin action underlying diurnal locomotor rhythms. To the best of our knowledge, this is the first report showing melatonin action on the regulation of neurosteroid synthesis. We further provide evidence for a clear sex difference in the synthesis of 7α-hydroxypregnenolone in connection with the sex difference in locomotor activity. This is also the first finding indicating a clear sex difference in neurosteroid synthesis in any vertebrate class.

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