# Comparison between Hippocampus-Synthesized and Circulation-Derived Sex Steroids in the Hippocampus

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Estradiol (E2) and other sex steroids play essential roles in the modulation of synaptic plasticity and neuroprotection in the hippocampus. To clarify the mechanisms for these events, it is important to determine the respective role of circulating *vs.* locally produced sex steroids in the male hippocampus. Liquid chromatography-tandem mass spectrometry in combination with novel derivatization was employed to determine the concentration of sex steroids in adult male rat hippocampus. The hippocampal levels of 17$\beta$-E2, testosterone (T), and dihydrotestosterone (DHT) were 8.4, 16.9, and 6.6 nM, respectively, and these levels were significantly higher than circulating levels. The hippocampal estrone (E1) level was, in contrast, very low around 0.015 nM. After castration to deplete circulating high level T, hippocampal levels of T and DHT decreased considerably to 18 and 3%, respectively, whereas E2 level only slightly decreased to 83%. The strong reduction in hippocampal DHT resulting from castration implies that circulating T may be a main origin of DHT. In combination with results obtained from metabolism analysis of [$^3$H]steroids, we suggest that male hippocampal E2 synthesis pathway may be androstenedione $\rightarrow$ T $\rightarrow$ E2 or dehydroepiandrosterone $\rightarrow$ androstenediol $\rightarrow$ T $\rightarrow$ E2 but not androstenedione $\rightarrow$ E1 $\rightarrow$ E2. **(*Endocrinology* 150: 5106–5112, 2009)**

Both circulation-derived sex steroids and endogenously synthesized sex steroids are present in the brain. However, comparison of these sex steroids with both origins is not well clarified about relative content or difference in function. Increasing evidence has accumulated to support the endogenous synthesis of brain-derived steroids (1–6). Synthesis of dehydroepiandrosterone (DHEA), testosterone (T), dihydrotestosterone (DHT) and 17$\beta$-estradiol (17$\beta$-E2) as well as expression of cytochromes P450(17$\alpha$) and P450aromatase (P450arom) are demonstrated in the isolated hippocampus from adult rats (7–9). If the level of brain-derived steroids is comparable to or more than that of circulation-derived steroids, then brain-derived steroids may have a significant role in modulation of brain function. A considerable decrease of T has been observed in the whole brain within 1 d after castration (1). It is interesting to examine a resultant content of T and E2 after castration, with an improved methodology, to estimate the level of brain-derived T and E2.

We focus on the hippocampus, which is a good target for the neuromodulatory actions of sex hormones. Extensive studies have been performed *in vivo* to investigate the role of circulation-derived 17$\beta$-E2 for female and DHT for male in slow modulation of hippocampal synaptic plasticity (10–12). Estrogens also have specific contributions to rapid action on the synaptic plasticity as neuromodulators (13).

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Abbreviations: ADione, androstenedione; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; E2, estradiol; E1, estrone; 17$\beta$-HSD, 17$\beta$-hydroxysteroid dehydrogenase; LC-MS/MS, liquid chromatography with tandem mass spectrometry; m/z, mass to charge ratio; P450arom, P450aromatase; PFBz, pentafluorobenzyl; PROG, progesterone; T, testosterone.

In addition to conventional RIA (7, 14), recently, mass spectrometry has been applied to detect brain steroids such as DHEA and T in the whole brain. However, the presence of 17β-E2 or DHT has not yet been observed with mass spectrometry (15–18).

Here, we determine the accurate concentrations of hippocampus-derived estrogens and androgens by employing novel derivatization methods to improve the sensitivity of liquid chromatography with tandem mass spectrometry (LC-MS/MS). The metabolisms and participating enzymes for sex steroid production are investigated to illustrate the complete synthesis pathways in the hippocampus.

## Materials and Methods

Animals, castration procedures, and chemicals used in the current study are described in supplemental material (published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Young adult male or female Wistar rats were used.

### Mass-spectrometric assay of steroids

Detailed procedures are described in supplemental material.

#### *Step 1: purification of steroids from hippocampi with normal-phase HPLC*

The preparation of hippocampal homogenates from slices and the extraction of steroids were performed as described in supplemental materials and elsewhere (7). [$^3$H]Steroids were added to homogenates as internal standards. The steroid extracts were applied to a $C_{18}$ Amprep solid-phase column (Amersham Biosciences, Arlington Heights, IL) to remove contaminating fats. Then the steroids were separated into fractions of E2, T, DHT plus DHEA, E1, androstenedione (ADione), and progesterone (PROG) using a normal-phase HPLC system (Jasco, Tokyo, Japan) with a silica gel column (Cosmosil 5SL; Nacalai Tesque, Kyoto, Japan). Plasma was prepared by centrifugation from trunk blood collected from decapitated rats (8).

#### *Step 2: derivatization of HPLC-purified steroids before application to LC (reverse phase)-MS/MS (19, 20)*

At first, 100 pg isotope-labeled steroids ([$^{13}C_4$]E2, T-$d_3$, DHT-$d_3$, [$^{13}C_4$]E1, PROG-$d_4$, ADione-$d_4$ and DHEA-$d_4$,) were added to steroid extracts prepared via step 1). For preparation of E2-pentafluorobenzyl (PFBz)-picolinoyl, evaporated E2 extracts from the hippocampus or evaporated total steroid extracts from plasma were reacted with 5% PFBz bromide/acetonitrile, under KOH/ethanol, for 1 h at 55 C. After evaporation, the products were reacted with 100 μl picolinoic acid solution (2% picolinoic acid, 2% 2-dimethylaminopyridine, 1% 2-methyl-6-nitrobenzoic acid in tetrahydrofuran) and 20 μl triethylamine for 0.5 h at room temperature. The reaction products dissolved in 1% acetic acid were purified using a Bond Elute $C_{18}$ column (Varian, Palo Alto, CA). The dried sample was dissolved in elution solvent of LC. For preparation of T-17-picolinoyl-ester, DHT-17-picolinoyl-ester, E1-17-picolinoyl-ester, and DHEA-3-picolinoyl-ester, evaporated steroid extracts from the hippocampus or plasma were reacted with 100 μl picolinoic acid solution and 20 μl triethylamine for 0.5 h at room temperature. The reaction products were purified with the $C_{18}$ column by using 80% acetonitrile. The purified T, DHT, E1, or DHEA derivative was dissolved in elution solvent of LC.

#### *Step 3: determination of the concentration for 17β-E2, T, DHT, E1, and other steroids using LC-MS/MS*

The LC-MS/MS system, which consisted of a reverse-phase LC (Agilent 1100, Agilent Technologies, Santa Clara, CA) coupled with an API 5000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA), was operated with electrospray ionization in the positive-ion mode.

To examine specificity of LC-MS/MS analysis, samples were spiked with steroid isotopes as internal standards. Although the mass to charge ratio (m/z) transitions are different between intact steroids (*e.g.* from m/z = 558 to 339 for E2) and their isotopes (*e.g.* from m/z = 562 to 343 for [$^{13}C_4$]E2), their retention times are the same, because the affinity of intact steroids for the LC column is the same as that for their isotopes (supplemental Fig. S1).

Isotope-labeled steroid derivatives were also used for internal standards to measure recovery of steroids The recoveries of 17β-E2, T, DHT, and E1 were determined as 89 ± 8, 75 ± 4, 73 ± 5, and 76 ± 4%, respectively, after derivatization and MS/MS detection. Total recovery during all the steps was determined via $^3$H- and isotope-labeled steroids in steps 1 and 2.

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 for 17β-E2, T, DHT, and E1 were 0.3, 1, 1, and 1 pg per 0.1 g hippocampal tissue or 1 ml plasma, respectively (supplemental Table S2). From the calibration curve using standard steroids dissolved in blank samples, the good linearity was observed (supplemental Fig. S2).

### *In situ* hybridization

The hippocampal frozen slices were treated with proteinase K and postfixed. The mRNAs in the hippocampal slices were hybridized with digoxigenin-labeled sense or antisense cRNA probes. The slices were treated with ribonuclease A and stringent washes after hybridization. Then the slices were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody. Target mRNAs were visualized by color development with reagent chemicals. Details are described in supplemental material.

### Statistical analysis

Data are expressed as mean ± SEM in tables. An unpaired, two-tailed *t* test, under the assumption of unequal variances, was used 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. Metabolism analysis and RT-PCRs are described in supplemental material.

## Results

### Mass-spectrometric analysis

The concentration of sex steroids was determined for adult male rat hippocampus using a chromatogram anal-

**TABLE 1.** Mass-spectrometric analysis of the concentration of steroids in the hippocampus and plasma of adult male rats

| | Hippocampus | | Plasma | |
|---|---|---|---|---|
| | Intact | Castrated | Intact | Castrated |
| 17β-E2 (ng/g wet weight or ml) | 2.3 ± 0.4 (n = 6) | 1.9 ± 0.2 (n = 16) | 0.004 ± 0.001 (n = 5) | 0.002 ± 0.000 (n = 14) |
| 17β-E2 (nM) | 8.4 ± 1.5 | 6.9 ± 0.8 | 0.014 ± 0.003 | 0.006 ± 0.001 |
| T (ng/g wet weight or ml) | 4.9 ± 0.7 (n = 8) | 0.9 ± 0.2 (n = 16) | 4.2 ± 0.5 (n = 8) | 0.06 ± 0.02 (n = 16) |
| T (nM) | 16.9 ± 2.3 | 3.1 ± 0.8 | 14.6 ± 1.7 | 0.20 ± 0.08 |
| DHT (ng/g wet weight or ml) | 1.9 ± 0.5 (n = 8) | 0.06 ± 0.01 (n = 16) | 0.18 ± 0.03 (n = 8) | 0.012 ± 0.003 (n = 16) |
| DHT (nM) | 6.6 ± 1.7 | 0.22 ± 0.04 | 0.63 ± 0.10 | 0.04 ± 0.01 |
| E1 (ng/g wet weight or ml) | 0.004 ± 0.001 (n = 4) | 0.003 ± 0.002 (n = 4) | 0.002 ± 0.001 (n = 4) | |
| E1 (nM) | 0.015 ± 0.003 | 0.014 ± 0.006 | 0.007 ± 0.003 | |
| ADione (ng/g wet weight or ml) | 0.43 ± 0.08 (n = 4) | 0.48 ± 0.00 (n = 4) | 0.17 ± 0.05 (n = 4) | |
| ADione (nM) | 1.5 ± 0.3 | 1.7 ± 0.0 | 0.61 ± 0.17 | |
| PROG (ng/g wet weight or ml) | 4.6 ± 1.1 (n = 4) | 2.2 ± 1.1 (n = 4) | 2.1 ± 0.6 (n = 4) | |
| PROG (nM) | 14.6 ± 3.5 | 7.1 ± 3.4 | 6.8 ± 1.8 | |
| DHEA (ng/g wet weight or ml) | 0.08 ± 0.01 (n = 4) | | 0.006 ± 0.003 (n = 4) | |
| DHEA (nM) | 0.27 ± 0.05 | | 0.02 ± 0.01 | |

Intact shows the averaged values from intact and sham-operated rats, because there were no significant differences between these two groups of rats. Data are expressed as mean ± SEM. Number of animals (*i.e.* the number of hippocampi) is shown in *parentheses*. Concentration in nanomolar is calculated using the average volume of 0.14 ml for one whole hippocampus that has 0.14 ± 0.02 g wet weight (n = 86). We assumed that tissue having 1 g wet weight has an approximate volume of 1 ml, because the major part of tissue consists of water whose 1 ml weight is 1 g. The volume should be decreased by less than 10%, due to the specific volumes of proteins and lipids (0.7–0.8 ml/g) (14).

ysis of the fragmented ions of steroid derivatives (supplemental Fig. S1). Results are summarized in Table 1.

Many steroids need derivatization before application to LC-MS/MS to determine their accurate concentrations in the brain where the absolute content of steroids is extremely low. We employ picolinoyl derivatization to improve limit of quantification (supplemental Table S2). In case of E2, PFBz derivatization was additionally performed simultaneously, to increase evaporation probability in electrospray ionization procedures.

Chromatographic profiles for the fragmented ions of E2-PFBz-picolinoyl showed a clear peak with the retention time of 7.01 min, which was the same as that of the standard 17β-E2 derivative (Fig. 1A). The peak corresponding to 17α-E2 was not observed around 6.79 min. In the chromatographic profiles of the fragmented ion of T-17-picolinoyl-ester, a major peak at 4.84 min and a small peak at 5.59 min were observed (Fig. 1B). In the profiles of the fragmented ion of DHT-17-picolinoyl-ester, a major peak at 5.94 min and a small peak at 6.36 min were observed for DHT-picolinoyl (Fig. 1C). In the chromatographic profiles of the fragmented ion of E1–3-picolinoyl-ester, a very small peak at 2.4 min was observed. For these steroids, the retention time of the observed steroid peak was the same as that of standard steroid-picolinoyl.

Using the average hippocampal volume of 0.14 ml (deduced from 0.14 ± 0.02 wet weight for one whole hippocampus of a 12-wk-old rat, n = 86), the average concentrations of 17β-E2, T, and DHT in the hippocampus of intact rats were calculated to be 2.3, 4.9, and 1.9 ng/g wet weight (*i.e.* 8.4, 16.9, and 6.6 nM), respectively (Table 1). In contrast, the hippocampal E1 level was extremely low, 0.004 ng/g (15 pM). The relative concentrations of 17β-E2, T, DHT, and E1 were T higher than 17β-E2 higher than DHT much higher than E1 in that order. Because no significant differences were observed in the concentrations of these steroids between intact and sham-operated rats, we pooled the data from both experimental groups as control values. The average concentration of 17β-E2, T, and DHT in plasma was 0.004, 4.2, and 0.18 ng/ml (*i.e.* 0.014, 14.6, and 0.63 nM), respectively (Table 1). Plasma E1 was approximately 0.002 ng/ml (7 pM).

The concentration of DHT was nearly 10-fold higher in the hippocampus than in plasma. The concentration of T in the hippocampus was higher than that in plasma. As shown in Fig. 1, E and F, for individual rats, a roughly linear relationship was observed between hippocampal T and plasma T and between hippocampal DHT and plasma T. No such linear relationship was observed between hippocampal E2 and plasma T.

Castration was performed to eliminate the contribution of testis-derived sex steroids (T and DHT) via the blood circulation (Table 1 and Fig. 1, D–F). After castration, the concentrations of T and DHT in plasma decreased to roughly 1/100 and 1/15 of their intact levels, respectively. Upon castration, the level of T and DHT in the hippocampus also decreased. T remained, however, at approximately 18% of the intact level (0.9 ng/g = 3.1 nM), implying that 18% of T is endogenously synthesized in the hippocampus. The sum of the castrated residual T level (3.1 nM) in the hippocampus and the intact plasma T level (14.6 nM) was roughly equal to the level of intact hippocampal T (16.9 nM) (Table 1 and Fig. 1E). A considerable decrease in DHT to 3% of the intact level (0.06 ng/g = 0.22 nM) was observed by castration (Table 1 and Fig. 1F). In contrast, castration induced only a slight de-

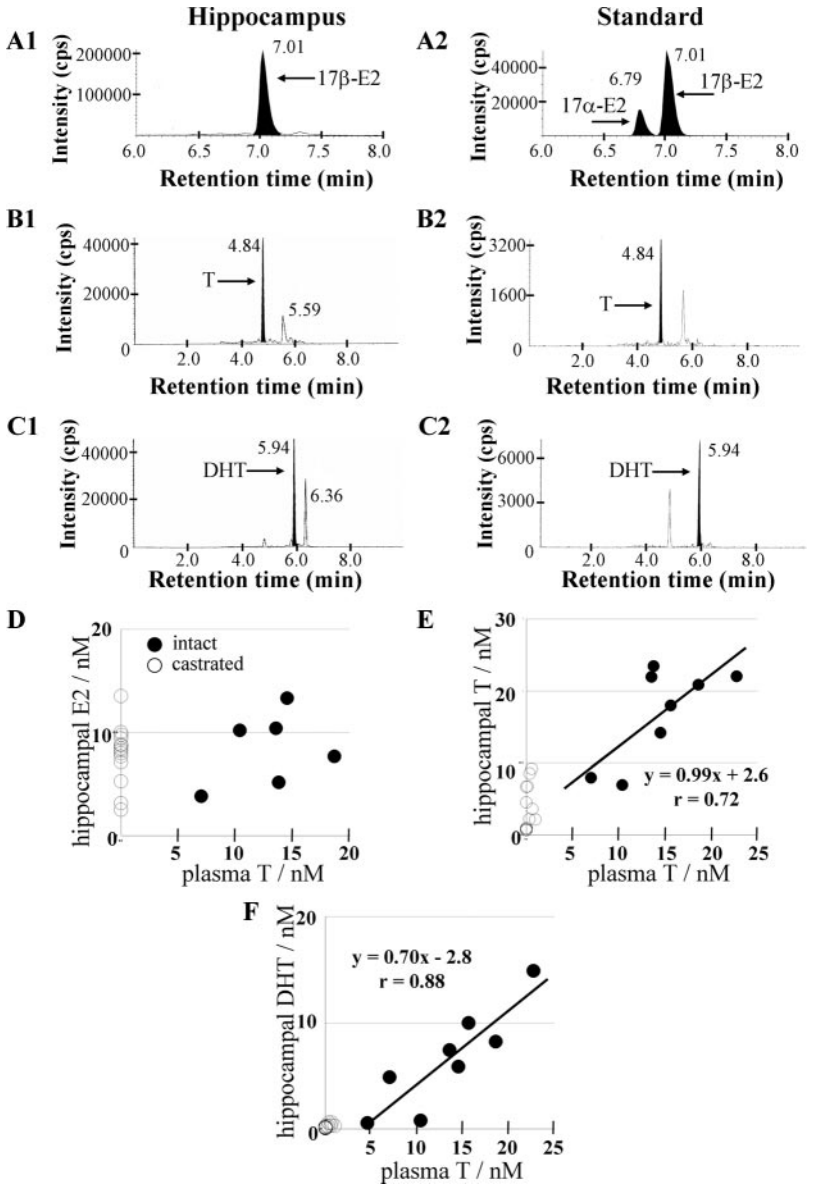


**FIG. 1.** Mass-spectrometric analysis of hippocampal sex steroids. A–C, LC-MS/MS ion chromatograms of E2 (A), T (B), and DHT (C). A1, B1, and C1 represent the chromatograms of the fragmented ions of each steroid derivative from the hippocampus of adult male rats (12 wk old). Shaded portions indicate the intensity of fragmented ions of E2-PFBz-picolinoyl (m/z = 339, A1), T-picolinoyl (m/z = 253, B1), and DHT-picolinoyl (m/z = 203, C1), respectively. A2, B2, and C2 represent the chromatograms of the fragmented ions of each of the standard steroid derivatives. The *vertical axis* indicates the detected intensity of the fragmented ions of each steroid derivative. The *horizontal axis* indicates the retention time of the fragmented ions; t = 7.01 min for 17$\beta$-E2 (A1), t = 4.84 min for T (B1), and t = 5.94 min for DHT (C1). The time of the injection to the LC system was defined as t = 0 min. D–F, Relationship between hippocampal sex steroids and plasma T for individual rats. *Vertical axis* is E2 (D), T (E), or DHT (F). ●, intact rats; ○, castrated rats. Note that a prepurification step using normal-phase HPLC before steroid derivatization was very important to achieve high precision and good reproducibility of LC-MS/MS determination to avoid contamination of other steroids and fats. Derivatized steroids were first separated with the LC column. In the multiple reaction monitoring mode, the instrument monitored the m/z transition (supplemental Table S2). In MS/MS procedures, the mother ion (17$\beta$-E2 derivative, m/z = 558) is selected using a first-stage mass spectrometer. This E2 derivative is broken by collision with $N_2$ gas, and the fragmented ion (m/z = 339) is selected using a second-stage mass spectrometer and detected (supplemental Fig. S1).

crease in the 17$\beta$-E2 to 1.9 ng/g (6.9 nM) in the hippocampus (Fig. 1D).

The observed high level of E2 as well as only slight decrease by castration in the male hippocampus are not an artifact of the determination. To prove this, we did determine the hippocampal level of E2 in 12-wk-old Wistar female rats. We could follow the change of the E2 level of the female rat hippocampus dependent on the estrous cycle such as 1.7 ± 0.4 nM at proestrus, 1.0 ± 0.3 nM at estrus, 0.5 ± 0.1 nM at diestrus-1, and 0.7 ± 0.2 nM at diestrus-2. We also observed estrous cycle-dependent plasma E2 level such as 0.120 ± 0.015 nM at proestrus, 0.020 ± 0.015 nM at estrus, 0.008 ± 0.005 nM at diestrus-1, and 0.026 ± 0.005 nM at diestrus-2. Three animals (hippocampi) were used for each condition. These results demonstrate that a nanomolar level of male hippocampal E2 truly exists.

The hippocampal levels of DHEA, PROG, and ADione were determined to be 0.08, 4.6, and 0.43 ng/g, respectively (*i.e.* 0.27, 14.6, and 1.5 nM) (Table 1). The plasma concentrations of these steroids were significantly lower than their hippocampal concentrations (Table 1).

To confirm the assay accuracy, the hippocampal homogenate spiked with known amounts of the steroids was prepared, and its concentration of steroid was determined (supplemental Table S1). Satisfactory accuracy was obtained, supporting the accuracy of determined hippocampal steroid content in Table 1. The limits of quantification were defined in supplemental Table S2 as the lowest value with an acceptable accuracy (90–110%) and precision [*i.e.* relative standard deviation (RSD) <10%]. The results of intra- and interassay were shown in supplemental Table S2. The RSD for intra- and interassay was less than 7.2 and 8.9%, respectively.

## Male hippocampal sex steroid metabolism

We needed to analyze the pathway of steroid metabolism (supplemental Fig. S3) because mass-spectromet-

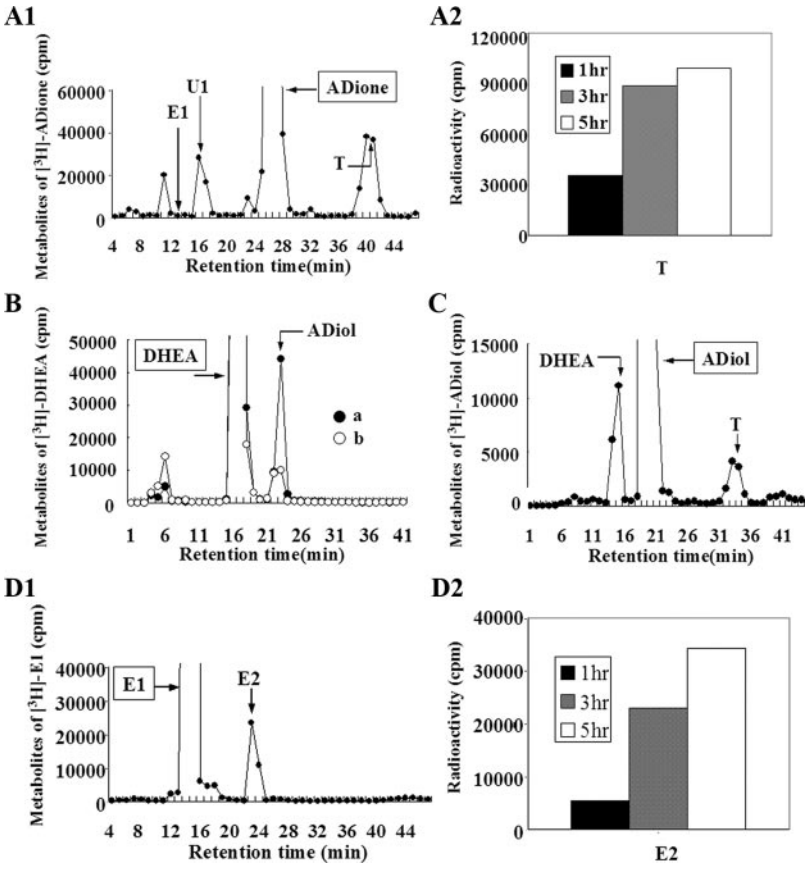


**FIG. 2.** Normal-phase HPLC analysis of steroid metabolism in hippocampal slices from adult male rat. Hippocampal slices from the adult male rats were incubated with $5 \times 10^6$ cpm $^3$H-labeled steroid substrate for 1, 3, and 5 h. A portion of the purified radioactive metabolites (total of $10^6$ cpm) was analyzed using an HPLC system. The rate of steroid production was normalized as products (cpm) per gram wet weight per 5 h. A1, HPLC profiles of [$^3$H]ADione metabolites. U1 indicates unknown product. As judged from the retention time, U1 (17 min) was not E1 (13 min). A2, Time dependence of the production for T from ADione. The production is the average of three experiments. B, HPLC profiles of [$^3$H]DHEA metabolites. Hippocampal slices were incubated in the absence of an inhibitor (line a) or in the presence of 40 $\mu$M RM352–26 (28), a specific inhibitor of 17$\beta$-HSD type 3 (line b). C, HPLC profiles of [$^3$H]androstenediol (ADiol) metabolites. D1, HPLC profiles of [$^3$H]E1 metabolites. D2, Time dependence of E2 production from E1. E2 production is the average of three experiments. The *arrows* designate the elution peak positions calibrated with standard [$^{14}$C]steroids. The retention time of the (same) standard [$^{14}$C]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.

ric determination shows only the contents of individual steroids. The metabolism of radioactive steroids in hippocampal slices was investigated using normal-phase HPLC. Typical results of HPLC analysis are illustrated in Fig. 2, and the production rates are summarized in supplemental Table S3. Detailed results and discussion are described in supplemental material (Table S3 and Figs. S4 and S5).

### Molecular biological analysis

Cellular localization and expression of each subtypes of 5$\alpha$-reductase, 17$\beta$-hydroxysteroid dehydrogenase (17$\beta$-HSD), 3$\beta$-HSD, and 3$\alpha$-HSD, responsible for sex steroidogenesis, were examined, because this important information had not been fully clarified. The expression level of mRNA for 17$\beta$-HSD (types 1 and 3), 3$\beta$-HSD type1, 5$\alpha$-reductase, and P450arom was not changed within 5% by castration, suggesting that the activity of local sex steroid synthesis may not be changed by castration. Typical RT-PCR patterns of mRNA transcripts are shown in supplemental Fig. S6. To identify the cellular localization of 5$\alpha$-reductase and 17$\beta$-HSD (type 1), *in situ* hybridization was performed. Significant expression of 5$\alpha$-reductase (types 1 and 2) as well as 17$\beta$-HSD was observed in pyramidal neurons and granule neurons (supplemental Fig. S7).

## Discussion

### Higher level of sex steroids in hippocampus than in circulation

Hippocampal levels of these sex steroids were higher than circulating levels (Table 1). Because the volume of hippocampus is very small (nearly 0.1 ml for one whole hippocampus), the calculated concentrations were relatively high in the nanomolar range except for E1. The absolute content of E2 was only around 0.23 ng in one hippocampus with 0.14 g (Table 1). Although steroid production capacity is strong in the gonads (except for E2 in male), circulation levels of steroids are also in the nanomolar range due to dilution in approximately 20 ml blood (200-fold of the hippocampal volume). Although the hippocampal expression levels of enzymes (such as P450arom) are approximately 1/200 of those in gonads (7, 13), they need to fill only a small hippocampal volume (1/200 of the blood volume). Taken together, sex steroid concentrations could be higher in the hippocampus than in the blood. Although 17$\alpha$-E2 exists in the mouse hippocampus and its level elevates upon castration (21), 17$\alpha$-E2 was not detected in the hippocampus of intact and castrated male rats in the current study (Fig. 1A). This discrepancy may be due to the difference between mouse and rat.

### Modulation of T, E2, and DHT levels by castration

Circulating T has been considered as a major source of male brain E2. Therefore, evaluation of the relative

amount of hippocampus-derived and testis-derived T in the male hippocampus was performed. Circulating T level (14.6 nM = 4.2 ng/ml) is very high in male animals as compared with other circulating sex steroids such as E1 and E2 (around 10 pM = 3 pg/ml) (22, 23). The sum of the residual hippocampal T level after castration and the intact plasma T level was roughly equal to the level of the intact hippocampal T (Table 1). The roughly linear relationship was also observed between plasma T and hippocampal T (Fig. 1E). Collectively, nearly all the circulating T may penetrate into the hippocampus.

The male hippocampal E2 level was only slightly decreased by castration, which significantly decreased hippocampal T (a substrate for E2 production) (Fig. 1, D and E). The high hippocampal E2 level (6.9 nM = 1.9 ng/g) after depletion of circulating T may be maintained by an efficient conversion of hippocampus-derived T (3.1 nM) to E2. Another reason for the high level of hippocampal E2 may be the high stability of E2 in the hippocampus. The conversion of E2 to other metabolites (such as E1) was extremely slow, and nearly 95% of [$^3$H]E2 remained stable even after 5 h (7). Because the levels of hippocampal E1 (current study) and circulating E1 (23) are extremely low in male rats, the T → E2, rather than the E1 → E2, pathway is deduced to be a main pathway in the male hippocampus.

### Previous determination of brain sex steroids

The concentrations of 17$\beta$-E2, T, and DHEA have been measured using RIA in the hippocampus (4, 7, 8, 29). By using mass spectrometry (15–18), the presence of various steroids such as DHEA, PROG, and T has been demonstrated for the whole-brain extracts of adult rats. However, 17$\beta$-E2, E1, and DHT have not yet been detected in those studies. The reported concentrations in whole male brain have been 0.3–2.5 ng/g for T, 0.08–0.27 ng/g for DHEA, and 0.7–1.9 ng/g for PROG (15, 17, 18). These concentrations are qualitatively similar to those observed in the current study. Concerning circulating sex steroids of male rats, the currently measured levels were in reasonable agreement with the reported levels (15, 22, 23).

### Functional significance of hippocampus-derived sex steroids

Most of the observed hippocampus-derived sex steroids are probably bioavailable and not just stored in cells. Hippocampus-derived steroids may act locally because they can bind to drive estrogen or androgen receptors within the hippocampal neurons (13). They are not necessary to be transported to outside of the hippocampus, different from the situation of circulating steroids.

Concerning hippocampal function, hippocampus-derived E2 regulates the density of spines and enhances long-term depression as a modulator (13, 24, 25). In pathological aspect, hippocampus-derived E2 may protect neurons from damages such as kainic acid administration (26, 27). We observed sex differences in the steroidogenic pathway (concerning E1, for example) in the hippocampus.

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## References

1. **Baulieu EE, Robel P** 1998 Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids. Proc Natl Acad Sci USA 95:4089–4091
2. **Corpéchot C, Robel P, Axelson M, Sjövall J, Baulieu EE** 1981 Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. Proc Natl Acad Sci USA 78:4704–4707
3. **Garcia-Segura LM, Melcangi RC** 2006 Steroids and glial cell function. Glia 54:485–498
4. **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
5. **Robel P, Bourreau E, Corpéchot C, Dang DC, Halberg F, Clarke C, Haug M, Schlegel ML, Synguelakis M, Vourch C** 1987 Neuro-steroids: 3$\beta$-hydroxy-$\Delta$5-derivatives in rat and monkey brain. J Steroid Biochem 27:649–655
6. **Zwain IH, Yen SS** 1999 Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. Endocrinology 140:3843–3852
7. **Hojo Y, Hattori TA, Enami T, Furukawa A, Suzuki K, Ishii HT, Mukai H, Morrison JH, Janssen WG, Kominami S, Harada N, Kimoto T, Kawato S** 2004 Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017$\alpha$ and P450 aromatase localized in neurons. Proc Natl Acad Sci USA 101:865–870
8. **Kawato S, Hojo Y, Kimoto T** 2002 Histological and metabolism analysis of P450 expression in the brain. Methods Enzymol 357:241–249
9. **Mukai H, Tsurugizawa T, Ogiue-Ikeda M, Murakami G, Hojo Y, Ishii H, Kimoto T, Kawato S** 2006 Local neurosteroid production in the hippocampus: influence on synaptic plasticity of memory. Neuroendocrinology 84:255–263
10. **Leranth C, Hajszan T, MacLusky NJ** 2004 Androgens increase spine

synapse density in the CA1 hippocampal subfield of ovariectomized female rats. J Neurosci 24:495–499

11. **Leranth C, Petnehazy O, MacLusky NJ** 2003 Gonadal hormones affect spine synaptic density in the CA1 hippocampal subfield of male rats. J Neurosci 23:1588–1592
12. **Woolley CS, McEwen BS** 1994 Estradiol regulates hippocampal dendritic spine density via an *N*-methyl-D-aspartate receptor-dependent mechanism. J Neurosci 14:7680–7687
13. **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
14. **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
15. **Ebner MJ, Corol DI, Havlíková H, Honour JW, Fry JP** 2006 Identification of neuroactive steroids and their precursors and metabolites in adult male rat brain. Endocrinology 147:179–190
16. **Higashi T, Ninomiya Y, Iwaki N, Yamauchi A, Takayama N, Shimada K** 2006 Studies on neurosteroids XVIII LC-MS analysis of changes in rat brain and serum testosterone levels induced by immobilization stress and ethanol administration. Steroids 71:609–617
17. **Liere P, Akwa Y, Weill-Engerer S, Eychenne B, Pianos A, Robel P, Sjövall J, Schumacher M, Baulieu EE** 2000 Validation of an analytical procedure to measure trace amounts of neurosteroids in brain tissue by gas chromatography-mass spectrometry. J Chromatogr B Biomed Sci Appl 739:301–312
18. **Liu S, Sjövall J, Griffiths WJ** 2003 Neurosteroids in rat brain: extraction, isolation, and analysis by nanoscale liquid chromatography-electrospray mass spectrometry. Anal Chem 75:5835–5846
19. **Yamashita K, Kobayashi S, Tsukamoto S, Numazawa M** 2007 Synthesis of pyridine-carboxylate derivatives of hydroxysteroids for liquid chromatography-electrospray ionization-mass spectrometry. Steroids 72:50–59
20. **Yamashita K, Okuyama M, Watanabe Y, Honma S, Kobayashi S, Numazawa M** 2007 Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography-electrospray ionization tandem mass spectrometry. Steroids 72:819–827
21. **Toran-Allerand CD, Tinnikov AA, Singh RJ, Nethrapalli IS** 2005 17$\alpha$-Estradiol: a brain-active estrogen? Endocrinology 146:3843–3850
22. **Meffre D, Pianos A, Liere P, Eychenne B, Cambourg A, Schumacher M, Stein DG, Guennoun R** 2007 Steroid profiling in brain and plasma of male and pseudopregnant female rats after traumatic brain injury: analysis by gas chromatography/mass spectrometry. Endocrinology 148:2505–2517
23. **Gao CQ, Dhooge WS, Kaufman JM, Weyne JJ, Eechaute WP** 2002 Hypothalamic 5$\alpha$-reductase and 3$\alpha$-oxidoreductase activity in the male rat. J Steroid Biochem Mol Biol 80:91–98
24. **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
25. **Pozzo-Miller LD, Inoue T, Murphy DD** 1999 Estradiol increases spine density and NMDA-dependent $Ca^{2+}$ transients in spines of CA1 pyramidal neurons from hippocampal slices. J Neurophysiol 81:1404–1411
26. **Azcoitia I, Sierra A, Veiga S, Honda S, Harada N, Garcia-Segura LM** 2001 Brain aromatase is neuroprotective. J Neurobiol 47:318–329
27. **Bi R, Broutman G, Foy MR, Thompson RF, Baudry M** 2000 The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus. Proc Natl Acad Sci USA 97:3602–3607
28. **Maltais R, Luu-The V, Poirier D** 2002 Synthesis and optimization of a new family of type 3 17$\beta$-hydroxysteroid dehydrogenase inhibitors by parallel liquid-phase chemistry. J Med Chem 45:640–653
29. **Munetsuna E, Hojo Y, Hattori M, Ishii H, Kawato S, Ishida A, Kominami SAJ, Yamazaki T** 2009 Retinoic acid stimulates 17$\beta$-estradiol and testosterone synthesis in rat hippocampal slice cultures. Endocrinology 150:4260–4269

## Supplementary material

## Introduction

### Brief history of brain sex steroid synthesis

In 1980's, a neurosteroid hypothesis was proposed, suggesting that pregnenolone, progesterone and DHEA may be endogenously synthesized in the brain due to the finding that the concentrations of pregnenolone and dehydroepiandrosterone (DHEA) in rodents are higher in the brain than in plasma (1, 2). The direct demonstration of steroidogenesis in the mammalian brain had, however, needed some decades, due to the extremely low levels of steroidogenic activity in the brain. In particular, sex steroids had not been considered to be brain-derived neurosteroids, and rather thought to reach the brain exclusively via blood circulation (3). This belief is supported by many reports suggesting the absence of cytochrome P450(17α) (DHEA synthase) in the adult mammalian brain (4, 5) and also by the observation of the disappearance of testosterone (T) in the brain within one day after castration (3). This decrease in T by castration, however, should be re-examined with an improved methodology, in order to analyze the resultant brain-derived T.

In recent years, increasing evidence has accumulated to support the local endogenous synthesis of estrogens and androgens in the mammalian brain, such as the hippocampus (6-8). DHEA, T, DHT and 17β-E2 are synthesized from cholesterol within the isolated hippocampus from adult rats (6, 7). The neuronal expression of cytochromes P450(17α) and P450aromatase is demonstrated. Ultrastructural analysis using immunogold electron microscopy indicates that P450(17α) and P450arom are localized not only in endoplasmic reticulum but also in synapses of glutamenergic neurons of adult hippocampus (6, 9). The hippocampal neurons are not only capable of steroid synthesis but also serve as the target for endogenous steroid hormones. In previous studies, the concentration of 17β-E2 in the hippocampus has often been determined using radioimmunoassay (RIA) with high sensitivity but relatively poor specificity (6). Recently, gas chromatography with mass-spectrometry (GC-MS/MS), liquid chromatography with mass-spectrometry (LC-MS) and liquid chromatography with tandem-mass-spectrometry (LC-MS/MS) have been applied to detect chemically distinct brain steroids. However, though DHEA and T have been observed in the whole brain extracts, the presence of

17β-E2, DHT and E1 has not yet been observed with mass-spectrometry (10-13).

## Materials and Methods

### Animals

Young adult Wistar rats (12 weeks old) were purchased from Saitama Experimental Animals Supply (Japan). All animals were maintained under a 12 h light/12 dark exposure and free access to food and water. Castration and sham operations were performed one-week before the experiments. The experimental procedure of this research was approved by the Committee for Animal Research of Univ of Tokyo. Male rats were used unless otherwise stated. Only in some experiments (see Results in the body of the manuscript), adult female Wistar rats were used. For this, the estrous cycle was confirmed by monitoring morning vaginal smears. Only those rats showing three consecutive 4-day cycles of proestrus, estrus, diestrus-1 and diestrus-2 were used.

### Chemicals

E2, T, DHT, estrone (E1), DHEA, androstenedione (ADione) and progesterone (PROG) were purchased from Sigma (USA). Picolinic acid was from Tokyo Chemical Industry (Japan) and [1,2,3,4-$^{13}C_4$]E2 and [1,2,3,4-$^{13}C_4$]E1 were from Hayashi Pure Chemical (Japan). T-$d_3$, DHT-$d_3$, DHEA-$d_4$, ADione-$d_4$, PROG-$d_4$ were from CDN Isotope Inc. (Canada). Finasteride was from Aska Pharma Medical Co., Ltd. [$^3$H] labeled steroids ([1,2,6,7-$^3$H]-DHEA, [1,2,6,7-$^3$H]-T, [1,2,6,7-$^3$H]-DHT, [2,4,6,7-$^3$H]-E1, [1,2,6,7-$^3$H]-PROG and [1,2,6,7-$^3$H]-ADione) were purchased from Perkin Elmer (USA). [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 $^{o}$C.

### Mass-spectrometric assay of steroids

*Step 1) Purification of steroids from hippocampi with normal phase HPLC.*

An adult male rat aged 12weeks was deeply anesthetized with ethyl ether and decapitated. The brain was removed, and hippocampal slices with 400 μm thickness were prepared with a vibratome (Dosaka, Japan), and then homogenized. To calculate the recovery of steroids, radioactive steroids

(20,000 cpm) were added as internal standards to hippocampal homogenate. 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 2,500×g and the organic layer was collected. After evaporation, the extracts were dissolved in 1ml 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 5ml of 40% methanol/$H_2O$ (hydrophilic phase), and then eluted with 5ml of 85% methanol/$H_2O$ (hydrophobic phase), and combined together, in order to collect all of the steroids. The combined organic extracts were dried, dissolved in an elution solvent of HPLC. The steroid metabolites were separated into E2, T , DHT plus DHEA, E1, ADione and PROG using a normal phase HPLC system (Jasco, Japan) with an elution solvent of hexane: isopropylalcohol: acetic acid = 98:2:1. A silica gel column (0.46×15 cm, Cosmosil 5SL, Nacalai Tesque, Japan) was used. Positions of eluted metabolites were determined using $^{14}$C-steroid, prior to the application of extracted steroid mixtures.
By monitoring $^{3}$H-steroids, the recoveries of 17β-E2, T, DHT, E1, DHEA, ADione and PROG were 44±4%, 49±3%, 35±3%, 42±3%, 45±2%, 44±4% and 42±1%, respectively, after extraction, $C_{18}$ column treatment and normal phase HPLC separation. Purification of steroids from the blood plasma was also performed. Plasma was prepared by centrifugation at 1,900×g, 4°C for 15 min of trunk blood collected from the same adult rats used for the measurements of hippocampal steroids. Extraction of sex steroids was performed by centrifugation at 2,500×g of the mixture of plasma with 100% ether, and the organic layer was collected and evaporated.

*Step 2) Derivatization of HPLC-purified steroids before application to LC (reverse phase)-MS/MS* (14, 15).
At first, 100 pg of isotope labeled steroids ($^{13}C_4$-E2, T-$d_3$, DHT-$d_3$, $^{13}C_4$-E1, PROG-$d_4$, ADione-$d_4$ and DHEA-$d_4$,) were added to steroid extracts prepared via *Step 1)*. For preparation of E2-PFBz-picolinoyl, evaporated E2 extracts from the hippocampus or evaporated total steroid extracts from plasma, were reacted with 5% pentafluorobenzyl bromide/acetonitrile, under KOH/ethanol, for 1 h 55 $^{o}$C. After evaporation, the products were reacted with 100 μl of picolinoic acid solution (2% picolinoic acid, 2% of 2-dimethylaminopyridine, 1% 2-methyl-6-nitrobenzoic acid in tetrahydrofuran) (i.e.,80mg of picolinoic acid, 80mg of 2-dimethylaminopyridine, 40mg of 2-methyl-6-nitrobenzoic

acid in 4 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 $C_{18}$ column (Varian, USA). The dried sample was dissolved in elution solvent of LC. For preparation of T-17-picolinoyl-ester, DHT-17-picolinoyl-ester, E1-17-picolinoyl-ester and DHEA-3-picolinoyl-ester, evaporated steroid extracts from the hippocampus or plasma were reacted with 100 μl of picolinoic acid solution (2% picolinoic acid, 2% of 2-dimethylaminopyridine, 1% 2-methyl-6-nitrobenzoic acid in tetrahydrofuran) and 20 μL of triethylamine, for 0.5 h at room temperature. The reaction products were purified with the $C_{18}$ column by using 80% acetonitrile. The purified T, DHT, E1 or DHEA-derivative was dissolved in elution solvent of LC.

*Step 3) Determination of the concentration for 17β-E2, T, DHT, E1 and other steroids using LC-MS/MS.*

For E2-PFBz-picolinoyl, the LC chromatographic separation was performed on a Cadenza CD-$C_{18}$ column (3×150 mm, 3 μm, Imtakt Japan). The mobile phase, composed of two solvents, solvent A (0.1% formic acid) and solvent B (acetonitrile:methanol, 50:50 v/v), was delivered at flow rate of 0.4 mL/min. Total run time was 9 min. The initial conditions were held at the mixture of solvent A and B (40:60 v/v). After injection of 0.02 ml sample, this was followed by a linear gradients to the mixture of solvent A and B (5:95 v/v) for 4 min, and to 100% solvent B for 2 min, and then these conditions were maintained for 1.5 min. This system was returned to the initial proportion of the mixture of solvent A and B (40:60 v/v) within 0.05 min and maintained for the final 1.45 min of each run.

For T-, DHT-, E1- and DHEA-picolinoyl-esters, the LC chromatographic separation was performed on a Cadenza CD-$C_{18}$ column. The mobile phase composed of two solvents, solvent A (0.1% formic acid) and solvent B (acetonitrile:methanol 50:50), was delivered at flow rate of 0.4 mL/min. Total run time was 10 min. The initial conditions were held at the mixture of solvent A and B (30:70 v/v). After injection of 0.02 ml sample, this was followed by a linear gradient to 100 % solvent B for 4 min and then these conditions were maintained for 3 min. This system was returned to the initial proportion of the mixture of solvent A and B (30:70 v/v) within 0.05 min and maintained for the final 2.95 min of each run.

The ionization conditions of LC separated derivatives were as follows: ion

spray voltage, 5 kV; turbo gas temperature, 600 ºC; ion source gas 1 (nebulizer gas), 45 psi (for picolinoyl derivatives); ion source gas 2 (turbo gas), 55 psi (for picolinoyl derivatives); declustering potential, 90V (for E2-picolinoyl), 80V (for T-picolinoyl), 95V (for DHT-picolinoyl) , 80V (for E1-picolinoyl), and 80V (for DHEA-picolinoyl). Nitrogen was used as the collision gas in the Q2 collision cell. The isotope-labeled steroid derivatives were used for calibration of retention time by monitoring the m/z transition, from 562 to 343 for E2-$^{13}C_4$-PFBz-picolinoyl, from 397 to 256 for T-$d_3$-picolinoyl-ester, from 399 to 206 for DHT-$d_3$-picolinoyl, from 380 to 161 for E1-$^{13}C_4$-picolinoyl, and from 398 to 179 for DHEA-$d_4$-picolinoyl, respectively.

In the multiple reaction monitoring mode, the instrument monitored the m/z transition, from 558 to 339 for E2-PFBz-picolinoyl, from 394 to 253 for T-picolinoyl, from 396 to 203 for DHT-picolinoyl, from 376 to 157 for E1-picolinoyl, from 394 to 175 for DHEA-picolinoyl, from 315 to 109 for PROG, and from 287 to 109 for ADione, respectively (Table S2). Here, m and z represent the mass and charge of a steroid derivative, respectively. In MS/MS procedures, for example, the mother ion (17β-E2 derivative, m/z = 558) is first selected using a 1st stage mass spectrometer. This E2-derivative is then broken by collision with $N_2$ gas, and the fragmented ion (m/z = 339) is selected using a 2nd stage mass spectrometer and detected (Fig. S1).

To examine specificity of LC-MS/MS analysis, samples were spiked with steroid isotopes as internal standards. Though the m/z transitions were different between intact steroids (e. g. from m/z = 558 to 339 for E2) and their isotopes (e.g. from m/z = 562 to 343 for $^{13}C_4$-E2 ), their retention times were the same, because the affinity of intact steroids for LC-column is the same as that for their isotopes (Fig.S1). We measured both m/z transitions almost simultaneously by rapidly switching m/z within 0.1 sec.

Isotope-labeled steroid derivatives were used for internal standards in order to measure recovery of steroids as well as to calibrate the retention time. By monitoring isotope steroids, the recoveries of 17β-E2, T, DHT, E1, DHEA, ADione and PROG were determined as 89±8%, 75±4%, 73±5%, 76±4%, 82±7%, 72±5% and 71±6%, respectively, after derivatization, purification and MS/MS detection.

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 for

17β-E2, T, DHT, E1 were 0.3 pg, 1 pg, 1 pg, and 1 pg per 0.1g of hippocampal tissue or 1 mL of plasma, respectively (Table S2). Such a good limit of quantification for E2, for example, was achieved by picolinoyl-derivatization (for induced ionization) (15) and pentafluorobenzyl-derivatization (to elevate evaporation probability). From the calibration curve using standard steroids dissolved in blank samples, the linearity was observed between 0.1 pg and 1000 pg for 17β-E2, between 0.5 pg and 1000 pg for T, DHT and 17α-E2, between 1 pg and 1000 pg for E1, and between 2 pg and 1000 pg for PROG, ADione and DHEA, respectively (Fig S2).

**Radioactive steroid metabolism assay with normal phase HPLC and reverse phase HPLC**

The procedures were essentially the same as previously described in elsewhere (6, 7). Hippocampus from one rat was sliced at 400 μm thickness with a vibratome and incubated with $5\times10^6$ cpm of [$^3$H]-steroids at 30 $^{o}$C for 5 h in 4 ml of physiological saline which contained 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. The procedures of steroid extraction from the hippocampal slices were the same as described above (*Step1* of Mass-spectrometric assay of steroids). The steroid extracts were pre-purified by a $C_{18}$ Amprep solid phase column (Amersham Biosciences, USA). The fractions containing the steroid metabolites were eluted with 5ml 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 of the steroids. Then the steroids were separated into fractions of E2, T, DHT plus DHEA, E1, PROG and ADione using a normal phase HPLC system (Jasco, Japan) with an elution solvent of hexane: isopropylalcohol: acetic acid = 98:2:1. A silica gel column (Cosmosil 5SL, Nacalai Tesque, Japan) was used. Reverse phase HPLC of $^3$H–steroid fractions was performed using acetonitrile: $H_2O$ = 40:60 as elution solvent. For reverse phase HPLC, an octadesyl–coated silica gel column (0.46×15 cm, Cosmosil 5$C_{18}$–MS, Nacalai Tesque, Japan) was used. Fraction radioactivity was measured using a liquid scintillation spectrometer LS6500 (Beckman,

USA). Care was taken to wash out trace amounts of fats and steroids from the silica gel column with the elution solvent of HPLC for 6 h between each analysis, in order to obtain a good reproducibility of steroid retention times.

**RT–PCR and Southern hybridization**

Total RNAs were isolated from rat tissues such as hippocampus, hypothalamus, 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 for genomic DNA contamination. The purified RNAs were reverse–transcribed, using a T–primed first–strand kit (Pharmacia, Sweden). The oligonucleotides for PCR amplification were designed as illustrated in Table S4. The PCR protocols comprised application of a 30 sec denaturation period at 95˚C, a 20 sec annealing period at 63˚C (5α-reductase type 1), 57˚C (5α-reductase type 2), 62˚C (3β–hydroxysteroid dehydrogenases (HSD) types 1-4), 55˚C (3α-HSD), and a 30 sec extension at 72˚C, for 28 cycles (5α-reductase type 1), 36 cycles (5α-reductase type 2), 40 cycles (3β-HSD type 1), 50 cycles (3β-HSD types 2-4), 34 cycles (3α-HSD). For semiquantitative analysis, the RT–PCR products were separated on 2.0 % 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 1/10000 in dilution), from liver, ovary, prostate or testis. To confirm the expression of 5α-reductase (type 1 and type 2), 3β–HSD (type 1) and 3α-HSD, Southern hybridization was performed. The amplified RT–PCR products of 5α-reductase, 3β–HSD and 3α-HSD 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 templates of DNA probes for Southern hybridization. After transfer of the RT–PCR products from agarose gels to nylon membrane, 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).

### *In situ* hybridization of hippocampal slices

The preparation of adult hippocampal slices fixed with 4% paraformaldehyde, was performed essentially as described elsewhere (6, 7, 16). Hippocampal slices were prepared from rat deeply anesthetized and perfused transcardially with a PBS (0.1 M phosphate buffer and 0.14 M NaCl, pH 7.3), followed by fixative solution of 4% paraformaldehyde. The hippocampi were postfixed, cryoprotected, and frozen-sliced coronally at 15 μm thickness with a cryostat microtome (Leica CM1510, Germany). Slices were immediately mounted on the slide glass at −18 ˚C. Digoxigenin (DIG)-labeled sense and antisense cRNA probes were in vitro transcribed from the PCR products of 5α−reductase or 17β-HSD (type 1) by using T7 or Sp6 promoters inherent in pGEM-T-Easy vector. The hippocampal slices were treated with 10 μg/mL of Proteinase K for 10-20 minutes, and then postfixed with fixative solution for 10 minutes. After acetylation with acetic anhydride and dehydration, the mRNA in the hippocampal slices were hybridized with 0.5 μg/mL of DIG-labeled sense or antisense cRNA probes. In order to digest and wash out the excess cRNA probes, the slices were treated with RNase A and stringent washes after hybridization. In order to visualize the hybridized cRNA probes, for 5α−reductase, the slices were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Chemicon, USA) (1/1000) for 30 min. After washing the hippocampal slices twice, target mRNAs were visualized by color development with 0.45 mg/mL of nitro blue tetrazolium chloride (NBT) and 0.175 mg/mL of 5-bromo-4-chloro-3-indoryl phosphate (BCIP) (Roche Diagnosis, USA) for 6 h (5α-reductase type 1) or 18 h (5α-reductase type 2). For 17β-HSD, the sections were incubated with horseradish peroxidase-conjugated anti-DIG antibody (Roche, USA) (1/1000) for 30 min. To amplify signals of in situ hybridization, the slides were incubated for 48 h with dinitrophenyl labeled amplification reagent using the TSA Plus DNP (AP) System (Perkin Elmer, USA) and stained with NBT/BCIP.

## Results

### Male hippocampal sex-steroid metabolism

We need to analyze the pathway of steroid metabolism (Fig. S3), because mass-spectrometric determination shows only the contents of individual steroids. The metabolism of radioactive steroids in hippocampal slices was

investigated using normal phase HPLC. Typical results of HPLC analysis are illustrated in Fig. 2 and the production rates are summarized in Table S3. A significant production of T from $^{3}$H-ADione was observed (Fig. 2A). The production of T was increased in time dependent manner. The E1 production from $^{3}$H-ADione was not observed (Fig 2A). Though E2 is a metabolite of $^{3}$H-T, DHT was another major metabolite of $^{3}$H-T (see Fig. 7 in (6)). The application of finasteride, a specific inhibitor of 5α-reductase, at 10 nM considerably suppressed the production of DHT down to approx. 3% of the control value, indicating the participation of 5α-reductase. A significant inactivation of DHT, catalyzed by 3α-HSD, was observed, resulting in androstanediol (Table S3, Fig S4). When $^{3}$H-DHEA was used as a substrate, androstenediol (ADiol) was produced as a major metabolite (Fig.2B). The conversion of $^{3}$H-ADiol to T was shown in Fig. 2C. The ADiol production was significantly inhibited by the presence of 40 μM of RM352-26, a specific inhibitor of 17β-HSD type 3 (Table S3) (17). When $^{3}$H-E1 was used as a substrate, a significant production of E2 was observed (Fig. 2D). The E2 production was increased in time dependent manner (Fig. 2D). However, the E1 level was very low (around 15 pM) from LC-MS/MS determination, therefore this E1→ E2 process cannot be a main pathway in the physiological condition. Not only 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 (Fig. S5).

**Molecular biological analysis of enzymes synthesizing sex steroids**

Cellular localization and expression of each subtypes of 5α-reductase, 17β-HSD, 3β-HSD and 3α-HSD, responsible for sex-steroidogenesis, were examined, because this important information had not been fully clarified. Typical RT-PCR patterns of mRNA transcripts are shown in Fig. S6. Relative number of transcripts, expressed in the hippocampus of adult male rats, was roughly 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), approx. 1/5000 of that in the ovary for 3β-HSD (type1), and approx. 1/500 of that in the liver for 3α-HSD (Fig. S6). The other subtypes of 3β-HSD (types 2,3,4) were not detected even after 50 cycle of PCR amplification (Fig. S6). The mRNA expression levels in the hippocampus were lower for 5α-reductase (type 1), 3β-HSD and 3α-HSD than those in the hypothalamus, except for 5α-reductase

(type 2). To identify the cellular localization of 5α-reductase (DHT synthase) and 17β-HSD (type 1) (T synthase from ADione or ADiol), *in situ* hybridization was performed. Significant expressions of both type 1 and type 2 of 5α-reductase were observed in pyramidal neurons (CA1, CA3) and granule neurons (DG) (Fig. S7A and E). As judged from the density of nitro blue tetrazolium staining, the expression level of type 1 (observed after 6 h) was much higher than that of type 2 (observed after 48 h). The expression of 5α-reductase was significant in pyramidal and granule neurons and very weak in glial cells (Fig. S7A and E). The moderate expression of 17β-HSD (type 1) was observed in pyramidal and granule neurons (Fig. S7C). Glial cells did not express 17β-HSD (type 1) significantly. Because the expression level of mRNA for 17β-HSD (type 1) was even lower than that of 5α-reductase (type 2) (6), we used the peroxidase reactions and color development for 48 h in order to further amplify the signals. Cellular localization of 17β-HSD (type 3) was not clear (data not shown), because of a bad *in situ* signal due to the lower expression level of type 3 than that of type 1.

## Discussion

### DHT production is significantly dependent on circulating T

The fact that male hippocampal DHT was roughly 10 fold of plasma DHT suggests that DHT is locally produced in the hippocampus from either circulating T or hippocampus-derived T. The disappearance of circulating T upon castration decreased the level of DHT to 3.2% of that of intact hippocampus (Fig 1F). Though 18% of the T was still present in the hippocampus of castrated rats, this endogenous T was not efficiently used to synthesize DHT. A roughly linear relationship was observed between hippocampal DHT and plasma T (Fig. 1F). Taken collectively, DHT may be synthesized in the hippocampus considerably from testis-derived T.

### One week castration paradigm

For castration, we took one-week castration paradigm in the current study, different from conventional two-week castration paradigm. The concentration of E2, T and DHT did not decrease even at 4 weeks after the castration (data not shown), indicating that the sex steroid level in the hippocampus was reached equilibrium already at one week after the castration. By castration, no

significant change in the expression level of steroidogenic enzymes was observed by analyzing mRNAs of 5α-reductase, P450arom, 3β-HSD and 17β-HSD (data not shown). Therefore, the resting 18% of T level should be simply due to the depletion of circulating T.

**Levels of PROG, ADione and DHEA**

The contribution of other circulating steroids to the hippocampal E2 production is also considered, however their contributions may be small. Circulating E2 and E1 in male rats are of course very low (7-14 pM i.e., 2- 4 pg/mL) (Table 1). Contribution of adrenal steroids to E2 production is considered. Circulating PROG released from adrenal gland might contribute to E2 production via pathway of PROG→ADione→T→E2, though the level of circulating PROG (6.8 nM = 2.1 ng/mL) was lower than hippocampal PROG (14.6 nM = 4.6 ng/g). The rat adrenal cortex does not express cytochrome P450(17α), therefore does not secrete DHEA and ADione as substrates of sex steroids. The plasma DHEA level in rats is very low and much lower than that in humans which express P450(17α) in the adrenal cortex (Table 1).

In Table 1, it may be strange that the level of DHEA or ADione (substrate) was lower than that of T, E2, DHT (downstream metabolites). This may not be unreasonable if we consider adrenal steroids in the blood plasma in case of stressful condition, where the levels of upstream adrenal steroids are much lower than those of downstream metabolite, such as 6.8 nM (2.1 ng/mL) for PROG, 26.4 nM (8.7 ng/mL) for deoxicorticosterone and 1024 nM (355 ng/mL) for corticosterone from our mesurements (data not shown).

**Previous determination of brain sex-steroids**

The concentrations of 17β-E2, T and DHEA have been measured using RIA in adult rat hippocampus (6, 7), cultured hippocampal neurons or slices (18). Though RIA has a high sensitivity, RIA method presents problems in terms of relatively low accuracy and specificity due to the cross-reaction with other steroids or isomers. In addition, many studies did not employ pre-purification of brain E2 with HPLC in *Step 1)* procedures, to eliminate contaminating other steroids and fats, and this may result in wrong lower contents for E2. Our previously published value of 0.6 nM (0.2 ng/g) for 17β-E2 in the hippocampus, is probably not accurate, because we did not perform rigorous pre-purification of E2 using HPLC, performed in the current study, prior to RIA (6). By using GC-MS/MS (10, 12), LC-MS (13) or API-2000 LC-MS/MS

(11) in combination with derivatization, the presence of various steroids such as DHEA, PROG and T has been demonstrated for the whole brain extracts of adult rats. Derivatization has been performed using various chemicals, including methoxyamine, trimethylsilylimidazole, heptafluorobutylic acid, oxime, and hydrazine-methylpyridine. However, 17β-E2, E1 and DHT have not yet been detected in those studies. The reported concentrations in the whole male brain have been 0.3 - 2.5 ng /g for T, and 0.08 - 0.27 ng /g for DHEA, and 0.7-1.9 ng /g for PROG (10, 12, 13). These concentrations are qualitatively similar to those observed in the current study, supporting the reliability of our methods. Note that all the reported values have been obtained in the whole brain containing many different regions, and therefore they are not necessarily the same as the concentration in the hippocampus. The LC-MS/MS method has an improved specificity and precision, relative to LC-single MS, because LC-MS/MS uses not only mother ions but also fragmented ions in the identification of a distinct steroid. By monitoring the m/z transition, we are able to exclude the detection of contaminated ions having the same m/z as that of the mother E2-derivative. We achieved the excellent quantification limit of 0.3 pg/0.1 g hippocampus by employing the 3-pentafluorobenzoxy-17- picolinoyl derivatization for E2 in combination with API-5000 LC-MS/MS. Picolinoyl-derivatization was used for induced-inonization, and pentafluorobenzoxy-derivatization was used to increase the evaporation probability. The 17-picolinoyl derivatization was necessary to detect DHT and T in castrated rat hippocampus in order to obtain the excellent quantification limit of 1 pg/0.1 g hippocampus (Table S2). We observed 0.22 ng of 17β-E2, 0.09 ng of T and 0.006 ng of DHT per 0.1 g hippocampus from castrated rats, respectively (Table 1). These amounts were sufficiently higher than the detection thresholds. However, it should be noted that LC-MS/MS only is not enough to detect a trace amount of brain E2 and DHT. Pre-purification of E2 and DHT via normal phase HPLC is necessary prior to LC-MS/MS determination.

Other than the current investigations using LC-MS/MS, there is one report which has demonstrated the concentration of 17α-E2 and 17β-E2 in the hippocampus in combination with dansyl derivatization (19). In their report of adult mouse, only 17α-E2 has been observed at around 7.7 pg/mg protein (= 0.74 ng/g wet weight), while 17β-E2 was not observed at their limit of sensitivity (5 pg/g wet weight). Concerning circulating sex-steroids of male rats, the currently measured levels were in reasonable agreement with the

reported levels which are, for example, 4.8 ng/mL (RIA) or 1.2 ng/mL (GC-MS) for circulating T, 4.8 ng/mL(RIA) or 2.2 ng/mL (GC-MS) for PROG, and 0.1 ng/mL (GC-MS) for ADione (10, 20, 21). By the way, significant capacity of brain production of E2 and T has also been reported in bird brain studies (such as quail) that support endogenous synthesis of sex-steroids as well as an essential role of brain-derived sex steroids in reproductive brain function (22).

**Characteristics of metabolisms of sex-steroids in the hippocampus**

Growing number of reports support that the site of sex steroid synthesis/metabolism is neuron or glia (6, 18, 23, 24). The immunohistochemical study demonstrated that in the hippocampus, steroidogenic enzymes (such as StAR, P450scc, P450(17α) and P450arom) are mainly expressed in glutamatergic principal neurons and that they were expressed weakly in glial cells (6, 7, 16, 25). *In situ* hybridization study demonstrated that 5α-reductase (type 1 and type 2) was expressed in neurons of adult male rat hippocampus (Figs. S5), and also expressed in neurons of adult mouse hippocampus (26, 27). The extensive investigations of 3β-HSD have been performed because 3β-HSD is essential for production of PROG from pregnenolone, ADione from DHEA, or T from ADiol (28). The current study added new results that only 3β-HSD (type 1) was expressed, while subtypes 2-4 of 3β-HSD were not expressed. Previous studies have shown that a mixture of subtypes 1-4 of 3β-HSD are expressed and localized along pyramidal neurons (CA1, CA3) and granule cells (DG) (28). However, the extremely low expression level of 3β-HSD is shown, because high sensitive $^{35}$S-oligonucleotide probes and 5-8 weeks long exposure are needed for in situ hybridization (28).

In conclusion, we observed, for the first time, nanomolar high concentrations of E2, DHT and ADione but extremely low concentration of E1 in the male hippocampus from LC-MS/MS determination. Hippocampus-synthesized T and circulation-derived T may occupy 18% and 82%, respectively, of the total T in the intact male hippocampus. In combination with results obtained from metabolism analysis, for E2 synthesis pathway, PROG → ADione → T→ E2 and DHEA → ADiol → T→ E2 but not ADione→E1→E2 are suggested. However, ADione→E1→E2 pathway might work in female hippocampus as judged from our preliminary experiments. Because E2 is a stable end-product

due to extremely slow inactivation (6), depletion of circulating T did not significantly reduced the E2 level in the male hippocampus. The knowledge of hippocampal concentration of E2 is essential to explain effects of exogenous E2 on synaptic plasticity of neurons (9).

References


1. **Corpechot C, Robel P, Axelson M, Sjovall J, Baulieu EE** 1981 Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. Proc Natl Acad Sci U S A 78:4704-4707
2. **Robel P, Bourreau E, Corpechot C, Dang DC, Halberg F, Clarke C, Haug M, Schlegel ML, Synguelakis M, Vourch C, et al.** 1987 Neuro-steroids: 3 beta-hydroxy-delta 5-derivatives in rat and monkey brain. J Steroid Biochem 27:649-655
3. **Baulieu EE, Robel P** 1998 Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids. Proc Natl Acad Sci U S A 95:4089-4091
4. **Le Goascogne C, Sananes N, Gouezou M, Takemori S, Kominami S, Baulieu EE, Robel P** 1991 Immunoreactive cytochrome P-450(17 alpha) in rat and guinea-pig gonads, adrenal glands and brain. J Reprod Fertil 93:609-622
5. **Mellon SH, Deschepper CF** 1993 Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. Brain Res 629:283-292
6. **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 P45017alpha and P450 aromatase localized in neurons. Proc Natl Acad Sci U S A 101:865-870
7. **Kawato S, Hojo Y, Kimoto T** 2002 Histological and metabolism analysis of P450 expression in the brain. Methods Enzymol 357:241-249
8. **Mukai H, Tsurugizawa T, Ogiue-Ikeda M, Murakami G, Hojo Y, Ishii H, Kimoto T, Kawato S** 2006 Local neurosteroid production in the hippocampus: influence on synaptic plasticity of memory. Neuroendocrinology 84:255-263
9. **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

10. **Ebner MJ, Corol DI, Havlikova H, Honour JW, Fry JP** 2006 Identification of neuroactive steroids and their precursors and metabolites in adult male rat brain. Endocrinology 147:179-190
11. **Higashi T, Ninomiya Y, Iwaki N, Yamauchi A, Takayama N, Shimada K** 2006 Studies on neurosteroids XVIII LC-MS analysis of changes in rat brain and serum testosterone levels induced by immobilization stress and ethanol administration. Steroids 71:609-617
12. **Liere P, Akwa Y, Weill-Engerer S, Eychenne B, Pianos A, Robel P, Sjovall J, Schumacher M, Baulieu EE** 2000 Validation of an analytical procedure to measure trace amounts of neurosteroids in brain tissue by gas chromatography-mass spectrometry. J Chromatogr B Biomed Sci Appl 739:301-312
13. **Liu S, Sjovall J, Griffiths WJ** 2003 Neurosteroids in rat brain: extraction, isolation, and analysis by nanoscale liquid chromatography-electrospray mass spectrometry. Anal Chem 75:5835-5846
14. **Yamashita K, Kobayashi S, Tsukamoto S, Numazawa M** 2007 Synthesis of pyridine-carboxylate derivatives of hydroxysteroids for liquid chromatography-electrospray ionization-mass spectrometry. Steroids 72:50-59
15. **Yamashita K, Okuyama M, Watanabe Y, Honma S, Kobayashi S, Numazawa M** 2007 Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography-electrospray ionization tandem mass spectrometry. Steroids 72:819-827
16. **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
17. **Maltais R, Luu-The V, Poirier D** 2002 Synthesis and optimization of a new family of type 3 17 beta-hydroxysteroid dehydrogenase inhibitors by parallel liquid-phase chemistry. J Med Chem 45:640-653

18. **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
19. **Toran-Allerand CD, Tinnikov AA, Singh RJ, Nethrapalli IS** 2005 17alpha-estradiol: a brain-active estrogen? Endocrinology 146:3843-3850
20. **Gao CQ, Dhooge WS, Kaufman JM, Weyne JJ, Eechaute WP** 2002 Hypothalamic 5 alpha-reductase and 3 alpha-oxidoreductase activity in the male rat. J Steroid Biochem Mol Biol 80:91-98
21. **Meffre D, Pianos A, Liere P, Eychenne B, Cambourg A, Schumacher M, Stein DG, Guennoun R** 2007 Steroid profiling in brain and plasma of male and pseudopregnant female rats after traumatic brain injury: analysis by gas chromatography/mass spectrometry. Endocrinology 148:2505-2517
22. **Balthazart J, Ball GF** 2006 Is brain estradiol a hormone or a neurotransmitter? Trends Neurosci 29:241-249
23. **Garcia-Segura LM, Melcangi RC** 2006 Steroids and glial cell function. Glia 54:485-498
24. **Zwain IH, Yen SS** 1999 Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. Endocrinology 140:3843-3852
25. **Prange-Kiel J, Wehrenberg U, Jarry H, Rune GM** 2003 Para/autocrine regulation of estrogen receptors in hippocampal neurons. Hippocampus 13:226-234
26. **Agis-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 U S A 103:14602-14607
27. **Matsui D, Sakari M, Sato T, Murayama A, Takada I, Kim M, Takeyama K, Kato S** 2002 Transcriptional regulation of the mouse steroid 5alpha-reductase type II gene by progesterone in brain. Nucleic Acids Res 30:1387-1393
28. **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: 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase in the rat brain. Endocrinology

144:2902-2911

**Legends to figures**

**Figure S1**
Steroid derivatives and their fragmented ions used for analysis with LC-MS/MS. (A) E2-3-pentafluorobenzoxy-17-picolinoyl-ester (m/z = 558) and its fragmented ion (m/z = 339), (B) T-17-picolinoyl-ester (m/z = 394) and its fragmented ion (m/z = 253), (C) DHT-17-picolinoyl-ster (m/z = 396) and its fragmented ion (m/z = 203). Picolinoyl-ester is used for induced-inonization, and pentafluorobenzoxy-ester is particularly used to increase evaporation probability of E2. E1-17-picolinoyl-ester (m/z = 376) and its fragmented ion (m/z = 157) are illustrated in elsewhere (15). Note that PROG and ADione were measured without derivatization.

**Figure S2**
Calibration curves for LC-MS/MS using standard steroids dissolved in ethanol. Horizontal (x) axis indicates the concentration of added standard steroid. Vertical (y) axis indicates the relative intensity obtained from the chromatogram. (A) Calibration curve for E2. Linearity was observed between 0.1 pg/mL to 1000 pg/mL (in this figure only until 400 pg/mL is shown). (B) Calibration curve for T. Linearity was observed between 0.5 pg/mL to 1000 pg/mL. (C) Calibration curve for DHT. Linearity was observed between 0.5 pg/mL to 1000 pg/mL.

**Figure S3**
Pathways of steroidogenesis in the hippocampus. The abbreviated names of steroid (underline) and enzyme (italic) involved in each reaction are indicated. A chain arrow from ADione to E1 indicates an extremely weak conversion.

**Figure S4**
Normal phase HPLC elution profile of $^{3}$H-DHT metabolites. Only 3α,5α-androstanediol was observed. The arrow designates the elution peak position calibrated with standard $^{3}$H–3α,5α-androstanediol. The vertical axis indicates $^{3}$H radioactivity (cpm).

**Figure S5**
Re-chromatography of the peaks of [$^3$H]-steroid metabolites using reverse-phase HPLC using an octadesyl-coated silica gel column (0.46×15 cm (Cosmosil 5$C_{18}$-MS, Nacalai Tesque, Japan). Each fraction of [$^3$H]-steroid metabolites observed in normal phase HPLC was collected and reconstituted to the elution solvent (acetonitrile: $H_2O$ = 40:60) of reverse-phase HPLC. (A) Reverse-phase HPLC profiles of [$^3$H]-E2 fractions collected from peaks of E2 in Fig. 2D. (B) Reverse-phase HPLC profiles of [$^3$H]-T fractions collected from peaks of T in Fig. 2C. (C) Reverse-phase HPLC profiles of [$^3$H]-androstanediol fractions collected from peaks of androstanediol in Fig. S4. Arrows indicate the positions of standard radioactive steroids. Because the reverse-phase HPLC has a completely different condition from normal phase HPLC, these results demonstrate good identification of each steroid.

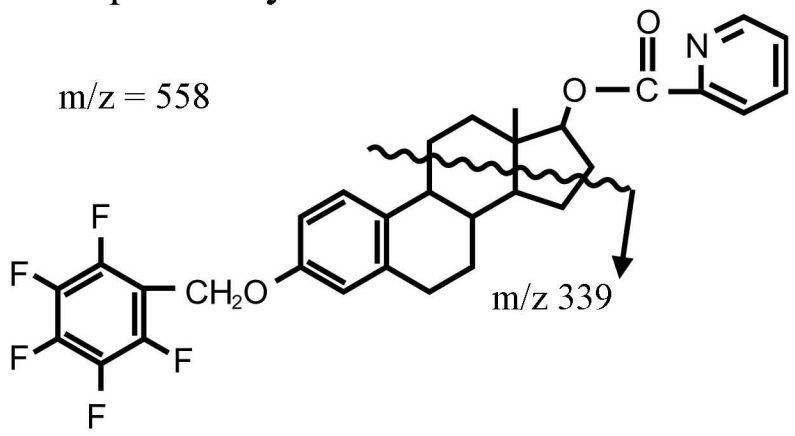
**Figure S6**
RT-PCR analysis of steroidogenic enzymes in the adult male hippocampus. For (A) 3β-HSD (type 1), (B) 3β-HSD (type 3), (C) 5α-reductase (type 1), (D) 5α-reductase type2 and (E) 3α-HSD, the RT−PCR products for mRNAs were visualized with ethidium bromide staining (EB). Southern hybridization (SH) of cDNA is also shown. from left to right, size marker (100 bp ladder), Hi(+) hippocampus with template DNA, Hi(-) hippocampus without template DNA, Hy (+) hypothalamus with template DNA, and Hy (-) hypothalamus without template DNA. The right most organ of (A)-(E) is the positive control. Ovary (Ov(+) and Ov(-)) for (A), liver (Li (+) and Li(-)) for (B), (C) and (E) and prostate (Pr(+) and Pr(-)) for (D), respectively. As an internal control, the ethidium bromide staining (EB) of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) is shown on the bottom of each panels. PCR cycles used are indicated for each panel. Total RNAs used were 200 ng for each enzyme.

**Figure S7**
*In situ* hybridization analysis of 5α-reductase (type 1) (E and F), 5α-reductase (type 2) (A and B) and 17β-HSD (type 1) (C and D). In each panel, left (A, C and E) with antisense probes, and right (B, D and F) with sense probes. Reaction time for color development was 6 h for 5α-reductase type1 and 48 h for 5α-reductase (type 2), respectively. For 17β-HSD (type 1), we used Tyramide Signal Amplification Plus DNP(AP) System (Perkin Elmer, USA)

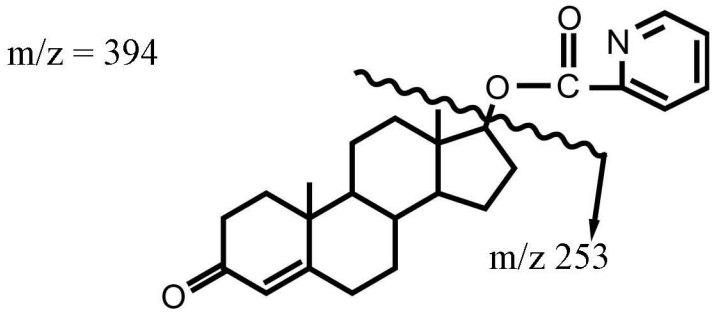
and color development for 48 h in order to further amplify the signals. 5α-reductase and 17β-HSD (type 1) were expressed in pyramidal neurons in CA1-CA3 region and granule cells in DG. As judged from the density of nitro blue tetrazolium staining, the expression level of type 1 (observed after 6 h) was much higher than that of type 2 (observed after 48 h). The expression of 5α-reductase was significant in pyramidal and granule neurons and very weak in glial cells (A and E). The moderate expression of 17β-HSD type 1 was observed in pyramidal neurons (CA1, CA3) and granule neurons (DG) (C). Glial cells did not express 17β-HSD type 1 significantly.

# Fig.S1

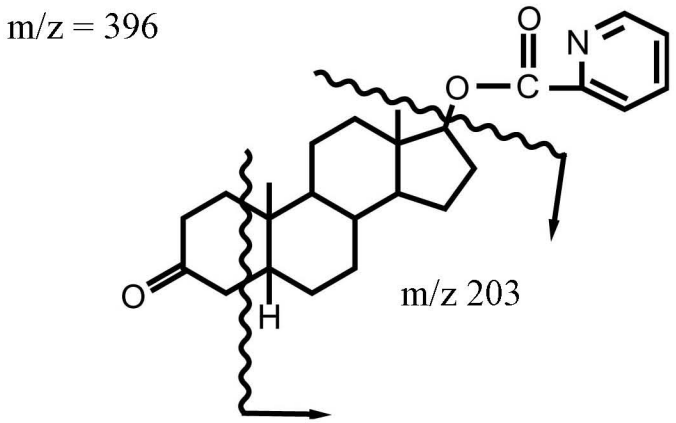
A E2-3-pentafluorobenzyl-17-picolinoyl-ester




B T-17-picolinoyl-ester




C DHT-17-picolinoyl-ester



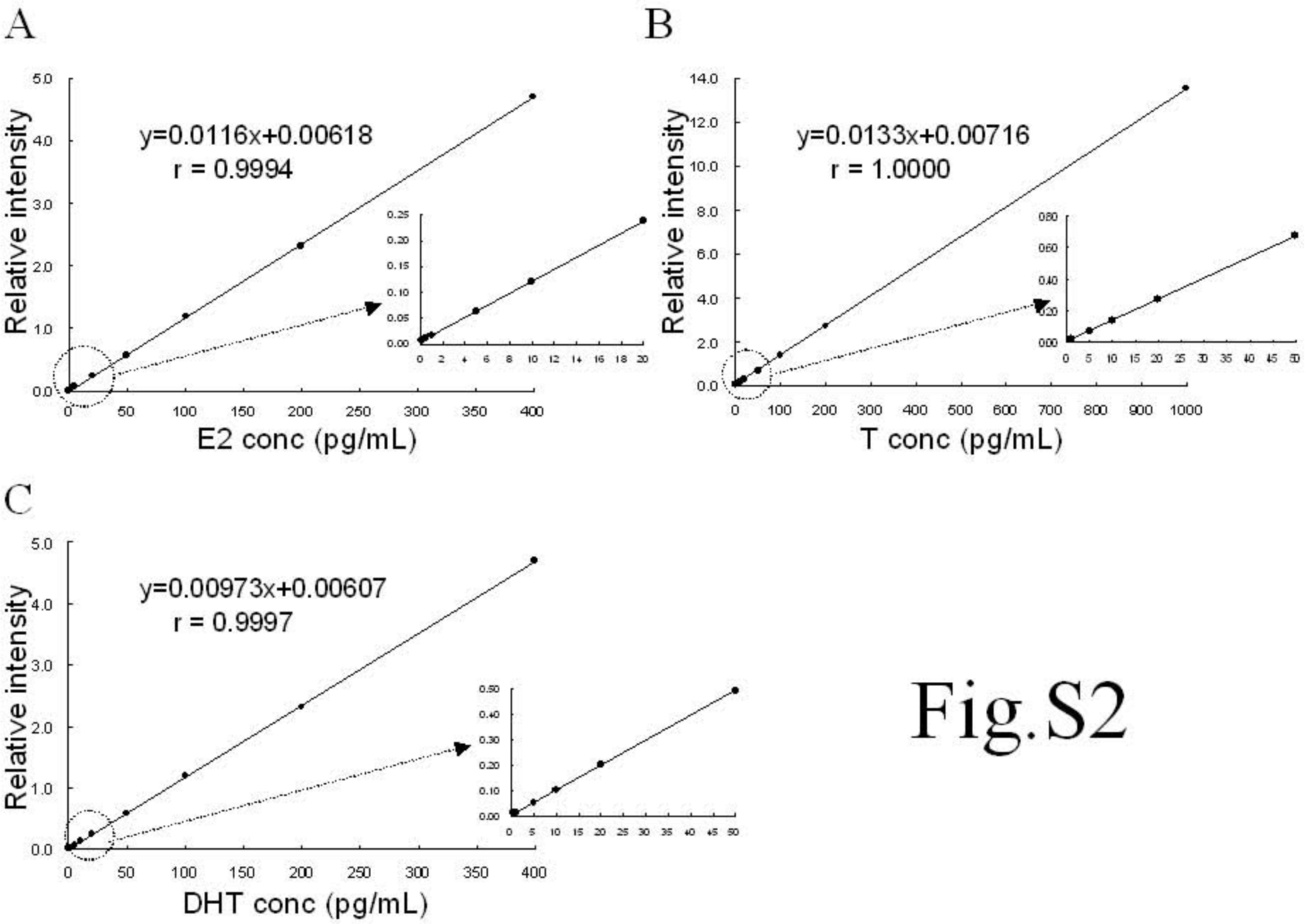
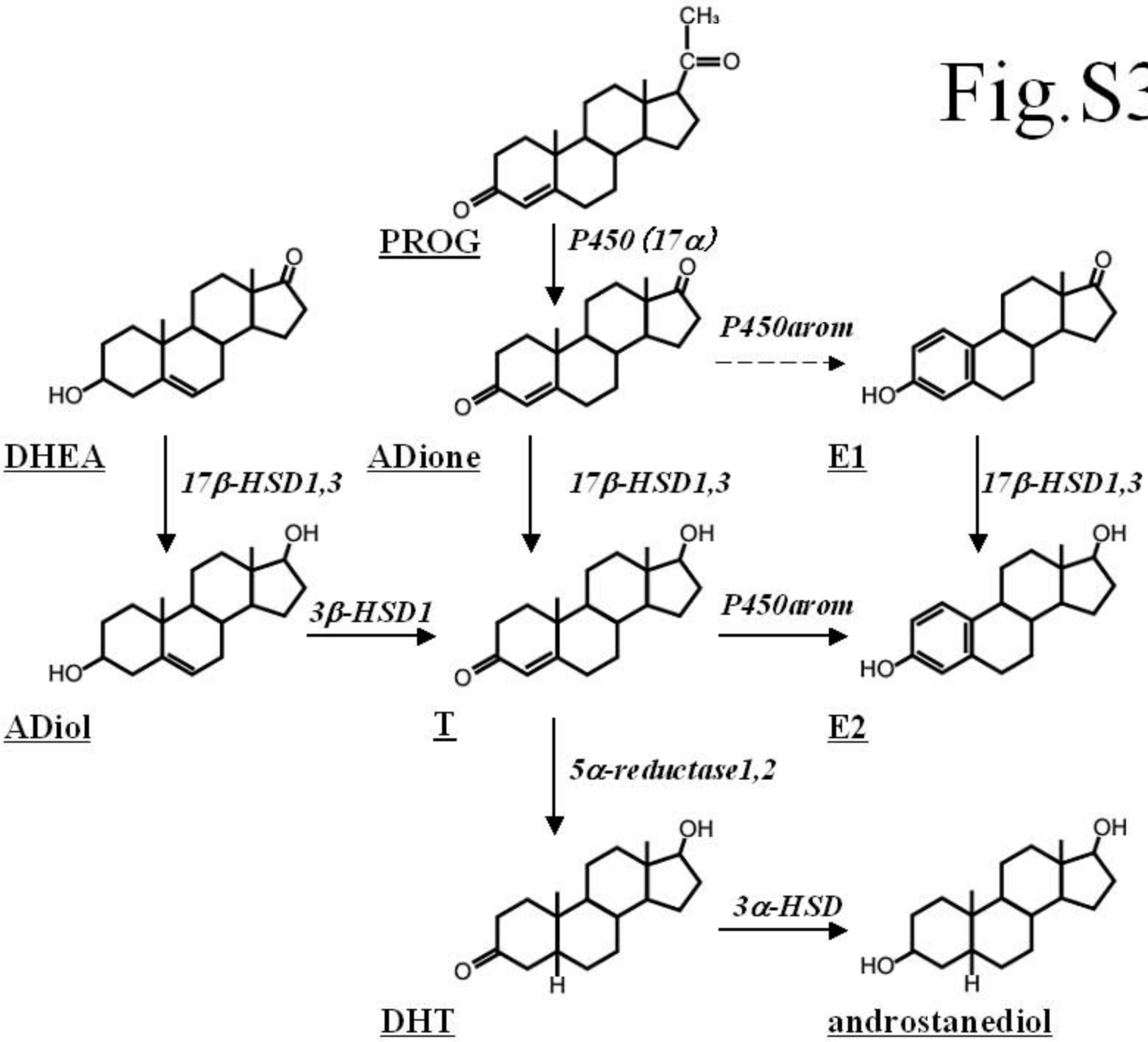

A
y=0.0116x+0.00618
r = 0.9994
Relative intensity
E2 conc (pg/mL)
B
y=0.0133x+0.00716
r = 1.0000
Relative intensity
T conc (pg/mL)
C
y=0.00973x+0.00607
r = 0.9997
Relative intensity
DHT conc (pg/mL)

Fig.S2

Fig.S3


PROG
P450 (17α)
ADione
P450arom
E1
DHEA
17β-HSD1,3
17β-HSD1,3
17β-HSD1,3
ADiol
3β-HSD1
T
P450arom
E2
5α-reductase1,2
DHT
3α-HSD
androstanediol

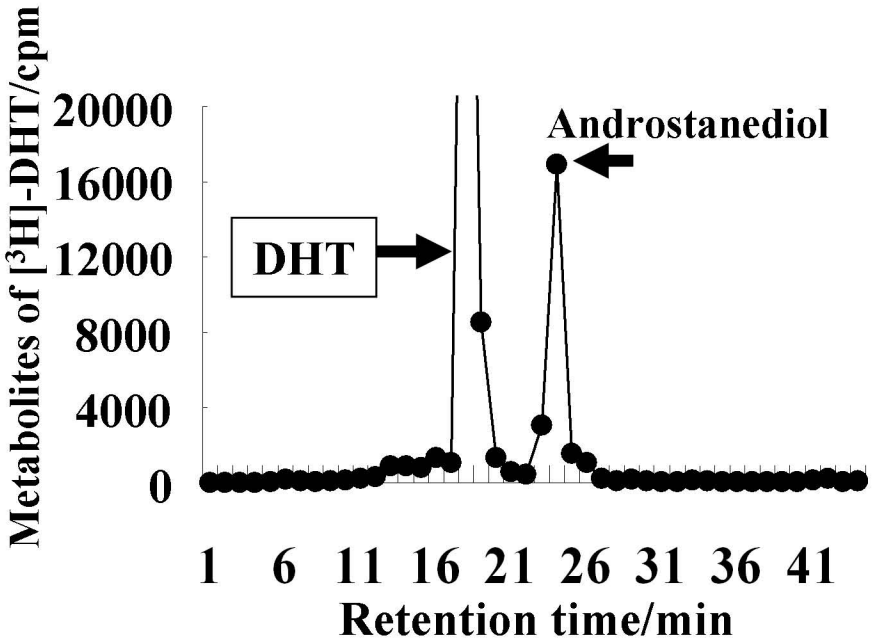
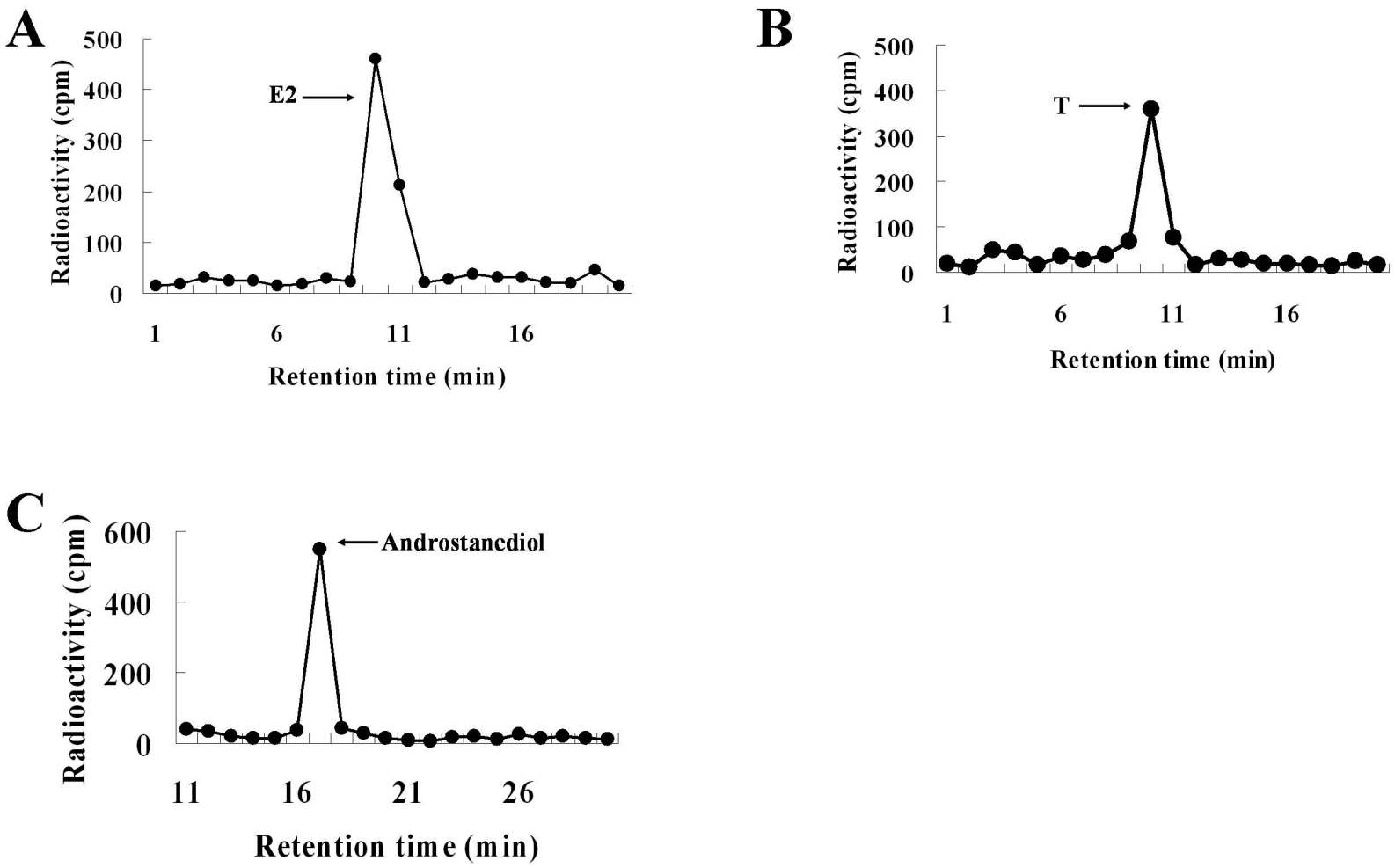

Metabolites of [3H]-DHT/cpm
20000
16000
12000
8000
4000
0
DHT
Androstanediol
1
6
11
16
21
26
31
36
41
Retention time/min

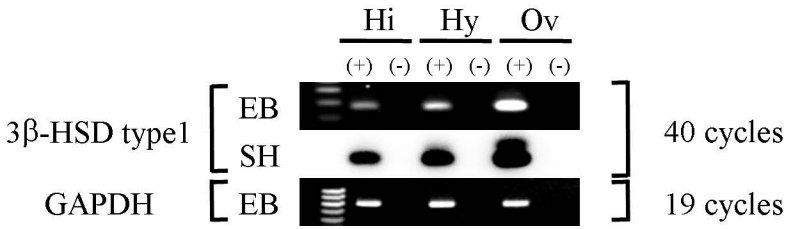
Fig.S4

# Fig.S5

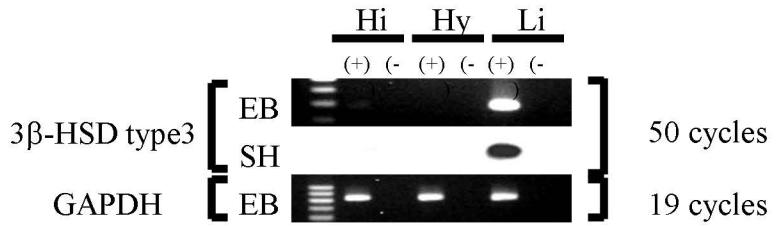

A
Radioactivity (cpm)
500
400
300
200
100
0
E2
1
6
11
16
Retention time (min)
B
Radioactivity (cpm)
500
400
300
200
100
0
T
1
6
11
16
Retention time (min)
C
Radioactivity (cpm)
600
400
200
0
Androstanediol
11
16
21
26
Retention time (min)

# Fig.S6

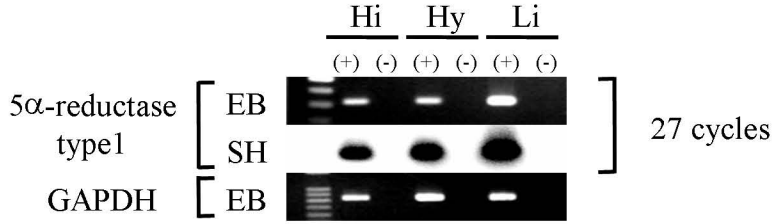
A


Hi
Hy
Ov
(+) (-) (+) (-) (+) (-)
3β-HSD type1
EB
SH
GAPDH
EB
40 cycles
19 cycles

B


Hi
Hy
Li
(+) (- (+) (- (+) (-
3β-HSD type3
EB
SH
GAPDH
EB
50 cycles
19 cycles

C


Hi
Hy
Li
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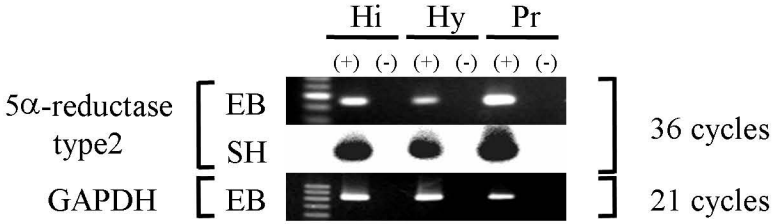
5α-reductase type1
EB
SH
GAPDH
EB
27 cycles

D


Hi
Hy
Pr
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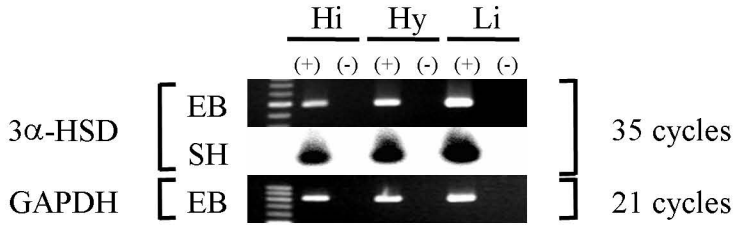
5α-reductase type2
EB
SH
GAPDH
EB
36 cycles
21 cycles

E


Hi
Hy
Li
(+) (-) (+) (-) (+) (-)
3α-HSD
EB
SH
GAPDH
EB
35 cycles
21 cycles

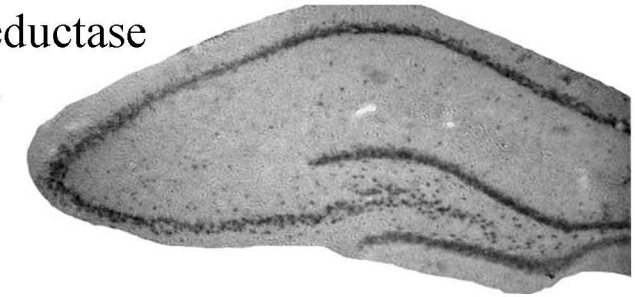
# Fig.S7

Antisense

Sense

A

5α-reductase type2

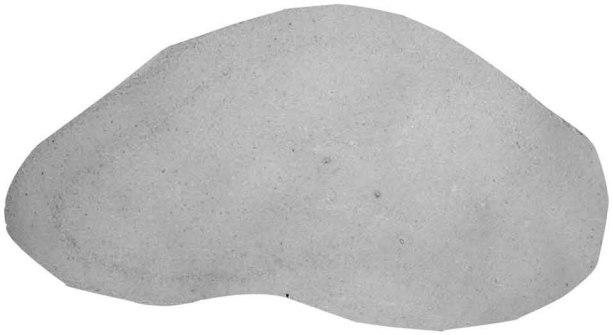


B

C

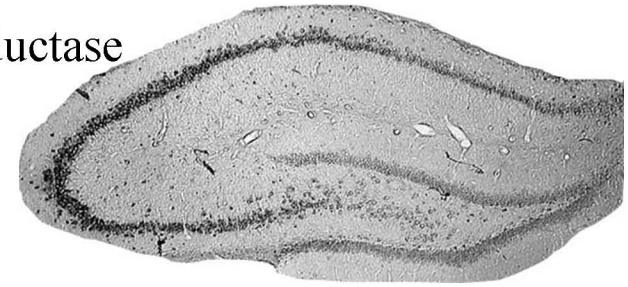
17β-HSD type1

D



E

5α-reductase type1



F

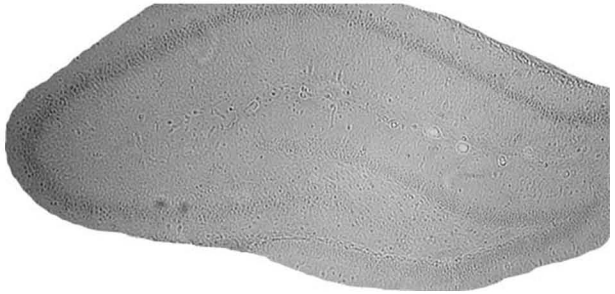


**Table S1**
The accuracy of steroid determination for hippocampal tissue spiked with exogenous steroids.

| | Steroid | Added (ng/g) | Found (ng/g) | Accuracy (%)[a] |
|---|---|---|---|---|
| Hippocampus | E2 | 0.0 | 2.302 | - |
| | | 0.5 | 2.838 (0.536)[b] | 107.2 |
| | | 1.0 | 3.349 (1.047)[b] | 104.7 |
| | T | 0.0 | 5.316 | - |
| | | 0.5 | 5.827 (0.511)[b] | 102.2 |
| | | 1.0 | 6.308 (0.998)[b] | 99.8 |
| | DHT | 0.0 | 2.388 | - |
| | | 0.5 | 2.878 (0.490)[b] | 98.0 |
| | | 1.0 | 3.403 (1.015)[b] | 101.5 |
| | E1 | 0.0 | 0.004 | - |
| | | 0.5 | 4.578 (4.574)[b] | 91.5 |
| | | 1.0 | 4.671 (4.667)[b] | 93.3 |
| | ADione | 0.0 | 0.444 | - |
| | | 0.5 | 0.989 (0.545)[b] | 108.8 |
| | | 1.0 | 1.476 (1.032)[b] | 103.2 |
| | PROG | 0.0 | 4.911 | - |
| | | 0.5 | 5.384 (0.473)[b] | 94.6 |
| | | 1.0 | 5.895 (0.984)[b] | 98.4 |
| | DHEA | 0.0 | 0.078 | - |
| | | 0.5 | 0.627 (0.549)[b] | 109.7 |
| | | 1.0 | 1.134 (1.056)[b] | 105.6 |

[a] Accuracy was expressed as a recovery rate (%) of the measured amount of steroid to the added amount of steroid.
[b] Value in parentheses was obtained by subtraction of the endogenous amount of steroid from the value obtained for each spiked steroid.

**Table S2**
The intra- and inter-assay of accuracy and precision as well as the limit of quantification (LOQ) for each steroid.

| | m/z[b] transition | Spike (pg) | Intraassay (n[a] = 5) Accuracy (%) | RSD[c] (%) | Interassay (n[a] = 3) Accuracy (%) | RSD (%) | LOQ[d] (pg/0.1g) |
|---|---|---|---|---|---|---|---|
| 17β-E2 | from 558 to 339 | 0.3 | 94.4 | 6.4 | 93.3 | 8.9 | 0.3 |
| | | 5 | 97.6 | 3.4 | 95.9 | 6.2 | |
| | | 20 | 100.3 | 2.7 | 101.2 | 1.9 | |
| T | from 394 to 253 | 1 | 108.8 | 3.0 | 96.1 | 3.2 | 1 |
| | | 10 | 94.4 | 2.8 | 98.7 | 3.3 | |
| | | 100 | 100.2 | 1.1 | 101.6 | 2.5 | |
| DHT | from 396 to 203 | 1 | 106.3 | 2.6 | 96.2 | 5.9 | 1 |
| | | 10 | 97.5 | 2.3 | 97.7 | 3.1 | |
| | | 100 | 99.6 | 1.9 | 100.7 | 2.9 | |
| E1 | from 376 to 157 | 1 | 106.1 | 4.6 | 98.7 | 3.6 | 1 |
| | | 10 | 105.2 | 3.7 | 101.1 | 3.0 | |
| | | 100 | 99.1 | 2.1 | 99.2 | 2.8 | |
| PROG | from 315 to 109 | 2 | 107.1 | 5.2 | 104.4 | 5.1 | 2 |
| | | 10 | 105.6 | 3.7 | 95.9 | 4.1 | |
| | | 100 | 102.3 | 1.8 | 96.1 | 1.2 | |
| ADione | from 287 to 109 | 2 | 92.8 | 3.3 | 105.6 | 6.6 | 2 |
| | | 10 | 96.0 | 3.3 | 103.7 | 3.1 | |
| | | 100 | 100.4 | 2.1 | 102.6 | 1.9 | |
| DHEA | from 394 to 175 | 2 | 103.8 | 7.2 | 96.5 | 5.5 | 2 |
| | | 10 | 97.9 | 3.8 | 102.1 | 4.7 | |
| | | 100 | 98.8 | 3.1 | 101.6 | 3.7 | |

Blank samples, prepared alongside hippocampal samples through the whole extraction and purification procedures, were spiked with E2 or other steroids at 0.1, 0.3, 1, 2, 5,10, 20 and 100 pg, and contents were determined by LC-MS/MS. Accuracy was expressed as a percentage of an analytical recovery rate of measured steroid content against spike amount.

[a]For each condition, intra- and interassay were performed five and three times, respectively.

[b]m and z represent the mass and charge of a steroid derivative, respectively.

[c]relative standard deviation.

[d]LOQ is expressed as pg/0.1 g. Because the average weight of one whole adult hippocampus (0.14 g) was close to 0.1 g, these LOQ values indicate the limit of quantification of steroids from nearly one hippocampus.

**Table S3**

The production rate of steroids from each substrate in hippocampal slices of adult male rats.

| metabolism (substrate→product) | enzyme | production rate ($10^4$cpm/g/5h)[a] | number of animals[b] |
|---|---|---|---|
| ADione→T | 17β-HSD (type1 and 3) | 75.5 ± 11.1 [c] | 5 |
| DHEA→ADiol | 17β-HSD (type1 and 3) | 40.5 ± 6.6 | 5 |
| DHEA→ADiol (+RM352-26) [d] | 17β-HSD (type1 and 3) | 15.8 ± 0.9*** [e] | 3 |
| ADiol→T | 3β-HSD (type 1) | 9.0 ± 1.5 | 3 |
| T→E2 | P450arom | 2.3 ± 0.1 | 5 |
| T→DHT | 5α-reductase | 133.0 ± 10.9 | 5 |
| DHT→androstanediol | 3α-HSD | 180.0 ± 13.9 | 3 |
| E1→E2 | 17β-HSD (type1 and 3) | 26.4 ± 3.6 | 5 |

[a] The production rate is given by:

$$\frac{\text{(Count of products from } 10^6 \text{ cpm of substrate) [cpm]}}{\text{(incubation time) [5hour]} \cdot \text{(hippocampal wet weight) [g]}}$$

[b] Number of animals (i.e. the number of hippocampi).

[c] Data are expressed as mean ± standard error.

[d] A specific inhibitor of 17β-HSD (type3).

[e] Statistical significance ***, $P < 0.005$, when compared with the production rate of DHEA→ADiol without RM352-26.

**Table S4**

The sequence of primer oligonucleotides for PCR amplification.

| target mRNA | | sequence |
|---|---|---|
| 5α–reductase (type1) | Forward | ACCGCGTCCTGCTGGCTATGTTT |
| | Reverse | GGCCTCCCCTGGGTATCTTGTATCC |
| 5α–reductase (type2) | Forward | AGGTGGCTTGTTTACGTATGTCTCTG |
| | Reverse | GGCCTCTGTGAAGCTCCAAAAG |
| 3β-HSD (type1) | Forward | AGGGCATCTCTGTTGTCATCCAC |
| | Reverse | TGCCTTCTCGGCCATCCTTT |
| 3β-HSD (type2) | Forward | ATCTCTGTTGTCATTCACACGGCTTC |
| | Reverse | CACTGCCTTCTCGGCCATCTT |
| 3β-HSD (type3) | Forward | CTTCCTCTGCCCCTGCTCTACTGG |
| | Reverse | GTCCCTGCCCTCTTCCCATCATTG |
| 3β-HSD (type4) | Forward | CTTCCTCTGCCCCTGCTCTACTGG |
| | Reverse | ATGTCCCTGCCCTCTTCCCATTAC |
| 3α-HSD | Forward | GGAATGTCACCTTTATCTCAACCA |
| | Reverse | ATGCATTCAGTCACCAGTATCCA |
| 17β-HSD (type1) | Forward | ACTCCGGGCGTGTGCTGGTGA |
| | Reverse | GGCGTGTCTGGATCCCCTGAAACTT |
| 17β-HSD (type3) | Forward | CTCCCCAACCTGCTCCCAAGTC |
| | Reverse | CAAGGCAGCACAGGTTTCAGC |