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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.