Semicomprehensive Analysis of the Postnatal Age-Related Changes in the mRNA Expression of Sex Steroidogenic Enzymes and Sex Steroid Receptors in the Male Rat Hippocampus

Tetsuya Kimoto,* Hirotaka Ishii,* Shimpei Higo, Yasushi Hojo, and Suguru Kawato

Department of Biophysics and Life Sciences (T.K., H.I., S.H., Y.H., S.K.), Graduate School of Arts and Sciences, and Core Research for Evolutional Science and Technology Project (T.K., Y.H., S.K.) and Bioinformatics Project (Y.H., S.K.), Japan Science and Technology Agency, The University of Tokyo, Tokyo 153-8902, Japan

Although sex steroids play a crucial role in the postnatal brain development, the age-related changes in the hippocampal steroidogenesis remain largely unknown. We performed comprehensive investigations for the mRNA expressions of 26 sex steroidogenic enzymes/proteins and three sex steroid receptors in the male rat hippocampus, at the ages of postnatal day (PD) 1, PD4, PD7, PD10, PD14, 4 wk, and 12 wk (adult), by RT-PCR/Southern blotting analysis. The relative expression levels of these enzymes/receptors at PD1 were *Srd5a1* > *Star* > *Ar* ~ *Hsd17b4* ~ *Hsd17b1* ~ *Hsd17b7* ~ *Esr1* ~ *Srd5a2* > *Hsd17b3* > *Esr2* > *Cyp11a1* > *Cyp17a1* > *Cyp19a1* ~ *Hsd17b2* > 3β-hydroxysteroid dehydrogenase I. The mRNA levels of essential enzymes for progesterone/testosterone/estradiol metabolisms (*Cyp17a1*, *Hsd17b7*, and *Cyp19a1*) were approximately constant between PD1 and PD14 and then declined toward the adult levels. *Cyp11a1* increased during PD4-PD14 and then considerably decreased toward the adult level (~8% of PD1). *Hsd17b1*, *Hsd17b2*, and 3β-hydroxysteroid dehydrogenase I mRNA decreased approximately monotonously. *Hsd17b3* increased to approximately 200% of PD1 during PD4-PD14 and was maintained at this high level. The 5α-reductase mRNA was maintained constant (*Srd5a1*) or decreased monotonically (*Srd5a2*) toward the adult level. The *Esr1* level peaked at PD4 and decreased toward the adult level, whereas *Ar* greatly increased during PD1-PD14 and was maintained at this high level. The *Star* and *Hsd17b4* levels were maintained constant from neonate to adult. These results suggest that the hippocampal sex steroidogenic properties are substantially altered during the postnatal development processes, which might contribute to brain sexual maturation. **(*Endocrinology* 151: 5795–5806, 2010)**

Brain architecture and neuronal properties are largely altered in an age-related manner, not only at the embryonic ontogeny stage but also in the postnatal development processes. It has been well documented that sex steroids play a crucial role in the postnatal development (1, 2). In some reproduction-related brain regions such as the hypothalamus, brain sex differentiation is supposed to be determined by whether the brain is exposed to testosterone (T) in male or not in female within the critical period of the perinatal/neonatal stage [for rats, from embryonic d 18 to postnatal day (PD) 10; (2)]. In addition to the conventional reproduction-related brain regions, recent studies revealed the profound effects of sex steroids in the neuronal development in the hippocampus, a center of learning and memory (3). Besides the perinatal/early postnatal development, changes in the cognitive ability and behavior, probably caused by the pronounced remodeling of the neuronal circuits, occur in the hippocampus during adolescence, which might be correlated with the T content (4, 5).

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-0581 Received May 21, 2010. Accepted September 24, 2010.
First Published Online November 3, 2010
* T.K. and H.I. contributed equally to the present work.

Abbreviations: DHEA, Dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; E2, 17β-estradiol; ER, estrogen receptor; EtBr, ethidium bromide; ΔG, Gibbs energy; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ΔG_{av}, mean value for all of the primer candidates; HSD, hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase; P450arom, P450 aromatase; PD, postnatal day; PREG, pregnenolone; PROG, progesterone; PW, postnatal week; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; T, testosterone.

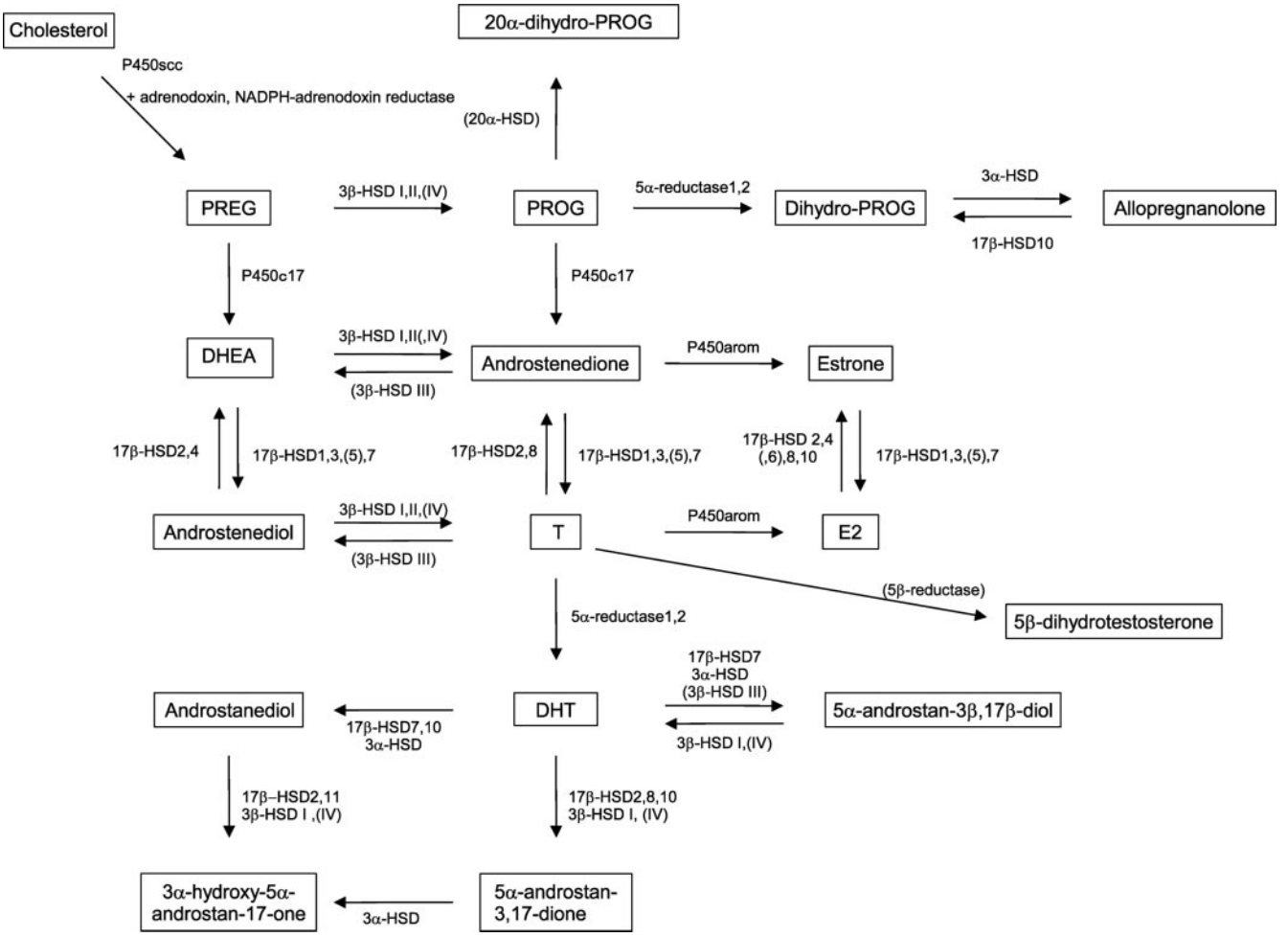


FIG. 1. Hippocampal steroid metabolism pathways and candidate enzymes/proteins responsible for each step, as described in introductory text. Names of steroids are denoted by *boxed character*. Enzymes not detected in the hippocampus are indicated in *parentheses*.

Although sex steroids can be provided to brain from gonads via circulation, they do not always exert their effects in the status quo because the significant levels of steroidogenic enzymes are expressed in the brain (4, 6, 7). In perinatal/neonatal brain sex-differentiation processes, the evidences have been accumulated showing that T is converted to 17β-estradiol (E2) by neuronal cytochrome P450 aromatase (P450arom; gene: *Cyp19a1*) and then exerts its effect (2, 8). Furthermore, sex steroids are also *de novo* synthesized in the hippocampus (9–11). The candidate enzymes or proteins probably involved in the hippocampal steroid metabolism are shown in Fig. 1. Steroidogenic acute regulatory protein (gene: *Star*) and/or translocator protein (peripheral benzodiazepine receptor) (12) transport free cholesterol to the inner mitochondrial membrane, in which cholesterol is converted to pregnenolone (PREG) by the monooxygenase system composed of nicotinamide adenine dinucleotide phosphate-adrenodoxin reductase (gene: *Fdxr*), adrenodoxin (gene: *Fdx1*), and cytochrome P450 side-chain cleavage (gene: *Cyp11a1*) (11, 13). PREG is metabolized to dehydroepiandrosterone (DHEA), progesterone (PROG), and/or androstenedione by cytochrome P450c17 (gene: *Cyp17a1*) and 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD) and further converted to sex steroids by the other enzymes such as 17β-hydroxysteroid dehydrogenase (17β-HSD; gene: *Hsd17b*), P450arom, and 5α-reductase (9).

For some enzymes, there are multiple isozymes having different activities and substrate affinities, which lead to the complex regulation of steroid metabolism. For 3β-HSD, four types of isozymes (3β-HSD I, II, III, and IV) have been identified in rat (14). For 17β-HSD, more than 10 isozymes have been identified so far, and they have very different enzymatic activities in the activation/inactivation of sex steroids (15). As illustrated in Fig. 1, 17β-HSD1, -3, -5, and -7 act as the reductases that convert inactive 17-ketohydroxysteroids to active 17β-hydroxysteroids (forward reactions), whereas 17β-HSD2, -4, -6, -8, -10, and -11 possess oxidative activities, inactivating 17β-hydroxysteroids by converting them into 17-ketosteroids (backward reactions). Note that *Hsd17b9*, originally cloned in mouse, is an ortholog of the *Hsd17b6* in rat. T is further

converted into 5α-dihydrotestosterone (DHT) by two isozymes of 5α-reductase, 5α-reductase 1 (gene: *Srd5a1*), and 5α-reductase 2 (*Srd5a2*) (16).

Considering the importance of sex steroids in the brain development, the elucidation of the age-related expression profiles of enzymes for sex steroid metabolism is critically important to understand the regulation of the postnatal development of the brain system. In fact, steroidogenic activities are largely altered, depending on the brain development, as we recently reported by comparing the hippocampal neurosteroidogenesis in male rats at PD10 with that at the adult stage (17). Nevertheless, the postnatal age-related differences in the hippocampal steroidogenesis still remain largely unknown. For *Cyp11a1* and *Cyp17a1*, little study has been performed focusing on their hippocampal age-related expression profiles. Age-related change in the expression of mRNA encoding for 3β-HSD was investigated in the hippocampus of rats with *in situ* hybridization and RT-PCR (18); however, the individual analysis for the isoforms of the enzyme has not been sufficiently conducted. For 17β-HSD, *Hsd17b1-4* are expressed in the hippocampus of adult rats (9). *Hsd17b10* is highly expressed in the hippocampus of Alzheimer's disease model mouse (19), suggesting that the product of the gene plays a significant role in the regulation of the hippocampal cognitive function. However, little information on the age-related expression profiles of the hippocampal *Hsd17b*s is available until today. Although *Cyp19a1* has been relatively well studied (20), poor information is available for the other sex steroid-metabolizing enzymes in rodents. For mRNAs encoding for 5α-reductases 1 and 2, their hippocampal expression was shown in the adult rat (21, 22). However, the age-related expression profile of *Srd5a2* has not been fully revealed until now, although the hippocampal ontogeny of *Srd5a1* mRNA expression was investigated in rats with *in situ* hybridization (23). For 3α-HSD (gene: *Akr1c9*), which is supposed to be involved in the inactivation of DHT, the age-related expression profile of the mRNA was investigated in the rat hippocampal dentate gyrus; however the time resolution was not sufficient to reveal the precise change at the neonate/juvenile stage (24). The hippocampal expression of mRNAs for rat 5β-reductase (*Akr1d2*) and 20α-HSD (*Akr1c8*), which might be responsible for the inactivation of T and PROG, respectively, remains unrevealed. The age-related expression profile of 20α-HSD mRNA has not been investigated in rodents, although the hippocampal localization of 20α-HSD mRNA was demonstrated in adult mouse (25).

In the present study, we have extensively investigated the temporal changes in the expression of mRNAs for these essential steroidogenic enzymes/proteins in the male rat hippocampus, in relation to the sexual maturation over the ages of neonate/juvenile (PD1, 4, 7, 10, and 14), postnatal week (PW) 4 and adult (PW12), using RT-PCR/Southern blotting. By means of the improved primer designing, we achieved the high sensitive RT-PCR analysis for the hippocampal steroidogenic genes whose expression is extremely low compared with that in the peripheral steroidogenic organs. Because the expression of steroid receptors should be elucidated to reveal the action of sex steroids, we also examined the age-related profiles of the hippocampal mRNA expression of estrogen receptor-α [ERα (NR3A1); gene: *Esr1*)], estrogen receptor-β [ERβ (NR3A2); *Esr2*)] and androgen receptor (NR3C4; *Ar*).

Materials and Methods

Animals

Wistar rats, purchased from Saitama Experimental Animal Supply (Saitama, Japan), were maintained in a 12-h light, 12-h dark cycle (lights on at 0800 h) with free access to water and pelleted food. All experiments using animals in this study were conducted according to the guidelines in the University of Tokyo and the institutional committee.

Total RNA extraction

Molecular biological experiments were carried out according to the methods previously described elsewhere (17, 26) with a slight modification. Briefly, animals were killed by cervical dislocation under ether anesthesia. Adrenal, kidney, liver, prostate, testis, and brain subregions (cerebral cortex, hippocampus, thalamus, hypothalamus, and cerebellum) were dissected from male rats at PW12 and immediately frozen and maintained in liquid nitrogen until use. Ovary was obtained from female rats at PW12. Hippocampi were also obtained from male rats at PD1, PD4, PD7, PD10, PD14, and PW4. Total RNA was extracted using AGPC reagent, Isogen (Nippongene, Japan). Purified total RNA was treated with ribonuclease-free deoxyribonuclease I (TaKaRa Bio, Shiga, Japan) to eliminate contamination of genomic DNA, and reextracted by AGPC reagent. The concentration and the purity of total RNA were quantitated by its absorbance at 260 and 280 nm.

Reverse transcription

Ten micrograms of total RNA were used to synthesize an oligo-dT primed first-strand cDNA in each sample. The reaction mixture composed of 10 μg of total RNA, 1× reverse transcription buffer, 0.5 mM deoxynucleotide triphosphate, 1 μg of oligo-(dT)15 primer (Promega, Madison, WI), 20 U of ribonuclease inhibitor (RNasin Plus; Promega), and 200 U of Moloney murine leukemia virus reverse transcriptase [RNase H (−), point mutant; Promega]. The reaction was carried out at 42 C for 60 min and stopped by heating at 75 C for 15 min. The cDNA solution was treated with 5 U of RNase H (TaKaRa Bio) at 37 C for 1 h and stored at −20 C until use. For the preparation of negative control samples, reverse transcriptase was omitted.

Primer design

Because the expression of brain steroidogenic genes is extremely low, PCR primers with high sensitivity and specificity are required

for the precise analysis of the expression. Reduction in the change in Gibbs energy (ΔG) during a primer-target hybridization tends to increase PCR sensitivity; however, it also increases the guanine-cytosine content of the primer, resulting in lower specificity. We designed the primers considering the balance of these factors as follows: 1) the ΔG of the whole primer ($\Delta G_{(whole)}$) was calculated with a nearest-neighbor model and set to be below the mean value for all of the primer candidates (ΔG_{av}) to obtain good stability in primer-target interaction; and 2) ΔG of the five 3′-terminal bases of the primer ($\Delta G_{(5base)}$) was set to be higher than ΔG_{av} for improved specificity.

Consequently, ΔG of the 5′-terminal bases was set to be less than the ΔG_{av}, resulting not only in the improved stability in 5′-terminal interaction but also in the improved DNA polymerase recognition. Primers were designed to avoid PCR debris such as primer dimers, and two (forward and reverse) primers were designed on separate exons.

Finally, the most optimal primers for each gene were selected by empirical screening based on the following criteria: 1) correct amplification of the target sequence, 2) absence of the nonspecific signal amplification, 3) absence of PCR debris, and 4) good amplification. Primer sequences used for the expression analysis are listed in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. The quality of the primers was compared with that of the primers used in the previous studies (27, 28) by PCR for *Cyp11a1* and *Cyp17a1* using the cDNA templates derived from the PW12 male rat hippocampus (Supplemental Fig. 1).

Polymerase chain reaction

The PCR reaction mixture composed of cDNA [corresponding to 200 ng (for PREG/DHEA/PROG synthesizing enzymes) or 50 ng (for others) of total RNA], 1× GC buffer I, 0.2 mM deoxynucleotide triphosphate mixture, 0.2 μM of each primer, and 1.25 U of TaKaRa LA Taq (TaKaRa Bio) in the total volume of 25 μl. PCR cycle condition was as follows: 1) 95 C denaturing step for 30 sec, 2) 55–65 C annealing step for 20 sec, and 3) 72 C elongation step for 30 sec with the first 95 C denaturing for 1 min and the last elongation for 15 min. To determine the optimal number of cycles for the hippocampal samples, a variable number of cycles was performed during PCR amplification. The primers used, annealing temperatures, and numbers of PCR cycles are shown in Supplemental Table 1. Five microliters of PCR products were electrophoresed on 2.0% agarose gels. Gels were stained with ethidium bromide (EtBr) and visualized under UV light. Images were recorded with Printgraph (ATTO, Tokyo, Japan). For quantitative estimation, images of the bands were analyzed using the ImageJ version 1.42 software (National Institutes of Health, Bethesda, MD). The expression of mRNA for steroidogenic enzymes/receptors was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. Relative abundance of different genes was estimated by adopting the expression level of *Gapdh* mRNA as an internal standard. First, OD value in each band was divided by the number of $(1 + e)^c$, where c is a PCR cycle number and e is an amplification efficacy obtained from the PCR amplification curves in the exponential amplification phase. PCR amplification curves were obtained by a number of PCR series using the hippocampal cDNA templates (equivalent to 50 or 200 ng of total RNA) from PD1 male rats. The range of e was 0.91–1.00 for all examined primer sets. For the *Gapdh* bands, the same calculation was performed, and then the obtained values were further divided by the ratio of the product length of the target gene PCR to that of the *Gapdh* PCR for the correction of the difference in the amount of EtBr bound to DNAs. Finally, the relative abundance was expressed as the ratio of the obtained value to that of the corresponding *Gapdh* band.

Sequencing

PCR products on the electrophoresed agarose gels were extracted using QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and cloned into pGEM-T-Easy vector (Promega). Dye terminator cycle sequencing was performed using Thermosequenase II sequencing kit (Amersham Biosciences, Piscataway, NJ), and sequenced by an ABI373A DNA sequencer (Applied Biosystems, Foster City, CA).

DNA probe preparation for Southern blotting

DNA fragments cloned in pGEM-T-Easy vector (Promega) was dissected with *Eco*RI or *Not*I, electrophoresed on an agarose gel, and purified using QIAquick gel extraction kit (QIAGEN). ^{32}P-labeled DNA probes were prepared from them using a random primer DNA labeling kit version 2 (TaKaRa Bio).

Southern blot analysis

PCR products were transferred to nylon membranes, Hybond N+ (Applied Biosystems), and cross-linked by UV irradiation. Southern hybridization was performed with 0.5–5 × 10^6 cpm of ^{32}P-labeled DNA probes in 5× standard saline citrate (SSC) [1× SSC (pH 7.0) is 150 mM NaCl, 15 mM sodium citrate], 1% sodium dodecyl sulfate (SDS), 5× Denhart's solution (1× Denhart's solution is 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400), and 100 μg/ml of salmon sperm DNA at 65 C for 16 h. After hybridization, the membranes were washed once each in 2× SSC and 0.2% SDS, in 0.5× SSC and 0.2% SDS, and then in 0.2× SSC and 0.2% SDS at 65 C for 15 min. Signals were exposed to imaging plates by autoradiography and scanned with BAS-2500 (Fujifilm, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SEM. The mRNA levels at different ages were compared by one-way ANOVA followed by Tukey's *post hoc* test when significant differences were found. A $P < 0.05$ was considered to be statistically significant.

Results

Postnatal age-related expression profiles of mRNAs encoding for sex-steroidogenic enzymes and sex steroid receptors in the hippocampus

To reveal the age-related changes in the expression levels of hippocampal mRNAs encoding for steroid-metabolizing enzymes/proteins and sex steroid receptors, we performed the RT-PCR analysis in the hippocampus of male rats at PD1, PD4, PD7, PD10, PD14, PW4, and PW12. The expression levels of each mRNA were normalized with *Gapdh* mRNA at each age, whose value at PD1 was set to be 100% (see Figs. 2–5). In all of the expression analyses, the sequence identity between PCR products and the target sequences was confirmed with Southern blotting and DNA sequencing. Primers

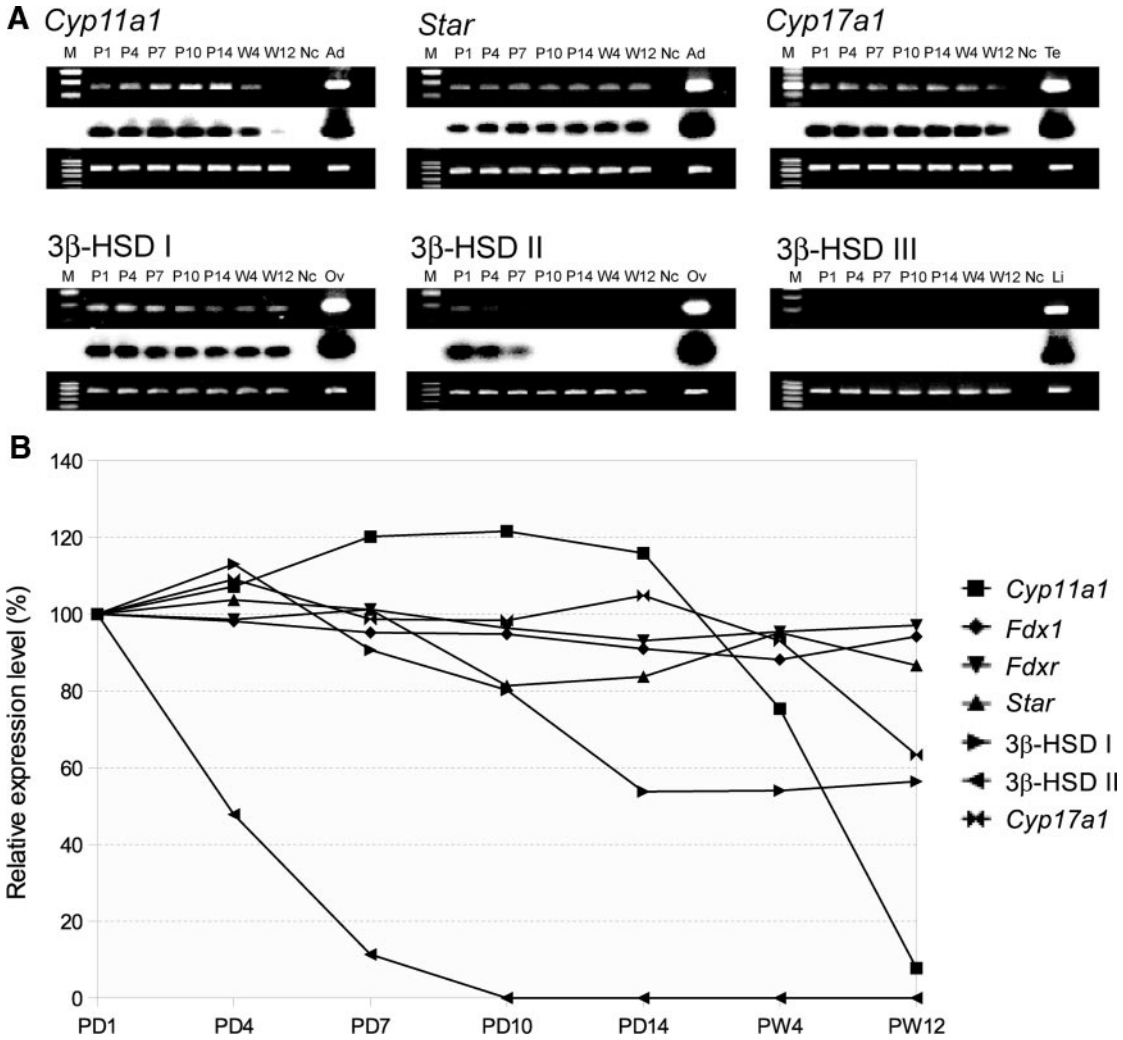


FIG. 2. Postnatal age-related changes in the mRNA expression of the PREG/DHEA/PROG-synthesizing enzymes. A, Representative image sets for the RT-PCR/Southern blot analysis for the mRNAs of PREG/DHEA/PROG-synthesizing enzymes (*Cyp11a1*, *Star*, *Cyp17a1*, and mRNAs for 3β-HSD I–III). In each image set discriminated by gene name, the *most upper image row* represents the gel image in which bands of the electrophoresed PCR products were stained with EtBr and visualized. The *second row in each image set* represents the Southern blots corresponding to the upper gel image. The *lowest row in each image set* shows the bands of *Gapdh*. Lanes, labeled with P1 (PD1), P4, P7, P10, P14, W4 (PW4), and W12, represent the PCR products derived from the male rat hippocampus at the corresponding ages. The left-most lane (labeled with M) is the DNA ladder marker lane. The right-most lane corresponds to the positive control derived from PW12 rats. Ad, Adrenal; Li, liver; Ov, ovary; Te, testis. The lane Nc represents the negative control. Final PCR products originated from 200 ng of total RNA were electrophoresed in each lane. The primer sequences and cycle numbers used in each PCR are indicated in Supplemental Table 1. Images for *Fdx1*, *Fdxr*, and 3β-HSD IV mRNA are shown in Supplemental Data (Supplemental Fig. 4). B, Graph showing the age-related temporal changes in the mRNA expression. Relative expression levels of mRNAs were evaluated with RT-PCR analysis. The OD value in each band in EtBr-stained gels was determined and normalized with that of *Gapdh* at the corresponding conditions, and the level was indicated as the ratio to that at PD1 (PD1 was set to be 100% for each gene). Data are duplicate determinations on six animals at each age. The temporal changes are shown only for the mRNAs detected in the hippocampus. Numerical values (mean ± SEM) of graph B are shown in Supplemental Table 2.

and PCR cycle numbers used in the present study are indicated in Supplemental Table 1.

Relative expression levels of mRNAs at PD1 (first heading of Table 1)

The relative expression levels of mRNAs at PD1 were analyzed. Data are expressed as the ratio to that of 3β-HSD I and summarized in the first heading of Table 1. The relative expression level of 3β-HSD I mRNA to that of *Gapdh* mRNA (internal standard) was estimated to be $(6.0 \pm 0.6) \times 10^{-7}$ (n = 6). As a whole, *Srd5a1* belonged to the most abundant group; on the other hand, 3β-HSD I, 3β-HSD II, and *Hsd17b2* belonged to the least abundant group.

Age-related changes for enzymes/proteins involved in PREG/DHEA/PROG metabolisms (Fig. 2 and Supplemental Table 2)

First, the expression of mRNAs encoding for PREG/DHEA/PROG-synthesizing enzymes/proteins (*Cyp11a1*,

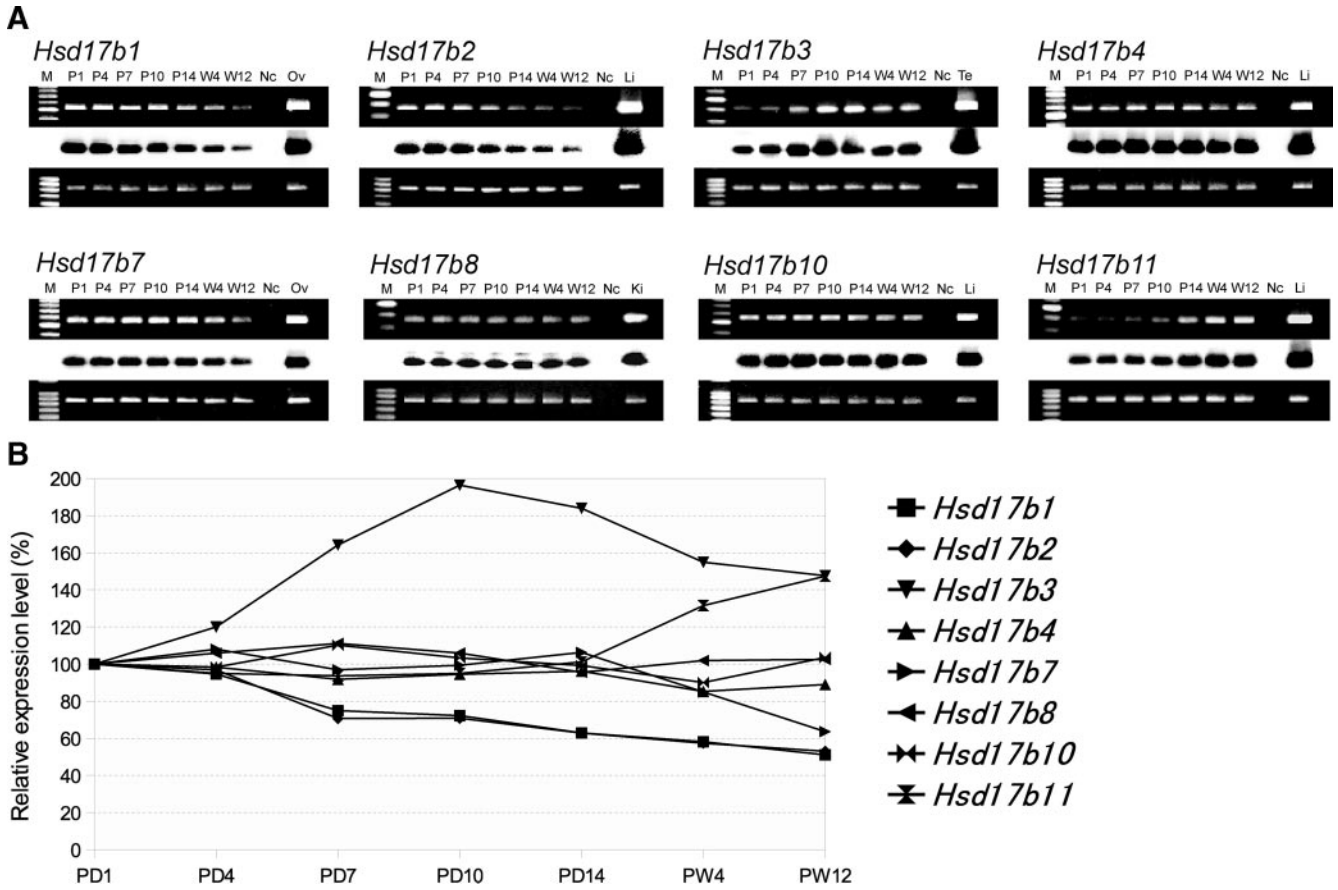


FIG. 3. Postnatal age-related changes in the expression of *Hsd17b1-11*. A, Representative image sets for the RT-PCR/Southern blot analysis for *Hsd17b1-11*. In each image set discriminated by gene name, the *most upper image row* represents EtBr staining; the *second row* represents Southern blots; and the *lowest row* represents *Gapdh*. Lanes, labeled with P1 (PD1), P4, P7, P10, P14, W4 (PW4), and W12, represent the PCR products derived from the male rat hippocampus at the corresponding ages. Final PCR products originated from 50 ng of total RNA were electrophoresed in each lane. The left-most lane (labeled with M) is the DNA ladder marker lane. The right-most lane corresponds to the positive control derived from PW12 rats. Ki, Kidney; Li, liver; Ov, ovary; Te, testis. The lane Nc represents the negative control. Primer sequences and cycle numbers used in each PCR are indicated in Supplemental Table 1. Images for *Hsd17b5* and *Hsd17b6* are shown in Supplemental Data (Supplemental Fig. 4). B, Graph showing the age-related temporal changes in the mRNA expression. Relative expression levels of mRNAs were evaluated with RT-PCR analysis. The OD value in each band in EtBr-stained gels was determined and normalized with that of *Gapdh* at the corresponding conditions, and the level was indicated as the ratio to that at PD1 (PD1 was set to be 100% for each gene). Data are duplicate determinations on six animals at each age. The temporal changes are shown only for the mRNAs detected in the hippocampus. Numerical values (mean ± SEM) of graph B are shown in Supplemental Table 2.

Fdx1, *Fdxr*, *Star*, *Cyp17a1*, and mRNAs for 3β-HSD I–IV) was examined. The relative abundance of mRNAs at PD1 hippocampus was in the following order: *Fdx1* > *Fdxr* > *Star* > *Cyp11a1* > *Cyp17a1* > 3β-HSD I > 3β-HSD II. The *Cyp11a1* expression was slightly elevated at PD7-PD14 (~120%) compared with that at PD1 and then considerably declined. The expression of *Fdx1*, *Fdxr*, and *Star* (examined with the primer set A indicated in Supplemental Table 1) did not change significantly over the period of PD1-PW12. The expression of *Cyp17a1* did not significantly alter during the period of PD1-PD14 but gradually decreased between PD14 and PW12. The expression level of 3β-HSD I mRNA was maintained constant from PD1 to PD7, then declined, and finally reached a next level kept at and after PD14. The expression of 3β-HSD II mRNA declined sharply during the period of PD1-PD7 and completely disappeared at and after PD10. The expression of mRNAs for 3β-HSD III and IV was not detected in the hippocampus at all examined ages.

Age-related changes for 17β-HSD (Fig. 3 and Supplemental Table 2)

The relative abundance of mRNAs at the PD1 hippocampus was in the following order: *Hsd17b11* > *Hsd17b8* ~ *Hsd17b10* > *Hsd17b4* ~ *Hsd17b1* ~ *Hsd17b7* > *Hsd17b3* > *Hsd17b2*. The expression changes of *Hsd17b1* and *Hsd17b2* were nearly parallel over the period of PD1-PW12. The levels decreased by 25–30% at PD7-PD10 compared with that at PD1 and then gradually decreased toward the level at PW12 (~50%). In contrast, the expression of *Hsd17b3* was

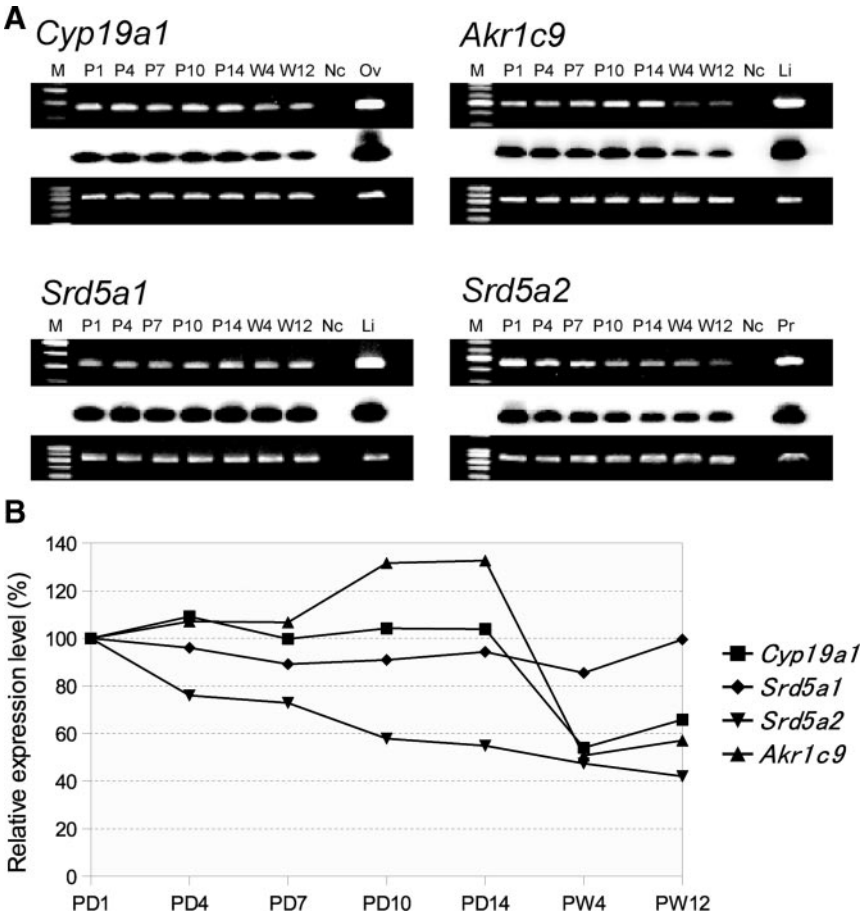


FIG. 4. Postnatal age-related changes in the expression of mRNAs for the enzymes catalyzing the androgen/estrogen metabolism other than 17β-HSD. A, Representative image sets for the RT-PCR/Southern blot analysis for *Cyp19a1*, *Akr1c9*, *Srd5a1*, and *Srd5a2*. In each image set discriminated by gene name, the *uppermost image row* represents EtBr staining; the *second row* represents Southern blots; and the *lowest row* represents *Gapdh*. Lanes, labeled with P1 (PD1), P4, P7, P10, P14, W4 (PW4), and W12, represent the electrophoresed PCR products derived from the male rat hippocampus at the corresponding ages. Final PCR products originated from 50 ng of total RNA were electrophoresed in each lane. The *left-most lane* (labeled with M) is the DNA ladder marker lane. The *right-most lane* corresponds to the positive control derived from PW12 rats. Li, Liver; Ov, ovary; Pr, prostate. The lane Nc represents the negative control. Primer sequences and cycle numbers used in each PCR are indicated in Supplemental Table 1. Images for *Akr1c8* and *Akr1d2* are shown in Supplemental Data (Supplemental Fig. 4). B, Graph showing the age-related temporal changes in the mRNA expression. Relative expression levels of mRNAs were evaluated with RT-PCR analysis. The OD value in each band in EtBr-stained gels was determined and normalized with that of *Gapdh* at the corresponding conditions, and the level was indicated as the ratio to that at PD1 (PD1 was set to be 100% for each gene). Data are duplicate determinations on six animals at each age. The temporal changes are shown only for the mRNAs detected in the hippocampus. Numerical values (mean ± SEM) of graph B are shown in Supplemental Table 2.

increased monotonically after PD1, peaked at PD10 (~200%), and then declined toward that at PW12 (~150%). *Hsd17b5* and *Hsd17b6* were not detected in the hippocampus at all ages. The level of *Hsd17b7* was maintained nearly constant between the ages of PD1 and PD14 and then decreased. The levels of *Hsd17b4*, *-8*, and *-10* were maintained at a constant level over the period of PD1-PW12. The expression of *Hsd17b11* was maintained at a constant level until PD14 and then increased toward PW12 (~150%).

Age-related changes for enzymes for androgen/estrogen metabolisms (Fig. 4 and Supplemental Table 2)

The relative abundance of mRNAs at the PD1 hippocampus was in the following order: *Srd5a1* > *Akr1c9* > *Srd5a2* > *Cyp19a1*. The expression of *Cyp19a1* mRNA was maintained at a nearly constant level between the ages of PD1 and PD14 and then decreased toward the next fixed level (~50–60% of that at PD1). The expression of *Srd5a1* was maintained nearly constant over the period of PD1-PW12. On the other hand, the expression of *Srd5a2* did monotonically decrease over the period of PD1-PW12.

For Aldo-keto reductases probably involved in the T metabolism, *Akr1c9*, *Akr1c8*, and *Akr1d2* were examined. The level of *Akr1c9* was nearly constant between the ages of PD1 and PD7, increased by about 30% during the period of PD10-PD14, and then decreased considerably toward the level at PW4 (~50%). *Akr1c8* and *Akr1d2* were not detected in the hippocampus for all the ages examined.

Age-related changes for receptors of estrogen and androgen (Fig. 5 and Supplemental Table 2)

The relative abundance of mRNAs at the PD1 hippocampus was in the following order: *Ar* > *Esr1* > *Esr2*. The expression of *Esr1* mRNA was significantly increased at PD4 (~150%) compared with that at PD1 and then declined gradually toward the level at PW4-PW12 (~70–80%). The *Esr2* expression, examined with the primer set Esr2-A (see Supplemental Table 1), was nearly constant during the period of PD1-PW12. The level of *Ar* increased monotonically during the period of PD1-PD14 (reaching ~350% at PD14) and then was maintained at this high level until PW12.

Age-related change for steroidogenic factor-1 mRNA (Supplemental Fig. 3)

As an important regulatory factor of neurosteroidogenesis, we also investigated the age-related expression of *Sf-1*. Hippocampal expression level of *Sf-1* at PD1 was 153 ± 10% of 3β-HSD I level. Developmental change of

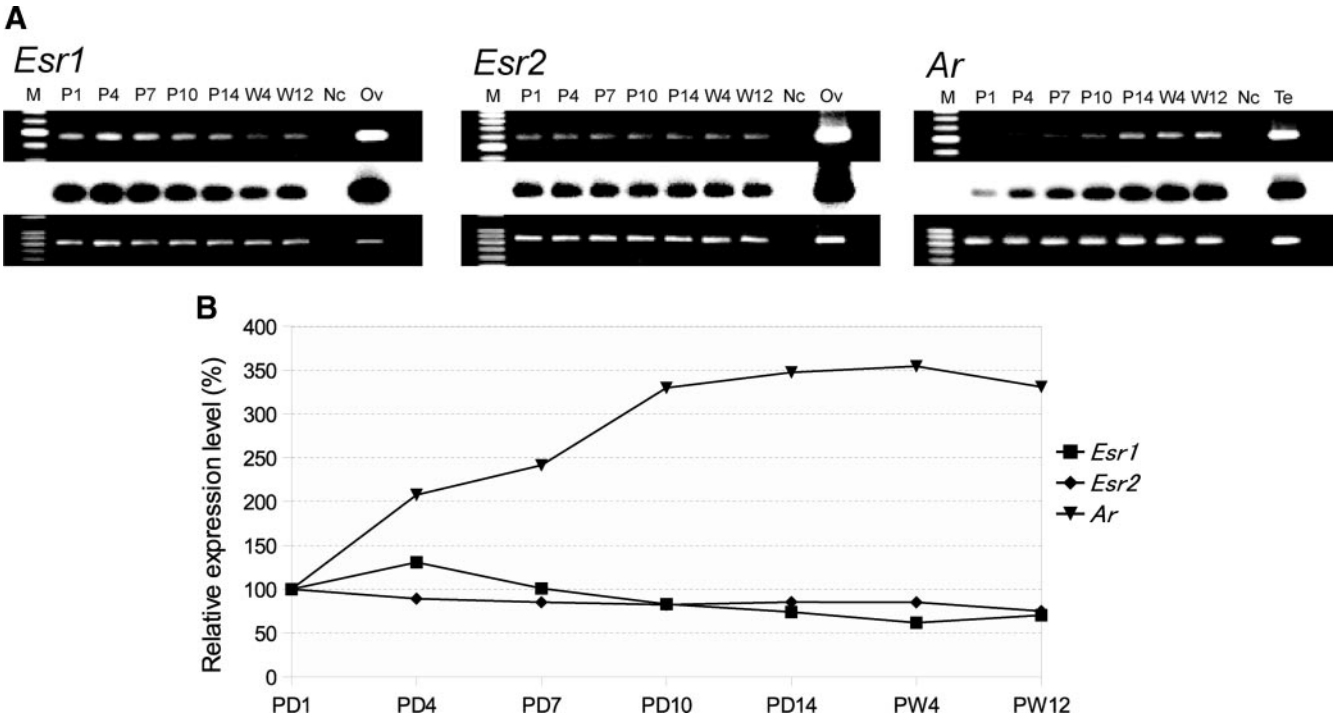


FIG. 5. Postnatal age-related changes in the expression of mRNAs for sex steroid receptors. A, Representative image sets for the RT-PCR/Southern blot analysis for *Esr1*, *Esr2*, and *Ar*. In each image set discriminated by gene name, the *uppermost image row* represents EtBr staining; the *second row* represents Southern blots; the *lowest row* represents *Gapdh*. Lanes, labeled with P1 (PD1), P4, P7, P10, P14, W4 (PW4), and W12, represent the PCR product derived from the male rat hippocampus at the corresponding ages. Final PCR products originated from 50 ng of total RNA were electrophoresed in each lane. The left-most lane (labeled with M) is the DNA ladder marker lane. The right-most lane corresponds to the positive control derived from PW12 rats. Ov, Ovary; Te, testis. The lane Nc represents the negative control. Primer sequences and cycle numbers used in each PCR are indicated in Supplemental Table 1. B, Graph showing the age-related temporal changes in the mRNA expression. Relative expression levels of mRNAs were evaluated with RT-PCR analysis. The OD value in each band in EtBr-stained gels was determined and normalized with that of *Gapdh* at the corresponding conditions, and the level was indicated as the ratio to that at PD1 (PD1 was set to be 100% for each gene). Data are duplicate determinations on six animals at each age. Numerical values (mean ± SEM) of graph B are shown in Supplemental Table 2.

relative expression level of *Sf-1* was as follows; 100 ± 7 (PD1), 93 ± 10 (PD4), 67 ± 9 (PD7), 69 ± 5 (PD10), 72 ± 2(PD14), 72 ± 4 (PW4), and 65 ± 8 (PW12).

Relative abundance of mRNAs in the different subregions of the adult brain (second and third headings of Table 1 and Supplemental Fig. 2)

We analyzed the relative abundance of mRNAs for sex steroidogenic enzymes and sex steroid receptors in different brain subregions (hippocampus, hypothalamus, thalamus, cerebral cortex, and cerebellum) of PW12 male rat with RT-PCR/Southern blot analysis. Results are summarized in the second heading of Table 1. Roughly speaking, the relative abundance of steroidogenic enzymes and steroid receptors are 50–100% as compared with those in hypothalamus in which these enzymes and receptors are most abundant. Relative abundance of mRNAs in the hippocampus of PW12 male rats compared with that in the reference organs is summarized in the third heading of Table 1. Note that all of the mRNAs including not detected ones in the hippocampus were successfully detected in the positive reference organs (liver for 3β-HSD III mRNA, *Hsd17b5*, *Hsd17b6*, and *Akr1d2*; ovary for 3β-HSD II and IV mRNAs and *Akr1c8*), except for *Star3*, which was not detected, even in the adrenal. The expression of *Star* was most abundant in the cerebellum, and the level in other subregions was almost identical.

Discussion

We analyzed the age-related changes in mRNA expression for sex steroidogenic enzymes/proteins and sex steroid receptors. Exhaustive data for mRNA contents may provide a global view of hippocampal sex steroidogenesis because the amount of mRNA may have some correlation with the activity of the enzymes and the protein contents. As an example, we previously demonstrated a relationship between steroidogenic activity and mRNA expression at PD10 and PW12 (17, 29). The mRNA levels of essential steroidogenic enzymes (*e.g. Cyp17a1*, *Hsd17b1*, *Hsd17b3*, 3β-HSD I, and *Cyp19a1*) decrease from PD10 levels (100%) to PW12 levels (~65–77%). The activities of steroidogenesis (PREG→DHEA→ Androstenediol →T→E2) also decrease from PD10 levels (100%) to PW12 levels (~15–50%), depending on each steroidogenesis step.

TABLE 1. Relative expression levels of mRNAs for enzymes/proteins and receptors related to sex steroidogenesis

	Comparison within PD1 hippocampus (%) Hippocampus PD1	Comparison between different brain subregions at PW12 for each specific enzyme/receptor					Relative abundance to reference organ Hippocampus PW12
		Hippocampus	Hypothalamus	Thalamus	Cerebral cortex	Cerebellum	
Cyp11a1	1,100 ± 90	++	++++	+	++	++	1:30,000 of adrenal
Fdx1	1,000,000 ± 60,000	++++	++++	++++	++++	++++	1:30 of adrenal
Fdxr	550,000 ± 50,000	++++	+++	++++	++++	++++	1:30 of adrenal
Star	72,000 ± 5,000	++	++	+++	++	++++	1:100 of adrenal
3β-HSD I	100 ± 10	+++	++++	+	Not detected	++	1:1,000 of ovary
3β-HSD II	8.2 ± 1.4	Not detected	++++	Not detected	Not detected	Not detected	Not detected
3β-HSD III	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
3β-HSD IV	Not detected	Not detected	++++	Not detected	Not detected	Not detected	Not detected
Cyp17a1	800 ± 30	++	++++	++	+	+++	1:300 of testis
Hsd17b1	24,000 ± 700	++	++++	++++	++	++++	1:200 of ovary
Hsd17b2	430 ± 30	+	++++	++++	+	+++	1:5,000 of liver
Hsd17b3	4,800 ± 700	+	++++	++	+	+	1:400 of testis
Hsd17b4	26,000 ± 1,000	++++	++++	++++	++++	++++	1:20 of liver
Hsd17b5	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Hsd17b6	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Hsd17b7	22,000 ± 1,600	++	++++	++++	+	++++	1:30 of ovary
Hsd17b8	400,000 ± 50,000	++++	++++	++++	++++	+++	1:5 of kidney
Hsd17b10	240,000 ± 30,000	+++	++++	++++	+++	++++	1:3 of liver
Hsd17b11	1,200,000 ± 100,000	++++	++++	++++	+++	+++	1:5 of liver
Srd5a1	820,000 ± 70,000	+++	++++	++++	++	+++	1:3 of liver
Srd5a2	12,000 ± 600	++++	+++	++	++	+++	1:300 of prostate
Cyp19a1	460 ± 50	++	++++	+	+	Not detected	1:600 of ovary
Akr1c8	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Akr1c9	30,000 ± 2,000	++	++++	+	++	+++	1:300 of liver
Akr1d2	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Esr1	16,000 ± 2,000	++	++++	++	++	+	1:30 of ovary
Esr2	1,600 ± 150	+	++++	++	+	++++	1:80 of ovary
Esr2a		+	++++	++	+	+++	1:40 of ovary
Esr2b		+	++++	++	++	++++	1:100 of ovary
Ar	38,000 ± 9,000	++++	++++	++	++	+	1:10 of testis

Note that the first two headings indicate different ways of comparison. The first heading shows comparison between different enzymes/receptors within the hippocampus. The second heading shows the comparison between different brain subregions for each specified enzyme/receptor. The second heading does not show comparison between different enzymes/receptors in the one specified subregion (*e.g.* hippocampus). Male rat mRNAs were evaluated with RT-PCR analysis. The OD value in each band in EtBr-stained electrophoresis gels was determined and normalized with that of *Gapdh*. For each mRNA, data are mean ± SEM of duplicate determinations on six animals. In the first heading, the relative abundance of mRNAs in the PD1 hippocampus is indicated with the ratio to that of 3β-HSD I mRNA. The expression level of 3β-HSD I mRNA is set to 100%. In the second heading, the normalized values for each mRNA and region were averaged over six animals and converted to the relative abundance ratios (RA) that is the ratio to that in the most abundant region. RAs were categorized into five groups according to the following definition: ++++, $1.00 \geq RA > 0.75$; +++, $0.75 \geq RA > 0.50$; ++, $0.50 \geq RA > 0.25$; +, $0.25 \geq RA > 0.00$; not detected. In the third heading, relative abundance of mRNAs in the PW12 hippocampus is compared with that in the PW12 reference organ.

The age-related expression patterns of mRNAs are complex and can be categorized into seven patterns: 1) maintained roughly constant or increased (within 30%) during PD1-PD14 and then decreased toward the adult level (*Cyp11a1*, *Cyp17a1*, *Hsd17b7*, *Cyp19a1*, *Akr1c9*); 2) maintained constant during PD1-PD4 and then declined (3β-HSD I, *Hsd17b1*, *Hsd17b2*, and *Esr1*); 3) greatly increased during PD1-PD14 and then maintained at a high level (*Hsd17b3* and *Ar*); 4) disappeared during PD1-PD7 (3β-HSD II); 5) increased after PD14 (*Hsd17b11*); 6) monotonically decreased from neonate to adult (*Srd5a2* and *Esr2*); and 7) maintained constant from neonate to adult (*e.g. Star*, *Hsd17b4*, and *Srd5a1*). Several enzymes were not detected in the hippocampus (3β-HSD III and IV, *Hsd17b5*,*6*, *Akr1c8*, and *Akr1d2*). There are some studies focusing on the ontogeny of a limited number of steroidogenic enzymes in some brain regions. However, the present study is the first one examining the hippocampal postnatal mRNA expression profiles of the crucial steroidogenic enzymes exhaustively and simultaneously, which is essentially required for the elucidation of the patterns of collaboration between the enzymes in the integrated steroidogenic system.

Enzymes for PREG/DHEA/PROG synthesis

So far, little study has been performed focusing on the hippocampal postnatal expression profiles of *Cyp11a1* and *Cyp17a1*, although the age-related expression changes of these genes are previously investigated in the whole cerebrum, diencephalon, cerebellum, and mesencephalon in which no clear-cut changes in the expression of *Cyp11a1* were observed during the neonate/juvenile period (PD1-PD14) (30). In the hippocampus, *Cyp11a1* showed an increase (~20%) during PD1-PD14. After the early postnatal stages, we observed the sharp decrease (but not reached zero) in the expression of *Cyp11a1*. This might reflect the sexual maturation-dependent increase in the supply of peripherally derived steroids into the hip-

pocampus, which results in the reduced needs of *de novo* PREG synthesis. Nevertheless, significant PREG synthesis remains, even in the adult hippocampus (11). In the adult brain, smaller amount of steroids might be required for the maintenance and modulations of the neuronal contacts and/or synaptic plasticity (31–33), which is different from the case of neonatal animals in which larger amount of steroids might be necessary for the large-scale alteration of the neuronal architecture. The expression of *Cyp17a1* also decreased toward PW12, which is again in agreement with the above view. Multiple subtypes of 3β-HSD should be distinguished. A high homology between 3β-HSD isozymes had made individual analysis difficult, which might lead to the contradictory observations: one showing only the expression of 3β-HSD I mRNA in the brain and another indicating the expression of mRNAs for 3β-HSD I, II, and IV (34, 35). Our careful investigations of the adult brain indicated that mRNAs for 3β-HSD II and IV were expressed only in the hypothalamus, and 3β-HSD I mRNA was expressed in the hippocampus, hypothalamus, and cerebellum. A monotonic decrease with aging for hippocampal 3β-HSD mRNA was previously demonstrated (18). As shown in Fig. 2, 3β-HSD I mRNA did not decrease during the period of PD1-PD7 (rather, increased at PD4), but 3β-HSD II decreased. This discrepancy might be resolved by considering the different expression patterns of mRNAs for 3β-HSD I and II. The stage PD4 might be important for the estrogen-related neuronal development because the hippocampal expression of 3β-HSD I mRNA and *Esr1* peaked at PD4 (essentially consistent with Ref. 36).

17β-HSD-related metabolism

The individual analysis of different subtypes is also important for 17β-HSD, for which more than 10 isozymes have been identified so far (15). We therefore performed the exhaustive analysis of *Hsd17b*s, which has not been done yet in the brain. The expression levels of *Hsd17b1* and *Hsd17b2* were observed to decrease monotonically after PD4. On the other hand, *Hsd17b3* showed the large increase in the expression during the neonate/juvenile stage. Because 17β-HSD3 has a stronger metabolic activity for the conversion of androstenedione to T than the other 17β-HSDs, the increase might reflect the increase in the needs of androstenedione metabolism. The hippocampal expression of *Hsd17b2* was very low, but *Hsd17b4* and *-10* were highly expressed; the enzymes encoded by *Hsd17b4* and *Hsd17b410* may protect neuronal cells from nondesired exposure to sex steroids. Another interesting subtype was the *Hsd17b11*, whose increase in the expression started at PW4. The high expression of *Hsd17b11* might reflect the increased supply of peripherally derived androgen into the hippocampus after sexual maturation; 17β-HSD11 together with 17β-HSD8 may perform the inactivation of the excess DHT. In addition to 17β-HSD1–11, new isozymes designated with 17β-HSD12–14 were recently identified (15). The role of these enzymes in the sex steroid metabolism is still obscure in the brain.

Enzymes for androgen/estrogen metabolism

For P450arom mRNA, there are a number of splice variants including cortical-type aromatase mRNA (37). The aromatization activity is correlated only with the expression level of the full-length P450arom mRNA but not with the amount of the cortical-type aromatase (38). In the present study, we therefore used the primer probes specific for the full-length P450arom mRNA to exclude the disturbance by cortical-type aromatase. By RT-PCR analysis using primers not distinguishing between mRNAs for the full-length P450arom and the cortical-type aromatase, the expression level of P450arom mRNA in the PW12 male rat hippocampus was evaluated to be approximately 1:300 of that in the PW12 ovary (9). In contrast, the expression of *Cyp19a1* (full length P450arom mRNA) in the PW12 hippocampus was estimated to be approximately 1:600 of that in the ovary by using the full-length P450arom-specific primers, which was essentially consistent with the hippocampal aromatization activity.

The level of *Cyp19a1* decreased after the juvenile stage, as observed for *Cyp11a1* and *Cyp17a1*. This might also reflect the sexual maturation-dependent increase in the supply of peripherally derived steroids into the hippocampus. Sustained elevation of the *Ar* expression might also reflect the increased supply of peripherally derived androgen into the hippocampus. Because *Srd5a1* is most abundant among the observed sex steroidogenic genes at PD1, DHT and allogregnanolone production may be very potent. *Srd5a2* was observed to be more highly expressed in the hippocampus than in the other brain subregions at PW12, suggesting that the relatively higher activity of hippocampal DHT synthesis. The metabolism analysis also revealed the hippocampal conversion of DHT to androstanediol, which is again consistent with the expression of *Hsd17b10* and *Akr1c9*. Because androstanediol has an antiepilepsy effect (39), the metabolism path might be involved in a novel neuronal regulation, in addition to the mere inactivation of DHT.

In the male rat during the first neonatal week, the brain is exposed to a high level of T supplied from the testis in addition to *de novo* synthesized T. A high expression of the hippocampal *Cyp19a1* and *Srd5a2* suggests the hippocampal needs of T metabolism at this stage. The relatively high expression of *Esr1* at this stage also suggests the

importance of E2 in the hippocampal development, which is in agreement with the aromatization hypothesis for brain sex differentiation (2). The relatively high expression of 3β-HSD I mRNA at the early neonatal stage suggests that the metabolism of PREG to PROG occurs to a higher extent compared with that in the adult hippocampus. PROG might be further converted to allopregnanolone by enzymes encoded by *Srd5a2* and *Akr1c9* and regulate secretion of γ-aminobutyric acid (GABA)-ergic transmissions at this stage. The expression of *Akr1c8* was not detected, implying that the inactivation of PROG by 20α-HSD does not occur in the rat hippocampus.

In the second postnatal week, the supply of T from gonad is attenuated. Even in this period, hippocampal *Cyp11a1*, *Akr1c9*, and *Cyp19a1* were maintained at a high level, suggesting that the sufficient sex steroids can be supplied by the endogenous neurosteroidogenic system. Not only PROG→androstenedione→T pathway (Δ^4 pathway) but also PREG→DHEA→androstenediol→T (Δ^5 pathway) work for T supply (17).

Although the relative expression levels of mRNAs for the hippocampal sex steroidogenic enzymes were approximately between 1:200 and 1:1000 as compared with their levels in gonads, these levels are not too low for them to produce sufficient amount of hippocampal sex steroids. They need to produce sex steroids that fill only a small hippocampal volume (~0.14 ml at PW12; ~1:200 of the blood volume of ~25 ml), whereas gonadal steroids need to fill the whole blood volume that is nearly 200-fold of the hippocampal volume. In fact, we observed that hippocampal levels of E2 and T (~8 and 17 nM, respectively) were even higher than circulating levels (~0.01 and 15 nM, respectively) (22).

The levels of mRNAs for 3β-HSD II and 5α-reductase 2, which catalyze the same reactions as 3β-HSD I and 5α-reductase 1, respectively, declined sharply, depending on ages, as compared with those of the latter enzymes. The expression levels of the mRNAs for 3β-HSD I and 5α-reductase 1 at PD1 were much higher (10- to 100-fold) than those of the 3β-HSD II and 5α-reductase 2, respectively, suggesting that the decrease of the reaction activity carried out by 3β-HSD II and 5α-reductase 2 might not significantly contribute the total reactions. Therefore, possible roles in the hippocampal development of 3β-HSD II and 5α-reductase 2 are still obscure. Although many isoenzymes are present in the 17β-HSD family, 17β-HSD1, -3, and -7 catalyze the forward reaction, whereas 17β-HSD2, -4, -8, -10, and -11catalyze the back reaction (Fig. 1). The mRNA levels for these enzyme gradually decreased, depending on ages, except for 17β-HSD3. Despite the relative order of the mRNA contents for enzymes responsible for the forward and backward reactions, the activity of the forward reaction might be greater than the backward enzymes because practically the steroid metabolism reactions proceed forward as a whole.

To obtain the comprehensive data set for sex steroidogenesis, additional investigations of translocator protein and PROG receptor may be necessary. Similar study for female hippocampus would give us a good example of sexual dimorphism of nonreproductive brain region.

Acknowledgments

We thank Dr. A. Furukawa (Kurihama Alcoholism Center) and Mr. Y. Sonoki for helpful discussions and technical assistance.

Address all correspondence and requests for reprints to: Dr. Suguru Kawato, Professor, Department of Biophysics and Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro, Tokyo 153-8902, Japan. E-mail: kawato@phys.c.u-tokyo.ac.jp.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Hajszan T, Milner TA, Leranth C** 2007 Sex steroids and the dentate gyrus. Prog Brain Res 163:399–415
2. **McCarthy MM** 2008 Estradiol and the developing brain. Physiol Rev 88:91–124
3. **Zhang JM, Konkle AT, Zup SL, McCarthy MM** 2008 Impact of sex and hormones on new cells in the developing rat hippocampus: a novel source of sex dimorphism? Eur J Neurosci 27:791–800
4. **Baulieu EE, Robel P, Schumacher M** 2001 Neurosteroids: beginning of the story. Int Rev Neurobiol 46:1–32
5. **Crews F, He J, Hodge C** 2007 Adolescent cortical development: a critical period of vulnerability for addiction. Pharmacol Biochem Behav 86:189–199
6. **Hojo Y, Murakami G, Mukai H, Higo S, Hatanaka Y, Ogiue-Ikeda M, Ishii H, Kimoto T, Kawato S** 2008 Estrogen synthesis in the brain—role in synaptic plasticity and memory. Mol Cell Endocrinol 290:31–43
7. **Kawato S, Hojo Y, Kimoto T** 2002 Histological and metabolism analysis of P450 expression in the brain. Methods Enzymol 357: 241–249
8. **Naftolin F, Ryan KJ, Davies IJ, Reddy VV, Flores F, Petro Z, Kuhn M, White RJ, Takaoka Y, Wolin L** 1975 The formation of estrogens by central neuroendocrine tissues. Recent Prog Horm Res 31:295–319
9. **Hojo Y, Hattori TA, Enami T, Furukawa A, Suzuki K, Ishii HT, Mukai H, Morrison JH, Janssen WG, Kominami S, Harada N, Kimoto T, Kawato S** 2004 Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017α and P450 aromatase localized in neurons. Proc Natl Acad Sci USA 101:865–870
10. **Ishii H, Tsurugizawa T, Ogiue-Ikeda M, Asashima M, Mukai H, Murakami G, Hojo Y, Kimoto T, Kawato S** 2007 Local production of sex hormones and their modulation of hippocampal synaptic plasticity. Neuroscientist 13:323–334
11. **Kimoto T, Tsurugizawa T, Ohta Y, Makino J, Tamura H, Hojo Y, Takata N, Kawato S** 2001 Neurosteroid synthesis by cytochrome p450-containing systems localized in the rat brain hippocampal neurons: *N*-methyl-D-aspartate and calcium-dependent synthesis. Endocrinology 142:3578–3589

12. **Papadopoulos V, Lecanu L** 2009 Translocator protein (18 kDa) TSPO: an emerging therapeutic target in neurotrauma. Exp Neurol 219:53–57
13. **Furukawa A, Miyatake A, Ohnishi T, Ichikawa Y** 1998 Steroidogenic acute regulatory protein (StAR) transcripts constitutively expressed in the adult rat central nervous system: colocalization of StAR, cytochrome P-450SCC (CYP XIA1), and 3β-hydroxysteroid dehydrogenase in the rat brain. J Neurochem 71:2231–2238
14. **Simard J, Ricketts ML, Gingras S, Soucy P, Feltus FA, Melner MH** 2005 Molecular biology of the 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase gene family. Endocr Rev 26:525–582
15. **Moeller G, Adamski J** 2009 Integrated view on 17β-hydroxysteroid dehydrogenases. Mol Cell Endocrinol 301:7–19
16. **Mahendroo MS, Russell DW** 1999 Male and female isoenzymes of steroid 5α-reductase. Rev Reprod 4:179–183
17. **Higo S, Hojo Y, Ishii H, Kominami T, Nakajima K, Poirier D, Kimoto T, Kawato S** 2009 Comparison of sex-steroid synthesis between neonatal and adult rat hippocampus. Biochem Biophys Res Commun 385:62–66
18. **Ibanez C, Guennoun R, Liere P, Eychenne B, Pianos A, El-Etr M, Baulieu EE, Schumacher M** 2003 Developmental expression of genes involved in neurosteroidogenesis: 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase in the rat brain. Endocrinology 144: 2902–2911
19. **He XY, Wen GY, Merz G, Lin D, Yang YZ, Mehta P, Schulz H, Yang SY** 2002 Abundant type 10 17β-hydroxysteroid dehydrogenase in the hippocampus of mouse Alzheimer's disease model. Brain Res Mol Brain Res 99:46–53
20. **Ivanova T, Beyer C** 2000 Ontogenetic expression and sex differences of aromatase and estrogen receptor-α/β mRNA in the mouse hippocampus. Cell Tissue Res 300:231–237
21. **Agís-Balboa RC, Pinna G, Zhubi A, Maloku E, Veldic M, Costa E, Guidotti A** 2006 Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. Proc Natl Acad Sci USA 103:14602–14607
22. **Hojo Y, Higo S, Ishii H, Ooishi Y, Mukai H, Murakami G, Kominami T, Kimoto T, Honma S, Poirier D, Kawato S** 2009 Comparison between hippocampus-synthesized and circulation-derived sex steroids in the hippocampus. Endocrinology 150:5106–5112
23. **Lauber ME, Lichtensteiger W** 1996 Ontogeny of 5α-reductase (type 1) messenger ribonucleic acid expression in rat brain: early presence in germinal zones. Endocrinology 137:2718–2730
24. **Mitev YA, Darwish M, Wolf SS, Holsboer F, Almeida OF, Patchev VK** 2003 Gender differences in the regulation of 3α-hydroxysteroid dehydrogenase in rat brain and sensitivity to neurosteroid-mediated stress protection. Neuroscience 120:541–549
25. **Pelletier G, Luu-The V, Li S, Labrie F** 2004 Localization of 20α-hydroxysteroid dehydrogenase mRNA in mouse brain by *in situ* hybridization. Brain Res Mol Brain Res 125:143–146
26. **Ishii H, Kobayashi M, Sakuma Y** 2010 Alternative promoter usage and alternative splicing of the rat estrogen receptor α gene generate numerous mRNA variants with distinct 5′-ends. J Steroid Biochem Mol Biol 118:59–69
27. **Mellon SH, Deschepper CF** 1993 Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. Brain Res 629:283–292
28. **Strömstedt M, Waterman MR** 1995 Messenger RNAs encoding steroidogenic enzymes are expressed in rodent brain. Brain Res Mol Brain Res 34:75–88
29. **Munetsuna E, Hojo Y, Hattori M, Ishii H, Kawato S, Ishida A, Kominami SA, Yamazaki T** 2009 Retinoic acid stimulates 17β-estradiol and testosterone synthesis in rat hippocampal slice cultures. Endocrinology 150:4260–4269
30. **Kohchi C, Ukena K, Tsutsui K** 1998 Age- and region-specific expressions of the messenger RNAs encoding for steroidogenic enzymes p450scc, P450c17 and 3β-HSD in the postnatal rat brain. Brain Res 801:233–238
31. **Kretz O, Fester L, Wehrenberg U, Zhou L, Brauckmann S, Zhao S, Prange-Kiel J, Naumann T, Jarry H, Frotscher M, Rune GM** 2004 Hippocampal synapses depend on hippocampal estrogen synthesis. J Neurosci 24:5913–5921
32. **Mukai H, Kimoto T, Hojo Y, Kawato S, Murakami G, Higo S, Hatanaka Y, Ogiue-Ikeda M** 2010 Modulation of synaptic plasticity by brain estrogen in the hippocampus. Biochim Biophys Acta 1800: 1030–1044
33. **Mukai H, Tsurugizawa T, Murakami G, Kominami S, Ishii H, Ogiue-Ikeda M, Takata N, Tanabe N, Furukawa A, Hojo Y, Ooishi Y, Morrison JH, Janssen WG, Rose JA, Chambon P, Kato S, Izumi S, Yamazaki T, Kimoto T, Kawato S** 2007 Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons. J Neurochem 100:950–967
34. **Guennoun R, Fiddes RJ, Gouézou M, Lombès M, Baulieu EE** 1995 A key enzyme in the biosynthesis of neurosteroids, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD), is expressed in rat brain. Brain Res Mol Brain Res 30:287–300
35. **Sanne JL, Krueger KE** 1995 Expression of cytochrome P450 side-chain cleavage enzyme and 3β-hydroxysteroid dehydrogenase in the rat central nervous system: a study by polymerase chain reaction and *in situ* hybridization. J Neurochem 65:528–536
36. **O'Keefe JA, Li Y, Burgess LH, Handa RJ** 1995 Estrogen receptor mRNA alterations in the developing rat hippocampus. Brain Res Mol Brain Res 30:115–124
37. **Yamada-Mouri N, Hirata S, Kato J, Hoshi K** 1997 Expression and distribution of cortical type aromatase mRNA variant in the adult rat brain. J Steroid Biochem Mol Biol 60:325–329
38. **Abdelgadir SE, Resko JA, Ojeda SR, Lephart ED, McPhaul MJ, Roselli CE** 1994 Androgens regulate aromatase cytochrome P450 messenger ribonucleic acid in rat brain. Endocrinology 135:395–401
39. **Reddy DS** 2004 Anticonvulsant activity of the testosterone-derived neurosteroid 3alpha-androstanediol. Neuroreport 15:515–518

Supplemental Data

Results and Discussion

Star ---- For rat *Star*, 4 types of sequences containing complete cds and poly A signal (AATAAA) appear in the NCBI Database to date (*Star1*: AB006007; *Star2*: AB001349 and AF044081; *Star3*: U76419; *Star4*: BC088859). Among all, *Star1*, *2*, and *4* are supposed to encode for the identical 284 amino-acid length protein with different poly A signals in their 3'-untranslated regions. Only the *Star3* predicts the different protein having 362 amino-acid residues. We therefore prepared 3 different PCR primer sets to amplify: (A) all of the *Star1-4*, (B) *Star1,2* and *4*, and (C) only the *Star3* (see Table S1). Almost the same results were obtained by using either set (A) or (B); However, when the primer set (C) was used, no *Star3* signal was detected in all of the brain subregions. Rat draft genome analysis indicates the single locus for STARD1 at 16q12.4. However, *Star3*-specific sequences are not found nearby the STARD1 locus in the rat draft genome map. Furthermore, BLAST search reveals that the sequence of 166 nt − 496 nt in U76419 is completely identical to 89 nt − 419 nt of the testis expressed gene 261 (BC091302). Taken together, *Star3* may be the product of an artifact.

Esr2 ---- The expression of *Esr2* was observed in all of the examined brain subregions by using the primer set "Esr2-A". Furthermore, splice variants of *Esr2*, *Esr2a* and *Esr2b*, were separated by using the primer set "Esr2-B" (Figure S2). Although both *Esr2a* and *Esr2b* were detected in the hippocampus, the hippocampal expression of *Esr2* was considerably lower compared with those in the other brain subregions. Furthermore, *Esr2a* was revealed to be the dominant component of the hippocampal *Esr2*, which was different from the cases of the other brain subregions. Estrogen receptors exert their effects as homo/hetero dimer complexes; therefore, the relative abundance of each ERβ isoform might be the important factor determining the function of ERs. The functional mode of ERβ in the hippocampus might be regulated in a different manner from that in the other brain subregions.

Figure Legends

Figure S1

In order to check the quality of the primer set, comparison of the characteristics of the primer set prepared in the present study with those used in the previous studies was performed. PCR for *Cyp11a1* and *Cyp17a1* was performed using the cDNA template derived from the same origin (total RNA extracted from PW12 male rat hippocampus).

Left panel: representative result for *Cyp11a1* PCR analysis; right panel: *Cyp17a1*. Upper image in each panel represents the electrophoresed bands of PCR product visualized as described in *Materials and Methods*. A, primer set prepared in the present study; B, primer set used in Ref.(1); C, primer set used in Ref. (2) (see Table S1). (+) for each primer set indicates the lane to which the PCR product obtained from the hippocampal cDNA was applied. PCR product originated from 200 ng of total RNA was electrophoresed in each lane. (-) represents the negative control. PCR cycle number was fixed to be 36 for both *Cyp11a1* and *Cyp17a1*. Red allow in each gel image indicates the target product; blue allows indicate non-desired bands. Essentially the same results were obtained in 3 independent PCR run for both *Cyp11a1* and *Cyp17a1*. $\Delta G_{(whole)}$ and $\Delta G_{(5base)}$ of each primer set are indicated in the tables below the corresponding gel image.

For *Cyp11a1* PCR, set A and C gave the clear single band whose electrophoretic mobility was in agreement with the predicted one. In contrast, set B gave many non-specific products. For *Cyp17a1* PCR, only the set A gave the clear single band whose mobility was in accordance with the predicted one. Set C gave one non-specific band. By using set B, only the very faint bands were observed. For PCR with set A, the logarithmic growth phase was observed in the cycle number of 36 - 40 and 32 - 36 for *Cyp11a1* and *Cyp17a1*, respectively. All primer set gave no bands for negative control (RTase-omitted), showing that the observed signals were not derived from genomic DNA contaminants. Note that no extra bands other than the target signal appeared even by the increase of the PCR cycle number up to be 50, when the primer set A was used for both *Cyp11a1* and *Cyp17a1*.

Figure S2

Expression analysis of *Esr2* splice variants in the brain of PW12 male rat.

A, Representative gel image for electrophoresed RT-PCR products of *Esr2* splice variants (upper image) and *Gapdh* (lower image). PCR was performed using the primer set "Esr2-B" for the separation of *Esr2a* and *Esr2b*. Electrophoresed gels were stained with EtBr, then bands were visualized by UV illumination as described in *Materials and Methods*. Upper and lower bands are corresponding to *Esr2a* and *Esr2b*, respectively. Final PCR products originated from 50 ng of total RNA was electrophoresed in each lane, except for the positive control lane (PCR products originated from 5 ng of total RNA of PW12 ovary). PCR cycle number was 39. M, 100 bp ladder marker; Cc, cerebral cortex; Hi, hippocampus; Th, thalamus; Hy, hypothalamus, Ce, cerebellum, Nc, negative control; Ov, ovary (positive control). Essentially the same results were obtained for the analysis performed on 4 animals.

B, Electroferogram for PCR products corresponding to *Esr2a* and *Esr2b*. PCR products were cloned in pGEM-T-Easy vector, and then DNA sequencing was performed as described in *Materials and Methods*. Only the sequences nearby the 54 nt insert of *Esr2a* are represented.

Figure S3

Postnatal age-related change in the expression of mRNA for steroidogenic factor-1(*Sf-1*).

A, Representative image for the RT-PCR/Southern blot analysis for *Sf-1*. The most upper image row: EtBr staining; second row: Southern blots; lowest row: *Gapdh*. Lanes, labeled with P1(=PD1), P4, P7, P10, P14, W4(=PW4) and W12, represent the electrophoresed PCR product derived from the male rat hippocampus at the corresponding ages. Final PCR products originated from 200 ng of total RNA was electrophoresed in each lane. M, DNA ladder marker; Nc, negative control; Ad, adrenal gland: positive control derived from PW12 rats. Primer sequences and cycle numbers used in each PCR are indicated in Table S1.

B, Graph showing the age-related temporal changes in the mRNA expression. Relative expression level of mRNA are evaluated with RT-PCR analysis. OD value in each band in EtBr-stained gels was determined and normalized with that of *Gapdh* at the corresponding conditions, and the level was indicated as the ratio to that at PD1 (PD1 was set to be 100% for each gene).

C, Representative image for the RT-PCR/Southern blot analysis for brain subregions at PW12. Lanes, from

left to right, size marker (M), cortex (CC), hippocampus (Hi), thalamus (Th), hypothalamus (Hy), cerebellum (Ce), negative control (Nc), and adrenal gland (Ad) as positive control.

Figure S4

Representative image sets for the RT-PCR/Southern blot analysis for *Fdx1*, *Fdxr*, 3β-HSD IV, *Hsd17b5*, *Hsd17b6*, *Akr1c8* and *Akr1d2*. In each image set discriminated by gene name, the most upper image row: EtBr staining; second row: Southern blots; lowest row: *Gapdh*. Lanes, labeled with P1(=PD1), P4, P7, P10, P14, W4(=PW4) and W12, represent the PCR products derived from the male rat hippocampus at the corresponding ages. Final PCR products originated from 200 ng (for 3β-HSD IV) or 50 ng (for other genes) of total RNA was electrophoresed in each lane. The left most lane (labeled with M) is the DNA ladder marker lane. The right most lane corresponds to the positive control derived from PW12 rats (Ad, adrenal; Li, liver; Ov, ovary). The lane (Nc) represents the negative control. Primer sequences and cycle numbers used in each PCR are indicated in Table S1.

References

1. **Mellon SH, Deschepper CF** 1993 Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. Brain Res 629:283-292
2. **Stromstedt M, Waterman MR** 1995 Messenger RNAs encoding steroidogenic enzymes are expressed in rodent brain. Brain Res Mol Brain Res 34:75-88

Table S1 Primers

Enzyme/Protein name	Gene name	Accession No.	Primer Set	Direction	Primer Sequence (5' to 3')	Product Length (bp[a])	T_a[b] (C)	PCR cycles[c] PW12 Brain	PCR cycles[c] Age-related Analysis	PCR cycles[c] Exponential[d] Amplification
P450scc	*Cyp11a1*	NM_017286	A[e]	forward	5'-TGACCTATTCCGCTTTGCCTTTGAGT-3'	363	62	39	36	36 - 42
				reverse	5'-TCTGTGATGTTGGCCTGGATGTTCTT-3'					
			B[e]	forward	5'-CTGTGGGGACAGTATGCTGGC-3'	400	62	39		
				reverse	5'-CTGGTGATAGGCCACCCAGG-3'					
			C[e]	forward	5'-CAACATCACAGAGATGCTGGCAGG-3'	559	62	39		
				reverse	5'-CTCAGGCATCAGGATGAGGTTGAA-3'					
Adrenodoxin	*Fdx1*	NM_017126		forward	5'-GGACCCCGAGCCCCCACAC-3'	418	65	28	28	24 - 30
				reverse	5'-TGTCGGACATCTGCCACTGCTTCA-3'					
NADPH-adrenodoxin reductase	*Fdxr*	NM_024153		forward	5'-CGCGTGGCCTGGGGTTCG-3'	283	68	28	28	24 - 30
				reverse	5'-GTCTGAGCGGGCTGTCTGTGTAAATGTG-3'					
StAR	*Star*	Star1:AB006007; Star2:AB001349/ AF044081; Star3: U76419; Star4: BC088859	A[f]	forward	5'-CTGGTGGGGCCCCGAGACTT-3'	360	60	32	32	28 - 34
				reverse	5'-CAATGGCGTGCAGGTAGATGTGGT-3'					
			B[f]	forward	5'-TGGGAGCAGCAGCAGCAACT-3'	379	60	32		
				reverse	5'-TGGCACCACCTTACTTAGCACTTCAT-3'					
			C[f]	forward	5'-GCATTTCAGTTTTTTGCGGAAGAAT-3'	384	60	34		
				reverse	5'-TGGCACCACCTTACTTAGCACTTCAT-3'					
P450c17	*Cyp17a1*	NM_012753	A[e]	forward	5'-TGGGGCGGGCATAGAGACAACT-3'	477	62	36	35	32 - 38
				reverse	5'-AGCAAGGCCGTGAAGACAAAGAGC-3'					
			B[e]	forward	5'-GAATTCTCTGGTCGGCCCCAG-3'	542	62	36		
				reverse	5'-GAATTCATCTTGGCTTGAATCAG-3'					
			C[e]	forward	5'-CCCATCTATTCTCTTCGCCTGGGTA-3'	719	62	36		
				reverse	5'-GCCCCAAAGATGTCTCCCACCGTG-3'					
3β-HSD I		NM_001007719		forward	5'-AGGGCATCTCTGTTGTCATCCAC-3'	275	62	40	38	36 - 42
				reverse	5'-TGCCTTCTCGGCCATCCTTT-3'					
3β-HSD II		NM_001042619		forward	5'-ATCTCTGTTGTCATCCACACGGCTTC-3'	273	62	40	40	38 - 44
				reverse	5'-CACTGCCTTCTCGGCCATCTT-3'					
3β-HSD III		NM_012584		forward	5'-AGGGCATGTCCGTCATCATCCAC-3'	275	62	40	40	
				reverse	5'-TGCCTTCTCGGCCATCCTTT-3'					
3β-HSD IV		NM_017265		forward	5'-CCACACTGCCGCCCTCATTGA-3'	259	62	40	40	
				reverse	5'-CACTGCCTTCTCGGCCATCTT-3'					
17β-HSD 1	*Hsd17b1*	NM_012851		forward	5'-ACTCCGGGCGTGTGCTGGTGA-3'	517	65	34	33	30 - 36
				reverse	5'-GGCGTGTCTGGATCCCCTGAAACTT-3'					
17β-HSD 2	*Hsd17b2*	NM_024391		forward	5'-GCCGCCTACGCCTCCACGAA-3'	367	65	40	39	36 - 42
				reverse	5'-CCGCGAAGCAGATCCACAGGTAAGTC-3'					
17β-HSD 3	*Hsd17b3*	NM_054007		forward	5'-CTCCCCAACCTGCTCCCAAGTCATTT-3'	408	65	35	35	32 - 38
				reverse	5'-AGCAAGGCAGCCACAGGTTTCAGC-3'					
17β-HSD 4	*Hsd17b4*	NM_024392		forward	5'-TCGGAGTAGGAGCTTCAGTCAAAAAT-3'	578	65	32	32	28 - 34
				reverse	5'-TGCAGAAAACCCAAAAGTACATAATCC-3'					

17β-HSD 5	*Hsd17b5*	NM_001033697		forward	5'-GGGAAGCCACCGTGAAACAAG-3'	345	55	45	45	
				reverse	5'-TGGCATAAAGCATAACACAGACCTC-3'					
17β-HSD 6	*Hsd17b6*	NM_173305		forward	5'-TGTTTTGGGAAGAGTTGCATTGTTTG-3'	484	62	45	45	
				reverse	5'-GAGCCGCCGACCCAGGAT-3'					
17β-HSD 7	*Hsd17b7*	NM_017235		forward	5'-GAACGCCGGAATCATGCCTAACC-3'	551	63	32	32	28 - 34
				reverse	5'-GGAAAAGCCACACCAATGCCTCTG-3'					
17β-HSD 8	*Hsd17b8*	NM_212529		forward	5'-TTTTTCGCCCGCCATCTGTCG-3'	366	64	31	30	26 - 32
				reverse	5'-TCACTTTCTCTGGCATTTTCTGGGTCAT-3'					
17β-HSD 10	*Hsd17b10*	NM_031682		forward	5'-CGGCGTGTCGGAGCGTGAAG-3'	551	64	30	30	26 - 32
				reverse	5'-CAGATCTCGAGCAATGGGCAGTGTC-3'					
17β-HSD 11	*Hsd17b11*	NM_001004209		forward	5'-AGCAGGGCACACCGTCGTTCC-3'	391	63	28	27	24 - 30
				reverse	5'-AGGCGGTCTGCGTCATTTATCTTTGTAG-3'					
5α-reductase 1	*Srd5a1*	NM_017070		forward	5'-ACCGCGTCCTGCTGGCTATGTTT-3'	318	63	28	28	24 - 30
				reverse	5'-GGCCTCCCCTGGGTATCTTGTATCC-3'					
5α-reductase 2	*Srd5a2*	NM_022711		forward	5'-AGGTGGCTTGTTTACGTATGTCTCTG-3'	453	57	36	34	30 - 36
				reverse	5'-GGCCTCTGTGAAGCTCCAAAAG-3'					
P450arom	*Cyp19a1*	NM_017085		forward	5'-CTGATCATGGGCCTCCTCCTC-3'	276	58	38	37	30 - 38
				reverse	5'-CCCACGCTTGCTGCCGAATCT-3'					
20α-HSD	*Akr1c8*	NM_138510		forward	5'-TGAAGATGGCACTGTGAAAAGGGAAGAT-3'	451	63	45	45	
				reverse	5'-CAGGGCACCATAGGCAACCAGAAC-3'					
3α–HSD	*Akr1c9*	NM_138547 (D17310)		forward	5'-GGAATGTCACCTTTATCTCAACCA-3'	489	55	34	34	30 - 36
				reverse	5'-ATGCGTTCAGTCACCAGTATCCA-3'					
5β-reductase	*Akr1d2*	NM_138884		forward	5'-CACTGCAAACCACCACATACCCCTAAAT-3'	552	63	45	45	
				reverse	5'-GGCTTGTACTTGAGTCCTGGCTTGTTC-3'					
ERα	*Esr1*	NM_012689		forward	5'-GCCGGCTGCGCAAGTGTTACG-3'	467	68	34	33	30 - 36
				reverse	5'-GGAGCGCCAGACCAGACCAATCA-3'					
ERβ	*Esr2*	NM_012754	Esr2-A[g]	forward	5'-GCAAACCAGGAGGCAGAAAGTAGC-3'	591	58	38	38	34 - 40
				reverse	5'-AAGTGGGCAAGGAGACAGAAAGTAAGTA-3'					
ERβ2 & ERβ1	*Esr2a* & *Esr2b*	*Esr2a*: AB190770 *Esr2b*: AB190769	Esr2-B[g]	forward	5'-GACCACCCCGGCAAGCTCATTTTC-3'	*Esr2a*: 496	65	39		
				reverse	5'-TCAGCATCTCCAGCAGCAGGTCATACAC-3'	*Esr2b*: 442				
Androgen receptor	*Ar*	NM_012502		forward	5'-CAACTTTCCGCTCGCTCTGTC-3'	536	56	33	31	28 - 34
				reverse	5'-TCTGGGGTGGGAAGTAATAGTCG-3'					
Steroidogenic factor-1	*Sf-1*	NM_001191099		forward	5'-TGTCTGTCTCAAGTTCCTCATCCTC-3'	272	55	38	38	34 - 40
				reverse	5'-TGGCCTGCAGCATCTCAAT-3'					
GAPDH	*Gapdh*	NM_017008		forward	5'-TATGACTCTACCCACGGCAAGTTCAA-3'	830	60	17 or 19[h]	17 or 19[h]	
				reverse	5'-ACCACCCTGTTGCTGTAGCCATATTCAT-3'					

[a] bp : base pair.
[b] T_a : annealing temperature.
[c] PCR cycle numbers used for the analysis of the subregion-dependent expression differences in the PW12 male rat brain and those used for the hippocampal age-related expression profiles.
[d] PCR cycle numbers for the exponential amplification, estimated from the PCR amplification curves obtained by a number of PCR series using the hippocampal cDNA templates (equivalent to 200 ng (for *Cyp11a1, Fdx1, Fdxr, Star,* mRNAs for 3β-HSD I-IV, *Cyp17a1* and *Sf-1*) and 50 ng (for others) of total RNA) from PD1 male rats.
[e] A, designed in the present study; B, used in Ref. (1); and C, used in Ref. (2).
[f] For rat *Star*, 4 types of sequences containing complete cds and poly A signal (AATAAA) appear in the NCBI Database; only the *Star3* predicts the distinct protein. 3 different PCR primer sets were designed to amplify: A, all of the *Star1-4*; B, *Star1,2* and *4*; and C, only the *Star3*.
[g] The primer set "Esr2-A" was designed to generate the same PCR product for both *Esr2a* and *Esr2b*. Set "Esr2-B" was able to separate *Esr2a* from *Esr2b*.
[h] 17-cycle was used for the samples containing 200 ng of total RNA, and 19-cycle was for 50 ng of total RNA.

Table S2 Relative expression levels of mRNAs shown in Fig. 2-5 evaluated with RT-PCR analysis.

Enzymes for PREG/DHEA/PROG metabolism

	Cyp11a1	*Fdx1*	*Fdxr*	*Star*	*Cyp17a1*	3β-HSD I	3β-HSD II	3β-HSD III	3β-HSD IV
PD1	100 ± 8 [a,b]	100 ± 6	100 ± 9	100 ± 7	100 ± 2 [a]	100 ± 10 [a]	100 ± 17 [a]	not detected	not detected
PD4	109 ± 12 [a,b]	98 ± 5	102 ± 10	104 ± 6	106 ± 12 [a]	113 ± 6 [a]	45 ± 12 [b]	not detected	not detected
PD7	120 ± 9 [a]	95 ± 5	101 ± 6	101 ± 5	96 ± 5 [a]	91 ± 11 [a]	11 ± 8 [b]	not detected	not detected
PD10	122 ± 7 [a]	95 ± 5	96 ± 8	81 ± 5	87 ± 5 [a,b]	80 ± 8 [a,b]	not detected	not detected	not detected
PD14	116 ± 11 [a]	91 ± 5	93 ± 7	84 ± 5	102 ± 6 [a]	54 ± 10 [b]	not detected	not detected	not detected
PW4	75 ± 9 [b]	88 ± 6	95 ± 5	95 ± 10	90 ± 8 [a]	54 ± 6 [b]	not detected	not detected	not detected
PW12	8 ± 2 [c]	94 ± 5	103 ± 3	81 ± 3	62 ± 7 [b]	56 ± 3 [b]	not detected	not detected	not detected

17β-HSD

	Hsd17b1	*Hsd17b2*	*Hsd17b3*	*Hsd17b4*	*Hsd17b5*
PD1	100 ± 3 [a]	100 ± 6 [a]	100 ± 15 [a]	100 ± 4	not detected
PD4	96 ± 4 [a]	97 ± 7 [a]	120 ± 9 [a]	102 ± 4	not detected
PD7	71 ± 3 [b]	71 ± 6 [a,b]	152 ± 24 [a,b]	96 ± 6	not detected
PD10	76 ± 3 [b]	71 ± 13 [a,b]	196 ± 17 [b]	98 ± 6	not detected
PD14	64 ± 4 [b,c]	63 ± 7 [a,b]	184 ± 10 [b]	99 ± 6	not detected
PW4	59 ± 3 [b,c]	57 ± 12 [b]	155 ± 10 [a,b]	83 ± 1	not detected
PW12	54 ± 3 [c]	53 ± 12 [b]	148 ± 15 [a,b]	94 ± 6	not detected

	Hsd17b6	*Hsd17b7*	*Hsd17b8*	*Hsd17b10*	*Hsd17b11*
PD1	not detected	100 ± 7 [a]	100 ± 12	100 ± 11	100 ± 9 [a,b]
PD4	not detected	108 ± 7 [a]	106 ± 11	98 ± 7	102 ± 4 [a,b]
PD7	not detected	97 ± 10 [a]	111 ± 13	110 ± 10	94 ± 6 [a]
PD10	not detected	99 ± 9 [a]	106 ± 13	103 ± 10	95 ± 9 [a]
PD14	not detected	106 ± 5 [a]	96 ± 11	99 ± 10	101 ± 8 [a,b]
PW4	not detected	85 ± 9 [a,b]	102 ± 11	90 ± 10	132 ± 12 [b,c]
PW12	not detected	64 ± 6 [b]	103 ± 10	104 ± 13	147 ± 9 [c]

Enzymes for androgen/estrogen metabolism

	Cyp19a1	*Srd5a1*	*Srd5a2*	*Akr1c8*	*Akr1c9*	*Akr1d2*
PD1	100 ± 7 [a]	100 ± 8	100 ± 5 [a]	not detected	100 ± 7 [a]	not detected
PD4	102 ± 7 [a]	96 ± 9	76 ± 3 [b]	not detected	107 ± 9 [a,b]	not detected
PD7	86 ± 5 [a,b]	89 ± 2	73 ± 4 [b]	not detected	107 ± 4 [a,b]	not detected
PD10	99 ± 7 [a]	91 ± 5	58 ± 4 [c]	not detected	132 ± 9 [b]	not detected
PD14	97 ± 9 [a]	87 ± 4	55 ± 2 [c,d]	not detected	133 ± 6 [b]	not detected
PW4	51 ± 4 [c]	85 ± 10	47 ± 2 [c,d]	not detected	51 ± 5 [c]	not detected
PW12	63 ± 4 [b,c]	93 ± 1	42 ± 3 [d]	not detected	57 ± 3 [c]	not detected

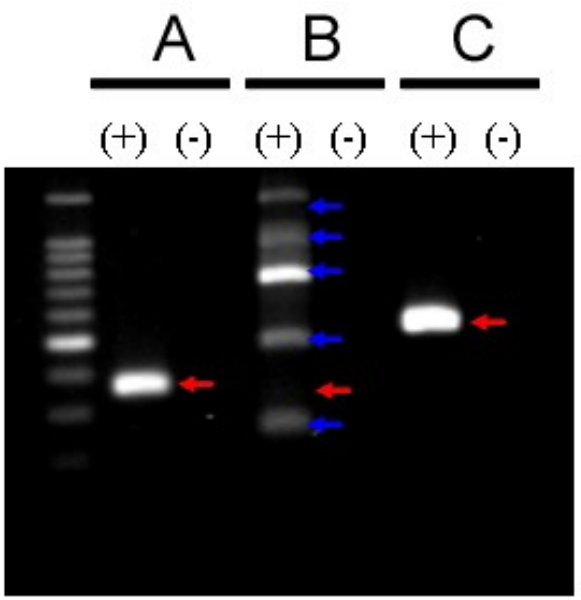
Receptors for estrogen and androgen

	Esr1	*Esr2*	*Ar*
PD1	100 ± 6 [a]	100 ± 10	100 ± 16 [a]
PD4	146 ± 11 [b]	89 ± 4	208 ± 24 [a,b]
PD7	101 ± 7 [a]	85 ± 13	242 ± 34 [a,b,c]
PD10	96 ± 12 [a,c]	82 ± 8	330 ± 13 [b,c]
PD14	71 ± 4 [a,c]	85 ± 15	348 ± 35 [c]
PW4	67 ± 5 [c]	85 ± 16	355 ± 11 [c]
PW12	79 ± 6 [a,c]	75 ± 12	331 ± 30 [c]

Superscripts that are different within each gene column indicate significant differences ($P < 0.05$). The level was indicated as the ratio to that at PD1 (PD1 was set to be 100% for each gene). Data are mean ± SEM of duplicate determinations on 6 animals at each age.

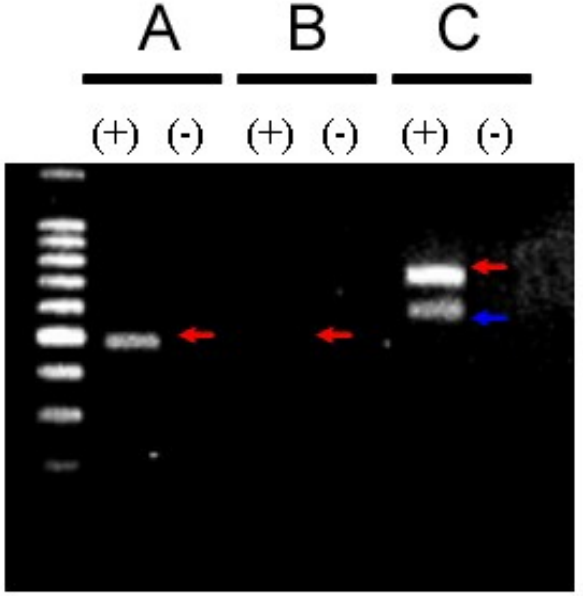
Figure S1

Cyp11a1



		$\Delta G_{(whole)}$ [kcal/mol]	$\Delta G_{(5base)}$ [kcal/mol]
A	forward	-50.5	-8.3
	reverse	-47.6	-8.3
B	forward	-42.5	-12.8
	reverse	-40.7	-12.8
C	forward	-44.4	-12.8
	reverse	-44.4	-8.6

Cyp17a1



		$\Delta G_{(whole)}$ [kcal/mol]	$\Delta G_{(5base)}$ [kcal/mol]
A	forward	-45.9	-8.0
	reverse	-47.8	-9.8
B	forward	-43.8	-12.8
	reverse	-40.6	-8.5
C	forward	-48.7	-10.3
	reverse	-50.1	-11.2

(+) : Hippocampal cDNA

(-) : total RNA

← Target Product

← Non-specific Product

Figure S2

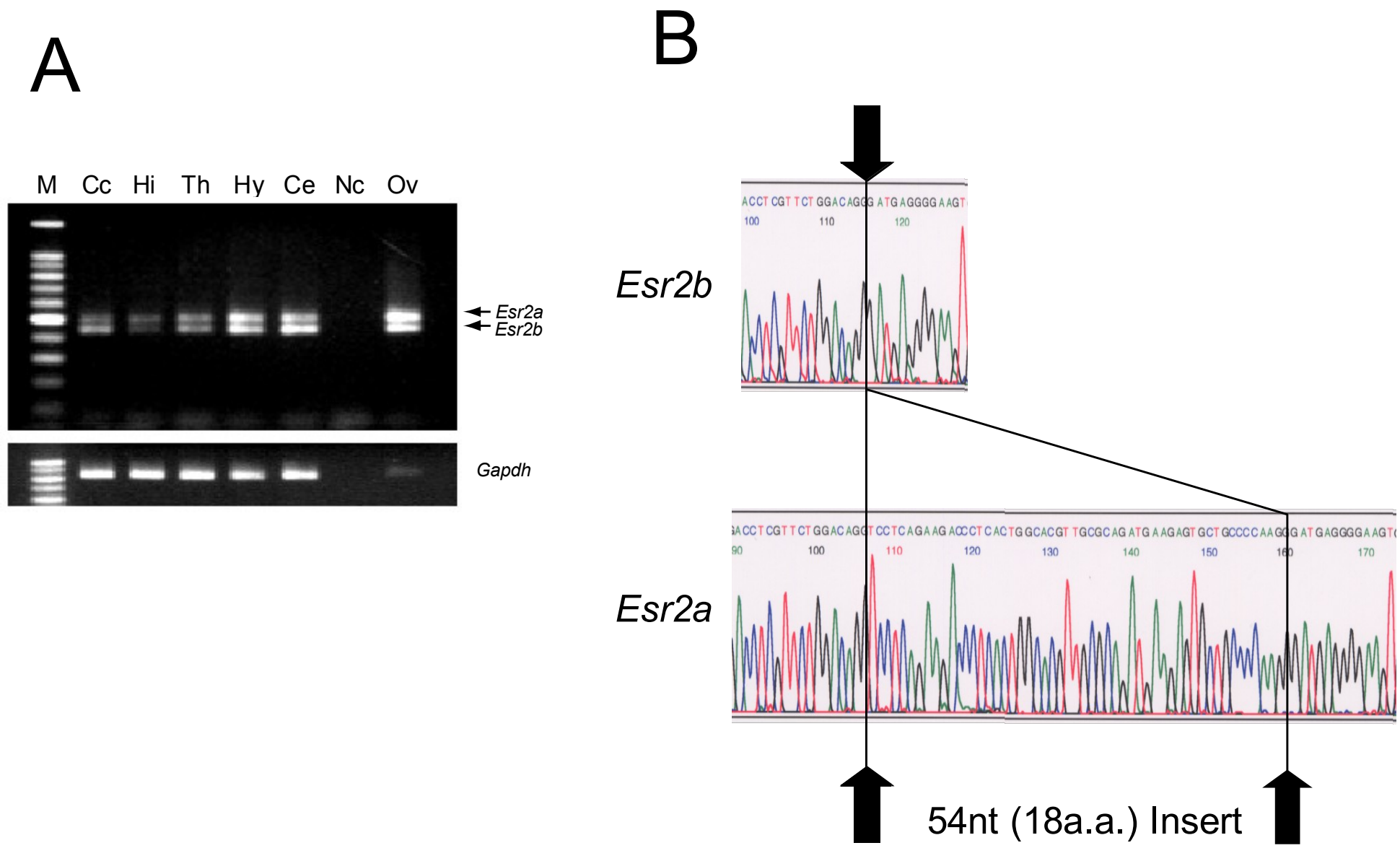
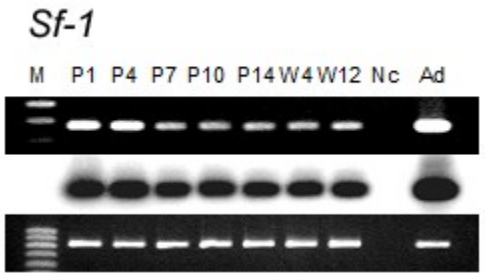
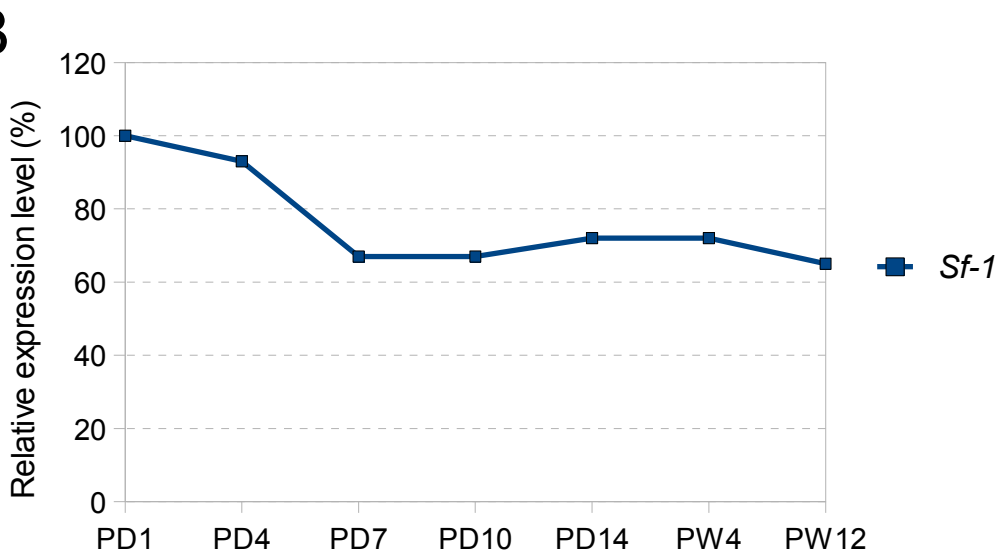

A
M
Cc
Hi
Th
Hy
Ce
Nc
Ov
Esr2a
Esr2b
Gapdh
B
Esr2b
Esr2a
54nt (18a.a.) Insert

Figure S3

A



B



C

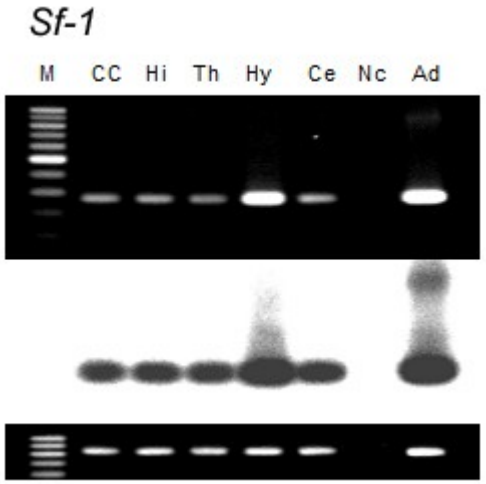
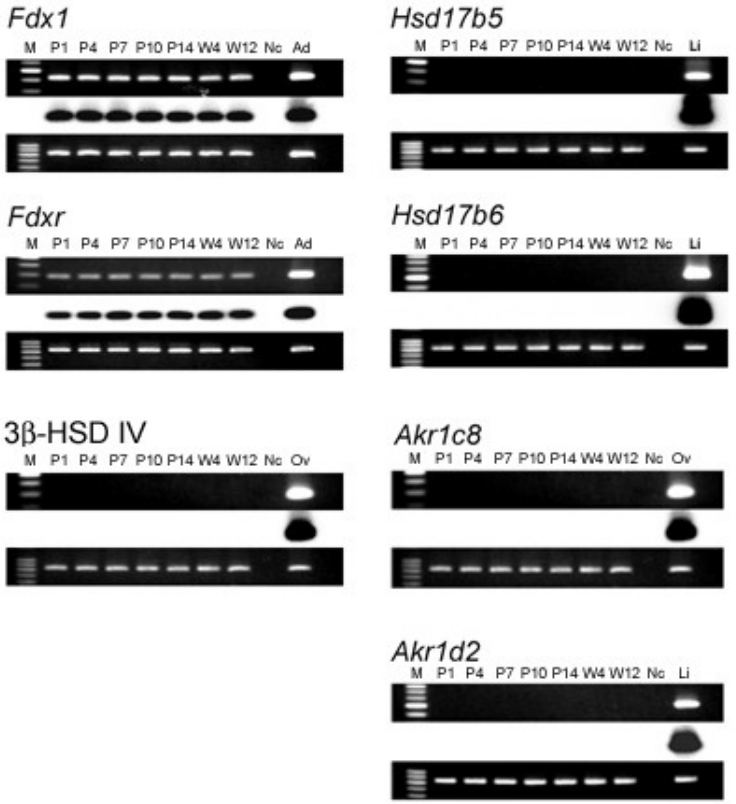


Figure S4


Fdx1
M P1 P4 P7 P10 P14 W4 W12 Nc Ad
Hsd17b5
M P1 P4 P7 P10 P14 W4 W12 Nc Li
Fdxr
M P1 P4 P7 P10 P14 W4 W12 Nc Ad
Hsd17b6
M P1 P4 P7 P10 P14 W4 W12 Nc Li
3β-HSD IV
M P1 P4 P7 P10 P14 W4 W12 Nc Ov
Akr1c8
M P1 P4 P7 P10 P14 W4 W12 Nc Ov
Akr1d2
M P1 P4 P7 P10 P14 W4 W12 Nc Li