Neurosteroid Synthesis in Adult Female Rat Hippocampus, Including Androgens and Allopregnanolone

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Abstract

Female rat hippocampus synthesizes significant amount of estrogens, including progesterone (PROG), estrone and estradiol (E2). Hippocampal level of PROG and E2 are considerably higher than those in plasma. Female hippocampal estrogens play a significant role in the fluctuation of dendritic spine density across the estrous cycle. Here we extend the study to the investigation of female androgens, including testosterone (T) and Dihydrotestosterone (DHT), in the female rat hippocampus, since female androgens had been largely known. By combination of mass-spectrometric analysis with HPLC-purification and picolinoyl-derivatization of sex steroids, we determined the accurate concentration of T and DHT in the hippocampus. The levels of T and DHT in female hippocampus at Proestrus were approximately 1.1 nM and 0.6 nM, respectively, suggesting a significant synthesis of T and DHT. The level of plasma T was approx. 0.1 nM, implying almost no contribution of plasma T to hippocampal T. The concentration of hippocampal DHT had a good correlation with that of hippocampal T, suggesting a significant activity of 5α-reductase (DHT synthase) in the female. Allopregnanolone level was also determined as a useful indicator of 5α-reductase activity. Interestingly mRNA expression level of 5α-reductase and androgen receptor (AR) was not significantly different between the different estrous cycle stages, or between female and male. Nevertheless, sex difference existed with respect to the levels of T, DHT and Allo in hippocampus. Although physiological significance of female hippocampal androgens awaits further investigations, the female hippocampus produces T or DHT which may be useful to suppress anxiety, for example.

Keywords: Dihydrotestosterone; Female hippocampus; Androgens; Testosterone; Allopregnanolone

Abbreviations: Allo: Allopregnanolone; AR: Androgen Receptor; D1: Diestrus 1; D2: Diestrus 2; DHT: Dihydro Testosterone; E2: Estradiol; ER: Estrogen Receptor; Est: Estrus; LC-MS/MS: Liquid Chromatography with Tandem-Mass-Spectrometry; Pro: Proestrus; PROG: Progesterone; PFBz: Pentafluorobenzoxy; STAR: Steroidogenic Acute Regulatory Protein; T: Testosterone

Introduction

In female hippocampus, synthesis and function of androgens have not been well investigated, although estrogens have been extensively studied about synthesis and function. We recently demonstrated that female rat hippocampus synthesizes 17β-estradiol (E2), progesterone (PROG) and estrone [1], which is similar to hippocampus-synthesized steroids in male [2-5]. The estrous cycle-dependent fluctuation of the spine density in female rat hippocampus had a good correlation with the cyclic fluctuation of hippocampal E2 or PROG level [1]. Hippocampal testosterone (T) level in female rats was almost as high as hippocampal E2 level (~1 nM) [1]. The level of Dihydrotestosterone (DHT), the most potent androgen, in female hippocampus, however, remains to be undetermined, although one study reported the presence of very low level DHT (the numerical concentrations were not written) with radioimmunoassay (RIA) [6]. We should determine the accurate concentration of DHT by improvement of the sensitivity of measurements in female hippocampus, since androgen application to the aged female hippocampus decrease a depression-like behavior of aged mice [7]. Concerning the steroid synthesis systems in female rat hippocampus, mRNA for T synthesis including P450 (17α) and 17β-HSD (type 1 and 3) are expressed [1]. It remains unclear whether 5α-reductase (DHT synthase) and androgen receptor (AR) are expressed in female hippocampus, although one report showed 5α-reductase (type 2) expression with in situ hybridization in female mice brain [8]. In male rat hippocampus, steroidogenic enzymes and steroid receptors are mainly localized in pyramidal neurons in CA1 and CA3, and granule neurons in dentate gyrus [2,4,9-12]. Their neuronal localization of female should be clarified.

5α-reductase produces not only DHT from T, but also allopregnanolone [13] from PROG. Concentration of Allo has been demonstrated using RIA [6,14], GC-MS [15,16] and liquid chromatography with tandem-mass-spectrometry (LC-MS/MS) [17]. Although Allo had been extensively investigated with regard to its anxiolytic and anti-depressive effects [18,19] via modulation of gamma-aminobutyric acid A (GABAA) receptors [20], the physiological significance of DHT in female hippocampus is still unknown and should be investigated. We here examine whether DHT synthase or AR exists in female rat hippocampus. Localization of P450 (17α), steroidogenic acute regulatory protein (STAR) and AR in female hippocampus is also examined with Immunohistochemical

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staining. By combination of mass-spectrometric analysis with HPLC-purification and picolinoyl-derivatization of steroids [1,10,21], the accurate concentration of hippocampal DHT and T is determined. Allo in female hippocampus is determined to compare with male Allo [6,14-17]. By comparison of hippocampal androgens and Allo between in male and female, we clarified the profile of steroid synthesis in female rat hippocampus, which is different from male.

Materials and Methods

Animals

Wistar rats (10 weeks old) were purchased from Saitama Experimental Animals Supply (Japan). The estrous cycle of female rats was monitored with morning vaginal smears. Only those rats showing three consecutive 4-day cycles of Pro, Est, D1 and D2 were used at the age of 12 weeks old. Ovariectomy (OVX) and sham operations were performed two weeks before (at 10w old) the experiments. Male rats were also used at the age of 12 weeks old. 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 the University of Tokyo.

Chemicals

T and DHT were purchased from Sigma [22]. Picolinic acid was from Tokyo Chemical Industry (Japan) and, [1,2,6,7-3H]-T, [1,2,6,7-3H]-DHT and Allo-d4, were from CDN Isotope Inc. (Canada). [3H] labeled steroids ([1,2,6,7-3H]-T, [1,2,6,7-3H]-DHT and [1,2,6,7-3H]-Allo) were purchased from Perkin Elmer [22].

RT-PCR

The detailed procedures of mRNA analyses are described elsewhere [2,4,23]. Total RNAs were isolated from the hippocampus adrenal gland, liver, prostate and testes of adult rats, using a SV total RNA Isolation System (Promega, USA). The purified RNAs were treated with RNase-free DNase to eliminate the possibility of genomic DNA contamination, and quantified on the basis of the absorbance at 260/280 nm. The purified RNAs (100 ng) were reverse-transcribed to obtain cDNAs, using a M-MLV Reverse Transcriptase (Promega, USA). PCR was performed by using these cDNAs. The oligonucleotides for PCR amplification were designed as illustrated in Table S1. 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, and a 30 s extension at 72°C, for individual number of cycles for each enzyme (Table S1). For semi-quantitative 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 all the cases we first plotted amplification curves in order to choose the linear phase of PCR cycles. The comparison between T and DHT 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 5SIL, Nacalai Tesque, Japan) was used. By monitoring 3H-steroids, the recoveries of T, DHT and Allo were 41±7%, 29±5% and 50±8% respectively, after extraction, C4 column treatment and normal phase HPLC separation. As internal standards, 100 pg of isotope labeled steroids ([12C2]-T, [12C2]-DHT and Allo-d4) were added to steroid extracts.

Step 2: Derivatization of HPLC-purified steroids before application to LC (reverse 185 phase)-MS/MS.

Preparation and purification of T-17-picolinoyl-ester, DHT-17-picolinoyl-ester and Allo-3-picolinoyl-ester were performed with slight modification of previous methods [1,21]. For preparation of T-17-picolinoyl-ester, DHT-17-picolinoyl-ester and Allo-3-picolinoyl-ester, evaporated steroid extracts from the hippocampus or plasma were reacted with 50 µL of picolinic acid suspension (4% 191 picolinic acid, 4% of 4-dimethylamino.pyridine, 2% 2-methyl-6-nitrobenzoic anhydride in tetrahydrofuran anhydrous) for 0.5 h at room temperature. The reaction products dissolved in 1% acetic acid in 2 mL of tetrahydrofuran and 20 µL of triethylamine, for 0.5 h at room temperature. The reaction products dissolved in 1% acetic acid were purified using a Bond Elute C18 column (Varian, USA). The dried sample was dissolved in elution solvent of LC. The reaction products were purified with the C4 column by using 80% acetonitrile. The purified steroid-derivative was dissolved in elution solvent of LC.

Step 3: Determination of the concentration for T and DHT and Allo using LC-MS/MS.

For determination of the concentration of T and DHT, the LC-MS/MS system, which consists of a Shimadzu HPLC system and an API-5000 triple stage quadrupole mass spectrometer (Applied Biosystems, USA) were employed. LC chromatographic separation was performed on a Kinetex C18 column (2.1×150 mm, 1.7 µm, Phenomenex, USA) for T-picolinoyl ester and DHT-picolinoyl ester. For determination of the concentration of Allo, LC chromatographic separation was performed on a preparation column (Unison UK- Phenyl HT (3 µm, 2.0×50 mm, Intact, Japan)) and an analytical column (Cap cell core C18 (2.7 µm, 2.1×100 mm, Shiseido Japan)). After elution of Allo-picolinoyl-ester from the preparation column, Allo-picolinoyl-ester was subsequently loaded to the analytical column. The preparation column enables us to separate Allo (3a-hydroxy-5α-pregn-20-one) from isoforms with equivalent molecular weight (3a-hydroxy-5β-pregn-20-one, 3b-hydroxy-5α-pregn-20-one and 3b-hydroxy-5β-pregn-20-one). MS analysis was operated with electro spray ionization [25] in the positive-ion mode. The isotope-labeled steroid derivatives were
Hippocampi were postfixed, cryo-protected, and frozen-sliced coronally. Animals were deeply anesthetized and perfused transcardially with PBS (0.1 M phosphate buffer and 0.14 M NaCl, pH 7.3), followed by fixative solution (4% paraformaldehyde/PBS). The hippocampal tissue, respectively (Table S2). From the calibration curve quantification were 1 pg for T and DHT, and 0.5 pg for Allo per 0.1 g of samples, prepared alongside hippocampal samples through the whole extraction, fractionation and purification procedures. The limits of quantification for steroids were measured with blank samples, prepared alongside hippocampal samples through the whole extraction, fractionation and purification procedures. The limits of quantification were 1 pg for T and DHT, and 0.5 pg for Allo per 0.1 g of hippocampal tissue, respectively (Table S2). From the calibration curve using standard T or DHT dissolved in blank samples, the linearity was observed between 1.0 pg and 2000 pg for T and DHT, between 5 pg and 1000 pg for Allo, respectively (Figure S2).

**Immunohistochemical staining of hippocampal slices**

Detailed procedures are described elsewhere [2,4,10,11]. Hippocampal slices were prepared from 12 week-old female rats at Proestrus stage. Animals were deeply anesthetized and perfused transcardially with PBS (0.1 M phosphate buffer and 0.14 M NaCl, pH 7.3), followed by fixative solution (4% paraformaldehyde/PBS). The hippocampi were postfixed, cryo-protected, and frozen-sliced coronally with a cryostat (CM1510, Leica). Staining for steroidogenic enzymes (P450 (17a), P450arom and StAR) and receptors (AR and ERα) was performed with the avidin-biotin-peroxidase complex technique. Dilution of primary antibody was 1:1000 each for purified anti-guinea pig cytochrome P450(17a) IgG [26], purified anti-human P450arom IgG [27], anti-mouse StAR IgG [28], anti-AR IgG (PG-21, Millipore, USA), or purified anti-rat C terminal of ERα antibody (RC-19) [12]. After application of primary antibody, the slices were incubated for 18 h at 4°C, in the presence of 0.5% Triton X-100. Biotinylated anti-rabbit IgG (1:1000) and streptavidin-horseradish peroxidase complex (Vector Laboratories) was applied. Immuno-reactive cells were detected with a solution of diaminobenzidine with ammonium nickel sulfate. After embedding in Entellan Neu (Merck, Germany), the immuno-reactive cells were observed under microscope.

**Statistical analysis**

Data are expressed as mean ± SEM. For comparison of the mRNA level of steroidogenic enzymes and receptors, we used a 1-way ANOVA followed by Tukey-Kramer posthoc multiple comparisons. For comparison of the concentration of T or DHT in the hippocampus between male and female, we used Student’s t-test. A difference was considered significant at a value of *p<0.05, **p<0.01 or ***p<0.001.

**Results**

Molecular biological analysis of steroidogenic enzymes and steroid receptors in the female hippocampus

The hippocampal expression of steroidogenic enzymes (StAR, 5α-reductase (Types 1 and 2)) and AR were examined across the estrous cycle. No significant change in expression levels of mRNAs for sex steroidogenic enzymes and receptor (A: 5α-reductase type1, B: 5α-reductase type2, C: AR and D: StAR) in the female hippocampus across the estrous cycle. Upper panels show representative PCR images and lower panels show statistical comparisons. In each image, from left to right, size marker (100 bp ladder) (M), male hippocampus (male), female hippocampus at Pro (P), Est (E), D1 (D1), D2 (D2), and OVX female rats (OVX), the sample without template DNA as negative control (Nc). Each enzyme, the RT-PCR products for mRNAs are visualized with ethidium bromide staining on the top of each panel. As an internal control, the ethidium bromide staining of GAPDH is shown on the bottom of each panel. PCR was performed by using cDNA made by reverse transcription from 100 ng of hippocampal total RNA. Statistical comparisons show no estrous cycle-dependent changes of mRNA expression for sex-steroidogenic enzymes. The vertical axis indicates the expression level for each enzyme calculated from the intensity of EB bands. Each value is mean ± S.E.M. Data are taken from duplicate determinations for each rat of total 4 rats.
cycle. Expression levels of mRNA transcripts normalized by GAPDH are shown as bar graphs in Figure 1. Surprisingly, no significant cyclic fluctuations across the estrous cycle were observed for their expression levels. OVX did not change the expression level of any steroidogenic enzyme and AR. Relative number of transcripts, expressed in the hippocampus of adult female rats, was approx. 1/3 of that in the liver for 5α-reductase type 1, approx. 1/300 of that in the male prostate for 5α-reductase type 2, approx. 1/10 of that in the male prostate for AR, and approx. 1/100 of that in the adrenal for STAR (Table 1).

Mass-spectrometric determination of androgen and allopregnanolone levels in the female hippocampus

The concentration of T, DHT and Allo was determined for adult female and male rat hippocampus using a chromatogram analysis of the fragmented ions of steroid-derivatives. We chose the hippocampus at Proestrus stage for steroid determination since RT-PCR analysis revealed no estrous cycle-related fluctuation of the expression for 5α-reductase (types 1 and 2) which is required for synthesis of DHT and Allo. Results are summarized in Figures 2 and 3. T, DHT and Allo were derivatized with picolinoyl before application to LC-MS/MS to increase the accuracy of determination by improving the limit of quantification (LOQ) (Table S2).

In the chromatographic profiles of the fragmented ion of T-17-picolinoyl-ester, DHT-17-picolinoyl-ester and Allo-3-picolinoyl-ester, a single clear peak was observed at 4.12, 4.37 and 8.43 min, respectively (Figure 2A1, 2B1 and 2C1). For these steroids, the retention time of the observed steroid peak was the same as that of standard steroid. To confirm the assay accuracy, the hippocampal homogenate spiked with known amounts of the steroids was prepared and its concentration of steroid was determined (Table S3). The LOQs were defined in Table S2 as the lowest value with an acceptable accuracy (90-110%) and precision (i.e. RSD<10%). The results of intra- and inter-assay were shown in Table S2. The RSD for intra- and inter-assay was less than 5.1% and 7.2%, respectively.

Hippocampal level of androgens in female were 1.1 nM for T and

![Figure 2](mass-spectrometric-analysis-of-hippocampal-sex-steroids-12-week-old-female-rats.-lc-msms-ion-chromatograms-of-t-a-dhtb-and-alloc-c-a1-b1-and-c1-represent-the-chromatograms-of-the-fragmented-ions-of-each-steroid-from-the-hippocampus.-shaded-portions-indicate-the-intensity-of-fragmented-ions-of-t-mz=253,-dht-mz=203-and-allo-mz=283.3,-respectively.-a2-b2-and-c2-represent-the-chromatograms-of-the-fragmented-ions-of-the-standard-steroids.-the-vertical-axis-indicates-the-intensity-of-the-fragmented-ion-the-horizontal-axis-indicates-the-retention-time-of-the-fragmented-ion-n=4.12-min-for-t-4.37-min-for-dht-and-8.43-min-for-allo.-the-time-of-sample-injection-to-lc-system-was-defined-as-t=0-min.-note-that-pre-purification-step-using-normal-phase-hplc-before-injection-to-lc-system-is-very-important-to-achieve-high-precision-and-good-reproducibility-of-lc-msms-determination-in-order-to-avoid-contamination-of-other-steroids-and-fats.-steroid-derivatives-or-steroids-were-further-separated-with-reversed-phase-lc-column-before-ms/ms.-in-the-multiple-reaction-monitoring-modes,-the-instrument-monitored-the-m/z-transition-table-s2)
The current study revealed that female hippocampus is able to synthesize androgens (T and DHT) in addition to estrogens. In female, hippocampal T (~1 nM) is much higher (nearly 10-fold) than plasma T (~0.1 nM) [1], implying that the contribution of plasma T to hippocampal T is very weak. This is very different from male in which 70-80% of hippocampal T is derived from plasma T [21]. Since female plasma DHT would be lower than plasma T (~0.1 nM), plasma DHT cannot contribute to the production of hippocampal DHT (~0.6 nM) in female. Majority of female hippocampal DHT should be therefore synthesized from hippocampal-synthesized T. Although male hippocampus synthesizes androgens and estrogens [2,11,21,33,34], sex difference exists in the level of hippocampal steroids. The hippocampal level of androgens (T and DHT) is significantly high in female than in male [4]. These results suggest that effects by sacrifice-stress on the hippocampus may not be serious.

Sex difference in hippocampal androgen and allo levels

The localization and presence of P450 (17α), P450 arom and 17β-HSD (T synthase) also exhibited neither sex difference nor estrous cycle-dependent fluctuation [1], suggesting the constant catalytic activity for steroids in the hippocampus. It may be possible that the localization of enzymes or receptors is sex different even with the equal level of mRNA expression. Therefore, we investigated the localization of enzymes including P450 (17α) and P450arom or receptors including AR and ERα in female hippocampus with immunohistochemistry in current study, resulting in the same pattern as male (Figure 4) [2,4,11,12]. These results suggest that hippocampal synthesis activity of androgen or allo is independent of sex. In fact, 5α-reductase activity was almost equal between male and female from the data with high correlation between hippocampal T and hippocampal DHT (Figure 3C). All data of hippocampal T (x-axis) and DHT (y-axis) from both sexes were aligned in a straight line with high correlation (Figure 3C), implying that DHT production was only dependent on substrate T concentration. Here we present analysis only at Proestrus stage, and further analysis at other estrus stages may help better understanding. In addition, we also measured corticosterone (CORT) level in the female hippocampus, and CORT was not considerably elevated upon sacrifice or anesthetics treatment.

In order to compare hippocampal levels of steroids with plasma steroids, we converted ng/g wet weight to nM concentration via the following estimation (Table 2). First, 1 mL of plasma (93% is water) is assumed to have 1 g weight, as 1 mL of water has 1 g weight. Second, we assume that the hippocampal tissue having 1 g of wet weight has an approx. volume of 1 mL, as nearly 78% of the brain tissue consists of water [29]. Consideration of specific volume of protein and lipids (0.7-1.0 mL/g) in the brain further support this assumption [11,30,31]. After dividing by the individual hippocampal volume (0.124 ± 0.002 wet weight for one whole hippocampus of 12 weeks old female rat, n=44), the levels of T, DHT, Allo in the hippocampus were calculated.

Based on these considerations, 0.18 ng/g wet weight of DHT in the hippocampus at Pro corresponds to 0.62 nM (Table 2).

**Localizaton of steroidogenic enzymes and receptors in female hippocampus**

The localization and presence of P450 (17α), P450arom and StAR in the hippocampus of adult female rats were demonstrated by Immunohistochemical staining. P450 (17α), P450arom and StAR were mainly localized in pyramidal neurons in CA1-CA3 region as well as granule cells in dentate gyrus [9] (Figure 4A-4C). Immuno staining of glial cells for these enzymes was much weaker than that of principal neurons. In addition to steroidogenic enzymes, we also demonstrated the localization for AR and ERα in hippocampal slices of female. AR and ERα also localized in pyramidal and granule neurons (Figures 4D and 4E). It should be noted that AR immuno reactivity was the most prominent at CA1pyramidal neurons, whereas region difference in staining pattern of ERα was not observed. The staining pattern of steroidogenic enzymes and receptors in female hippocampus was almost the same as that of male hippocampus [2,4,11,12,32].

**Discussion**

No significant sex difference in hippocampal steroidogenic systems (mRNA and protein)

Surprisingly, the expression levels of sex steroidogenic enzymes including 5α-reductase (types 1 and 2) and StAR were not significantly different between female and male hippocampus (Figure 1). Furthermore, no cyclic fluctuation of expression levels of these enzymes was observed across the estrous cycle. Other steroidogenic enzymes which are required for androgen or estrogen synthesis including P450 (17α), P450arom and 17β-HSD (T synthase) also exhibited neither sex difference nor estrous cycle-dependent fluctuation [1], suggesting the constant catalytic activity for steroids in the hippocampus. It may be possible that the localization of enzymes or receptors is sex different even with the equal level of mRNA expression. Therefore, we investigated the localization of enzymes including P450 (17α) and P450arom or receptors including AR and ERα in female hippocampus with immunohistochemistry in current study, resulting in the same pattern as male (Figure 4) [2,4,11,12]. These results suggest that hippocampal synthesis activity of androgen or allo is independent of sex. In fact, 5α-reductase activity was almost equal between male and female from the data with high correlation between hippocampal T and hippocampal DHT (Figure 3C). All data of hippocampal T (x-axis) and DHT (y-axis) from both sexes were aligned in a straight line with high correlation (Figure 3C), implying that DHT production was only dependent on substrate T concentration. Here we present analysis only at Proestrus stage, and further analysis at other estrus stages may help better understanding. In addition, we also measured corticosterone (CORT) level in the female hippocampus, and CORT was not considerably elevated upon sacrifice or anesthetics treatment.

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steroids for synthesis of sex steroids in the hippocampus may be PROG (precursor of Allo) originally produced by ovary or adrenal gland (20-50 nM in plasma) in female, and T (precursor of DHT) from testis (~15 nM) in male, respectively [21,1]. Almost all hippocampal androgens (T and DHT) in female were hippocampus-synthesized from PROG, because the level of plasma T (~0.1 nM) in female was much lower than that of hippocampal T [1].

**Earlier studies on female androgens in the brain**

The concentration of T and DHT in adult female hippocampus had not yet been accurately determined in previous studies. Although one study reported the presence of androgens with RIA method [6], the numerical concentrations were not written due to very low levels. On the other hand, a significant concentration of Allo has been demonstrated using RIA [6,14], GC-MS [15,16] and LC-MS/MS [17]. The concentration of Allo was approximately 6 ng/g (~20 nM) in female hippocampus at Proestrus [6,14]. On the other hand Allo was very low approximately 0.4 ng/g (~1 nM) in male adult whole brain [15,16] or undetectable in male cultured hippocampus. These concentrations are qualitatively similar to those observed in the current study. Compared to male, physiological role of androgens in female hippocampus is not clear. One of the possible physiological roles might be suppression of depression, since supplementation of androgens in female decreased...
a depression-like behavior of aged mice [7]. Extensive studies on physiological roles of female androgen should be needed.

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