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Comparison of sex-steroid synthesis between neonatal and adult rat hippocampus

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ABSTRACT

Sex-steroid synthesis in the hippocampus had been thought to be much more active at the neonatal stage than at the adult stage. However, the detailed comparison between these two stages had not been demonstrated yet. Here we performed the comparison about the mRNA level of steroidogenic enzymes and the rate of steroid metabolism between these two stages of the hippocampus. The relative expression level of P450(17α), 17β- or 3β-hydroxysteroid dehydrogenase, or P450arom was approximately 1.3–1.5-fold higher at the neonatal than at the adult stage. The rate of sex-steroid metabolism (from dehydroepiandrosterone to estradiol) was 2–7-fold (depending on different steps) more rapid at the neonatal than at the adult stage. Taken together, neonatal steroidogenesis is moderately more active than adult steroidogenesis.

Introduction

Neurosteroidogenesis has been thought to be transiently active at the fetal and neonatal stages and become inactive at the adult stage, because of very low expression of steroidogenic enzymes in the adult [1–3]. The distinct activity of steroidogenesis has been shown in primary cultured astrocytes, oligodendrocytes and neurons [4] as well as brain tissues [3,5]. Expression of cytochromes P450(17α), P450arom as well as 17β-hydroxysteroid dehydrogenase (17β-HSD) and 3β-HSD in the developmental brain has been demonstrated [6]. Relatively high level of sex-steroid content (1–10 nM) has been observed at the neonatal stages of the brain [7].

In recent years, increasing evidence has accumulated to support the significant adult neurosteroidogenesis or sex-steroidogenesis in the hippocampus [8–11]. These results are achieved by improvement of sensitivity of analysis, for example, better primer pair design for RT-PCR analysis. Therefore, the quantitative comparison of neurosteroidogenesis between neonatal and adult stages should be performed.

So far, the comparison between neonatal and adult hippocampus had not been well demonstrated about mRNA or protein level for steroidogenic enzymes. No direct comparison of sex-steroid production between neonatal and adult stages had been shown.

We here compare sex-steroid metabolism in male rat hippocampus between postnatal 10-day old (P10) and young adult stages. We also compare the expression level of mRNA for steroidogenic enzymes.

Materials and methods

Animals. Postnatal 10-day male Wistar rats (P10) and young adult rats (12 weeks old) were purchased from Saitama Experimental Animals Supply (Japan). All animals were maintained under a 12 h light/12 h dark exposure and free access to food and water. The experimental procedure of this research was approved by the Committee for Animal Research of University of Tokyo.

Chemicals. Estradiol (E2), testosterone (T), dihydrotestosterone (DHT), estrone (E1), dehydroepiandrosterone (DHEA), androstenedione (ADione), pregnenolone (PREG) and progesterone (PROG) were purchased from Sigma (USA). Finasteride was from Aska Pharma Medical. [^{3}H] or [^{14}C] labeled steroids were purchased

Abbreviations: ADione, androstenedione; ADiol, androstenediol; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; E2, estradiol; E1, estrone; PREG, pregnenolone; PROG, progesterone; T, testosterone

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from Perkin Elmer (USA) and their specific activities were 17.5 Ci/mmol ([7-^{3}H]-PREG), 60 Ci/mmol ([1,2,6,7-^{3}H]-DHEA), 95 Ci/mmol ([1,2,6,7-^{3}H]-T), 51 Ci/mmol ([1,2,6,7-^{3}H]-DHT), 65 Ci/mmol ([2,4,6,7-^{3}H]-E1) and 105 Ci/mmol ([1,2,6,7-^{3}H]-ADione). [1,2,6,7-^{3}H]-androstenediol (ADiol, 105 Ci/mmol) was synthesized in our group from [^{3}H]-DHEA by reduction with $NaBH_4$ in methanol at 4 °C.

RT-PCR and southern hybridization. Total RNAs including mRNAs of P450(17α), 17β-HSD (types 1 and 3), and 3β-HSD (types 1–4), P450arom, 5α-reductase (types 1 and 2) and 3α-HSD were isolated from P10 or adult rat tissues such as hippocampus, liver, ovary, prostate and testis, using a total RNA Purification Kit (Nippongene, Japan). The purified RNAs were quantified on the basis of the absorbance at 260/280 nm, and treated with RNase-free DNase to eliminate the possibility of genomic DNA contamination. The purified RNAs were reverse-transcribed, using a M-MLV Reverse Transcriptase (Promega, USA). The oligonucleotides for PCR amplification were designed as illustrated in Table 1. The PCR protocols comprised application of a 30 s denaturation period at 95 °C, a 20 s annealing period at individual temperature for each enzyme (see Table 1), and a 30 s extension at 72 °C, for individual number of cycles for each enzyme (see Table 1). For semiquantitative analysis, the RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide, and analyzed with a fluorescence gel scanner (Atto, Japan) and Image J software, in comparison with standard curves obtained from PCR of diluted RT products (between 1/100 and 1/10,000 in dilution), from liver, ovary, prostate or testis.

To confirm the expression, Southern hybridization was performed. The amplified RT-PCR products of steroidogenic enzymes were directly cloned into TA-cloning vector (Promega, USA), and sequenced. The resulting sequence was identical to the reported cDNA sequences of these enzymes. These cloned products were used as the template of DNA probes for Southern hybridization. After transfer of the RT-PCR products from agarose gels to nylon membrane (Hybond N+, Amersham, USA), Southern hybridization was performed with ^{32}P-labeled cDNA probes for these enzymes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The Southern hybridization signals were then measured using a BAS−1000 Image analyzer (Fuji film, Japan).

Metabolism analysis of radioactive steroids using normal phase HPLC. Procedures were essentially the same as previously described elsewhere [8,10] with slight modification. Hippocampi from two (for P10) or one (for adult) rats were sliced into 400 μm thickness with a vibratome and incubated with 5×10^6 cpm of [^{3}H]-steroids at 30 °C for 5 h in 4 ml of physiological saline containing 1.2 mM Mg^{2+} (0.6 mM $MgSO_4$, 0.6 mM $MgCl_2$, 137 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.36 mM KCl, 22 mM glucose, and 5 mM HEPES (pH 7.2)). The incubation medium was gassed with 95% O_2 and 5% CO_2 during the incubation in order to maintain the activity of hippocampal neurons. After termination of the reaction, the slices were homogenized. To extract steroid metabolites, ethyl acetate/hexane (3:2 vol/vol) was applied to the homogenates which were then mixed. The mixture was centrifuged at 1800×*g* and the organic layer was collected. After evaporation, the extracts were dissolved in 1 ml of 40% methanol/H_2O and applied to a C_{18} Amprep solid phase column (Amersham Biosciences, USA) to remove contaminating fats. The fraction of steroid metabolites was eluted with 5 ml of 40% methanol/H_2O (hydrophilic phase), and then eluted with 5 ml of 85% methanol/H_2O (hydrophobic phase), and combined together, in order to collect all the steroids. The combined organic extracts were dried, dissolved in an elution solvent of HPLC. To identify the steroid metabolites, [^{14}C]-steroids were added. The fractions were collected using a normal phase HPLC system (Jasco, Japan) with an elution solvent of hexane: isopropyl-alcohol: acetic acid = 98:2:1. A silica gel column (0.46 × 15 cm, Cosmosil 5SL, Nacalai Tesque, Japan) was used. Fraction radioactivity was measured using a liquid scintillation spectrometer LS6500 (Beckman, USA). [^{3}H]-steroid metabolites were identified by comparing with the retention time of [^{14}C]-steroids.

Immunohistochemical staining of hippocampal slices. Immunohistochemical staining was performed essentially as described elsewhere [8,10,12]. The hippocampi were frozen-sliced with a cryostat. After application of each antibody (anti-P450(17α) IgG at 1/1000 dilution [13], anti-P450arom IgG at 1/1000 [14]), the slices were incubated for 18–36 h. Biotinylated anti-rabbit IgG and streptavidin–horseradish peroxidase complex (Vector Laboratories, USA) was applied. Immunoreactive cells were visualized with diaminobenzidine-nickel.

Table 1
The sequences of primer oligonucleotides, the annealing temperature and the number of cycles for PCR amplification.

Target mRNA		Sequence	T (°C)	Number of cycles
P450(17α)	Forward	TGGGGCGGGCATAGAGACAACT	62	35
	Reverse	AGCAAGGCCGTGAAGACAAAGAGC		
17β-HSD (type1)	Forward	ACTCCGGGCGTGTGCTGGTGA	65	33
	Reverse	GGCGTGTCTGGATCCCCTGAAACTT		
17β-HSD (type3)	Forward	CTCCCCAACCTGCTCCCAAGTC	65	36
	Reverse	CAAGGCAGCACAGGTTTCAGC		
3β-HSD (type1)	Forward	AGGGCATCTCTGTTGTCATCCAC	62	40
	Reverse	TGCCTTCTCGGCCATCCTTT		
3β-HSD (type2)	Forward	ATCTCTGTTGTCATTCACACGGCTTC	62	40
	Reverse	CACTGCCTTCTCGGCCATCTT		
3β-HSD (type3)	Forward	CTTCCTCTGCCCCTGCTCTACTGG	62	40
	Reverse	GTCCCTGCCCTCTTCCCATCATTG		
3β-HSD (type4)	Forward	CTTCCTCTGCCCCTGCTCTACTGG	62	40
	Reverse	ATGTCCCTGCCCTCTTCCCATTAC		
P450arom[a]	Forward	CTGATCATGGGCCTCCTCCTG	58	37
	Reverse	CCCACGCTTGCTGCCGAATCT		
5α-reductase (type 1)	Forward	ACCGCGTCCTGCTGGCTATGTTT	63	28
	Reverse	GGCCTCCCCTGGGTATCTTGTATCC		
5α-reductase (type 2)	Forward	AGGTGGCTTGTTTACGTATGTCTCTG	57	34
	Reverse	GGCCTCTGTGAAGCTCCAAAAG		
3α-HSD	Forward	GGAATGTCACCTTTATCTCAACCA	55	34
	Reverse	ATGCATTCAGTCACCAGTATCCA		

[a] Primers for P450arm are designed to amplify only active form of P450arom.

Statistical analysis

Data were expressed as means ± SEM. An unpaired, two-tailed *t*-test, under the assumption of unequal variances, was utilized to test the significance of observed differences between groups. Several numbers of independent experiments from different animals were used to determine the parameters of *t*-distribution for the test.

Results

Comparison of the expression of steroidogenic enzymes between P10 and adult

The level of mRNA transcripts for enzymes which were required for synthesis of sex steroids (Fig. S1) was investigated using semi-quantitative RT-PCR analysis. Typical RT-PCR patterns of mRNA transcripts from P10 male rat hippocampus are shown in Fig. 1. Relative number of transcripts, expressed in P10 rat hippocampus, was approx. 1/200 of that in the testis for P450(17α), approx. 1/100 of that in the ovary for 17β-HSD (type 1), approx. 1/200 of that in testis for 17β-HSD (type 3), approx. 1/400 of that in the ovary for P450arom, approx. 1/5 of that in the liver for 5α-reductase (type 1), approx. 1/200 of that in the prostate for 5α-reductase (type 2), and approx. 1/200 of that in the liver for 3α-HSD. The expression level of 3β-HSD (type 1) was exceptionally very low, approx. 1/4000 of that in the ovary. The other subtypes of 3β-HSD (types 2, 3 and 4) were not detected (Fig. S3).

Comparison of the expression level of each mRNA between P10 and adult hippocampus is summarized in Table 2. The level of P450(17α), 17β-HSD (type 1), P450arom and 5α-reductase (type 2) was significantly higher in P10 than in adult. The level of these enzymes in P10 was approx. 130–150% of that in adult. No significant difference was observed for 5α-reductase (type 1), however, 3α-HSD in P10 was 228% of that in adult.

Table 2
Comparison of expression level of mRNA for hippocampal steroidogenic enzymes between P10 and adult male rats.

Enzyme	Relative expression level in P10[a]
P450(17α)	140 ± 12%[b,c,*]
17β-HSD (type 1)	141 ± 8%[*]
17β-HSD (type 3)	133 ± 11%
3β-HSD (type 1)	130 ± 13%
P450arom	153 ± 10%[*]
5α-reductase (type 1)	91 ± 5%
5α-reductase (type 2)	138 ± 10%[*]
3α-HSD	228 ± 18%[**]

[a] The expression level of mRNA in adult is normalized as 100%. The expression of mRNA was measured with the fluorescence intensity of the band stained with ethidium bromide as shown in Fig. 1. Six P10 rats and 6 adult rats were used.
[b] Data are expressed as means ± standard error.
[c] Statistically significant difference from the value of adult is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$.

Metabolisms of sex steroids in P10 hippocampus

The metabolism of radioactive steroids in hippocampal slices from P10 male rats was investigated using normal phase HPLC. Typical results of HPLC analysis are illustrated in Fig. 2 and the production rates are summarized in Table 3. When ^{3}H-PREG was used as a substrate, DHEA was produced as a major metabolite (Fig. 2A). When ^{3}H-DHEA was used as a substrate, androstenediol (ADiol) was produced (Fig. S2A). The application of RM352-26, a specific inhibitor of 17β-HSD (type 3) [15], at 40 μM considerably suppressed the production of ADiol down to approx. 25% of the control value. The conversion of ^{3}H-ADione to T was shown in Fig. 2B. Although T was produced from ^{3}H-ADione, the E1 production from ^{3}H-ADione was not observed (Fig. 2B). T was also produced from ^{3}H-ADiol. Both E2 and DHT were produced from ^{3}H-T (Fig. 2C). The application of finasteride, a specific inhibitor of 5α-reductase, at 10 nM considerably suppressed the production of DHT down to approx. 33% of the control value, indicating the participation of 5α-reductase. A significant inactivation of DHT, catalyzed by 3α-HSD, was observed, resulting in androstanediol production (Fig. 2D). When ^{3}H-E1 was used as a substrate, a significant production of E2 was observed (Fig. S2B).

In addition to identification via normal phase HPLC, further specific identification of the metabolites was performed using reverse phase HPLC for the steroid peaks which were collected from normal phase HPLC.

Comparison of the rate of steroid production between P10 and adult

Results are summarized in Table 3. No significant difference was observed in the conversion rate of DHEA to ADiol ($26–33 \times 10^{4}$ cpm/g/5 h) between P10 and adult hippocampus.

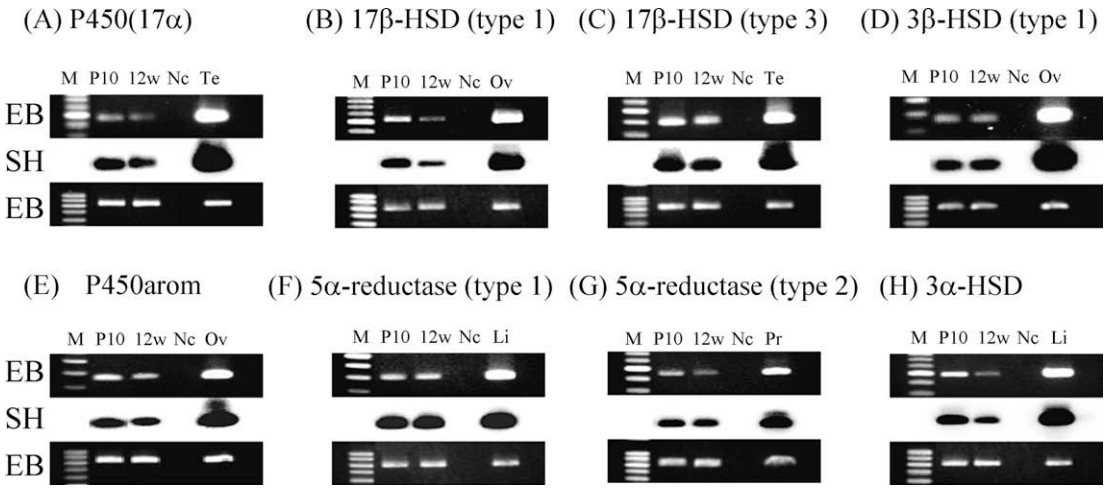

Fig. 1. RT-PCR analysis of steroidogenic enzymes in the P10 and adult (12-week old) male hippocampus. (A) P450(17α), (B) 17β-HSD (type 1), (C) 17β-HSD (type 3), (D) 3β-HSD (type 1), (E) P450arom, (F) 5α-reductase (type 1), (G) 5α-reductase (type 2), and (H) 3α-HSD. In each panel from left to right, size marker (M), P10 hippocampus (P10), adult hippocampus (12w), the sample without template DNA as negative control (Nc), and positive control. Testis (Te, 12w) for (A) and (C), ovary (Ov, 4w) for (B), (D) and (E), liver (Li, 12w) for (F) and (H), and prostate (Pr, 12w) for (G) were used as positive control samples. For each enzyme, the RT-PCR products for mRNAs were visualized with ethidium bromide staining (EB) on the top of each panel. Southern hybridization (SH) of cDNA is also shown on the middle of each panel. As an internal control, the ethidium bromide staining (EB) of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) is shown on the bottom of each panel. Total RNAs used were 200 ng for each enzyme.

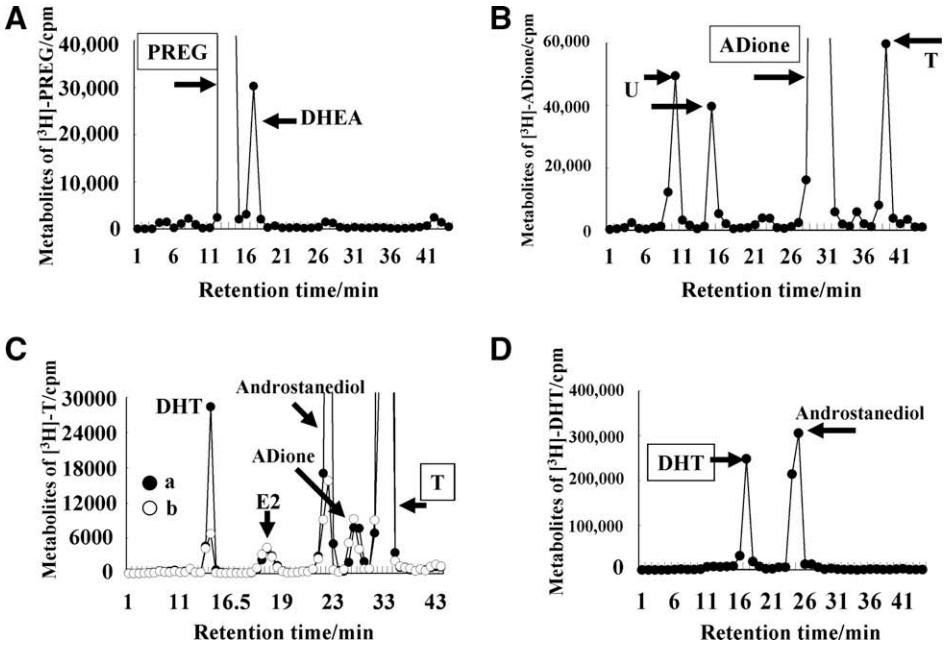

Fig. 2. Normal phase HPLC analysis of steroid metabolism in the hippocampal slices from P10 male rat. Hippocampal slices from the P10 male rats were incubated with 5×10^6 cpm of ^{3}H-labeled steroid substrate for 5 h. A portion of the purified radioactive metabolites (total of 10^6 cpm) was analyzed using an HPLC system. The rate of steroid production was normalized as products (cpm)/g wet weight/5 h. Closed rectangle indicates the steroid used as substrate. Others indicate products from substrate. The elution peak positions (arrows) were calibrated with standard 14C-steroids. (A) HPLC profiles of ^{3}H-PREG metabolites. (B) HPLC profiles of ^{3}H-ADione metabolites. U indicates unknown product. As judged from the retention time, U (10 min) and U (15 min) were not E1 (13 min). (C) HPLC profiles of 3H-T metabolites. Hippocampal slices were incubated in the absence of an inhibitor (line a) or in the presence of 10 nM of finasteride, a specific inhibitor of 5α-reductase (line b). (D) HPLC profiles of 3H-DHT metabolites. The retention time of the (same) standard 14C-steroid differed between each panel, due to the different elution experiments using the different silica gel columns. The vertical axis indicates ^{3}H radioactivity (cpm). More than three independent experiments were performed for each of these analyses.

The conversion rate of ADiol to T was very slow for both P10 (approx. 3×10^4 cpm/g/5 h) and adult (9×10^4 cpm/g/5 h). The rate of production of E2 from T was 7-fold more rapid in P10 (14×10^4 cpm/g/5 h) than in adult (2×10^4 cpm/g/5 h). Compared with E2 production, the production rate of DHT from T was more rapid in both P10 (142×10^4 cpm/g/5 h) and adult (133×10^4 cpm/g/5 h). The inactivation of DHT into androstanediol was very rapid, with approx. 3.6-fold difference between P10 (647×10^4 cpm/g/5 h) and adult (180×10^4 cpm/g/5 h).

Localization of P450 in P10 hippocampus

The localization and presence of P450(17α) and P450arom in the hippocampus of P10 male rats were confirmed by immunohistochemical staining. P450(17α) and P450arom were localized in pyramidal neurons in CA1-CA3 region as well as in granule cells in dentate gyrus (DG) (Fig. 3). The staining of glial cells was much weaker than that of neurons. The staining pattern in P10 hippocampus was nearly the same as that of adult.

Discussion

In the current study, the extensive comparison of the steroid productions between P10 and adult rats is performed. The difference in the rate of metabolism was less than 7-fold in all the metabolism steps analyzed. These results imply that sex-steroid synthesis is not transiently active at the fetal and neonatal stages, but the steroidogenic capacity does not decline considerably even at the adult stage.

The rate of metabolism for both androgens (T → DHT → androstanediol) and estrogen (T → E2) in P10 hippocampus was estimated to be roughly 2–7-fold higher than that in adult (Table 3). Interestingly, the rate of metabolism of androgens (T → DHT → androstanediol) was roughly 10–90-fold higher than that of estrogen (T → E2) in both P10 and adult hippocampus (Table 3). As already shown in our previous work, E2 was not significantly inactivated within 5 h [8]. These results suggest that E2 is produced very slowly but stably present, whereas DHT is produced and inactivated much more rapidly than E2.

Table 3
Comparison of production rate of steroids between P10 and adult hippocampus of male rats.

Metabolism (substrate → product)	Enzyme	Production rate (10^4 cpm /g/5 h)[a,b]	
		P10	Adult
PREG → DHEA	P450(17α)	37.3 ± 7.1[*,c,d]	27.9 ± 2.7
DHEA → ADiol	17β-HSD (types 1 and 3)	26.3 ± 3.7	32.8 ± 4.9
ADiol → T	3β-HSD (type 1)	2.8 ± 0.3[**]	9.0 ± 1.5
T → E2	P450arom	13.8 ± 3.4[*]	2.3 ± 0.1
T → DHT	5α-reductase (types 1 and 2)	142.1 ± 40.1	133.0 ± 10.9
DHT → androstanediol	3α-HSD	647.3 ± 43.9[*]	180.0 ± 13.9
ADione → T	17β-HSD (types 1 and 3)	27.6 ± 4.7[*]	54.2 ± 5.3
E1 → E2	17β-HSD (types 1 and 3)	146.5 ± 7.4[**]	25.5 ± 1.4

[a] The production rate is given by: (Count of products from 10^6 cpm of substrate) [cpm]/(incubation time) [5 h] × (hippocampal wet weight) [g].
[b] Number of experiments was between 3 and 5. Hippocampi from one (for adult) or two (for P10) rats were used in each experiment.
[c] Data are expressed as means ± standard error.
[d] Statistical significance $^*P < 0.05$, $^{**}P < 0.01$, when compared of production rate of each steroid between P10 and adult.

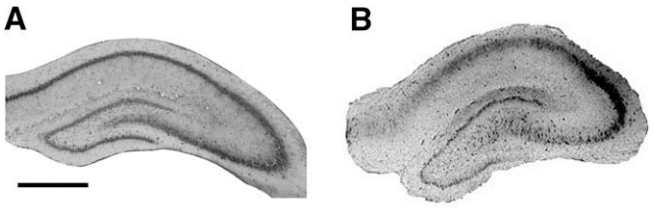

Fig. 3. Immunohistochemical staining for P450(17α) (A) and P450arom (B) in the hippocampus of P10 male rat. The coronal section of the whole hippocampus was used. Scale bar, 800 μm.

The observed significant production of androstanediol is interesting. Androstanediol is not merely an inactivated DHT but a physiologically very important steroid as a modulator of $GABA_A$ receptors. Androstanediol exerts protective effects on mice versus seizures induced by $GABA_A$ receptor antagonists, such as pentylenetetrazol, picrotoxin, and beta-carboline ester [16].

Interestingly, though the rate of steroid metabolism was several fold higher in P10 than in adult, the mRNA expressions of several steroidogenic enzymes (e.g., 3β-HSD, 17β-HSD, P450arom, P450(17α) and 5α-reductase (type 2)) were only 1.3–1.5-fold higher in P10 than in adult (Table 2). The protein level of these enzymes might be significantly higher in P10 than in adult (see Fig. S4 for P450arom). However, a quantitative comparison of protein level with Western blot is very difficult, due to extremely low level expression of these proteins as well as due to not adequate specificity of antibody presently available.

Comparison of mRNA levels for steroidogenic enzymes between neonatal and adult hippocampus is demonstrated only in several works. Concerning the mRNA level for P450arom, postnatal 7-day old (P7) male rat hippocampus has 2-fold higher expression than that in adult [17]. Hippocampal P450arom is expressed during P6 and P15 stages and disappeared in adult via in situ hybridization [18]. The mRNA level of 3β-HSD is 2–3-fold higher in P7 and P14 hippocampus than in adult [19]. In the cerebellum, 3β-HSD is expressed transiently during P7 and P14 and disappears in adult [3]. We observed much higher expression of mRNAs for steroidogenic enzymes in adult [8,10,12,20]. The reason why we are able to detect the higher expression level for steroidogenic enzymes in the adult hippocampus, higher than previous reports, is due to (i) an improvement of primer pairs design to increase the sensitivity and specificity for hybridization processes in PCR, by calculating the reduction of Gibbs free energy upon hybridization, (ii) considerable improvement in the last several years of the commercially available enzymes such as Taq polymerase used for RT-PCR, and (iii) the use of isolated fresh rat hippocampus, rather than the frozen whole brain stored for many days [8,20].

Which is the site of steroidogenesis, neurons or glial cells? In adult hippocampus, steroidogenic enzymes (such as steroidogenic acute regulatory protein (StAR), P450scc, P450(17α) and P450arom) are mainly expressed in glutamatergic principal neurons [8,10,12,21]. The current immunohistochemical study demonstrated that also in P10 hippocampus, P450(17α) and P450arom were primarily expressed in glutamatergic principal neurons, and that they were expressed weakly in glial cells (Fig. 3).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.005.

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