ESCC. The status of SXR immunoreactivity in carcinoma cells was correlated with several favorable clinicopathological parameters associated with a better clinical outcome for patients.

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Retinoic Acid Stimulates 17β-Estradiol and Testosterone Synthesis in Rat Hippocampal Slice Cultures

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The hippocampus is essentially involved in learning and memory processes. Its functions are affected by various neuromodulators, including 17β-estradiol, testosterone, and retinoid. Brain-synthesized steroid hormones act as autocrine and paracrine modulators. The regulatory mechanism underlying brain steroidogenesis has not been fully elucidated. Synthesis of sex steroids in the gonads is stimulated by retinoic acids. Therefore, we examined the effects of retinoic acids on estradiol and testosterone biosynthesis in the rat hippocampus. We used cultured hippocampal slices from 10- to 12-d-old male rats to investigate *de novo* steroidogenesis. The infant rat hippocampus possesses mRNAs for steroidogenic enzymes and retinoid receptors. Slices were used after 24 h of preculture to obtain maximal steroidogenic activity because steroidogenesis in cultured slices decreases with time. The mRNA levels for $P450_{17\alpha}$, P450 aromatase and estrogen receptor-β in the slices were increased by treatment with 9-*cis*-retinoic acid but not by all-*trans*-isomer. The magnitude of stimulation and the shape of the dose-response curve for the mRNA level for $P450_{17\alpha}$ were similar to those for cellular retinoid binding protein type 2, the transcription of which is activated by retinoid X receptor signaling. 9-*cis*-Retinoic acid also induced a 1.7-fold increase in the protein content of $P450_{17\alpha}$ and a 2-fold increase in *de novo* synthesis of 17β-estradiol and testosterone. These steroids may be synthesized from a steroid precursor(s), such as pregnenolone or other steroids, or from cholesterol, as so-called neurosteroids. The stimulation of estradiol and testosterone synthesis by 9-*cis*-retinoic acid might be caused by activation of $P450_{17\alpha}$ transcription via retinoid X receptor signaling. **(*Endocrinology* 150: 4260–4269, 2009)**

17β-Estradiol and testosterone are sex steroids that act on various organs, including the brain, to regulate reproductive behavior (1). In addition, there is evidence of sex steroid actions on nonreproductive functions in the developing, adult, and aging brain. Estradiol treatment has been observed to enhance spatial memory by what is considered to be stimulation of hippocampal function (2, 3). In rat hippocampal slices, the density of spines on the pyramidal neurons in the CA1 region of the hippocampus, and long-term depression in the CA1, CA3, and dentate gyrus regions, are rapidly enhanced by 1 nM 17β-estradiol (4, 5). It is also reported that 17β-estradiol has neuroprotective effects (6). More recently, androgen has been reported to play important roles in the hippocampus, such as modulation of syn-

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Abbreviations: CRBP-2, Cellular retinol binding protein type 2; ER, estrogen receptor; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase; 17β-HSD, steroid 17β-hydroxysteroid dehydrogenase; NMDA, *N*-methyl-D-aspartate; $P450_{11\beta}$, CYP11B1, cytochrome P450 having steroid 11β-hydroxylation activity; $P450_{17\alpha}$, CYP17, cytochrome P450 having steroid 17α-hydroxylation and C17-C20 side-chain cleavage activity; P450arom, CYP19, cytochrome P450 having steroid aromatization activity; $P450_{C21}$, CYP21, cytochrome P450 having steroid C21-hydroxylation activity; P450scc, CYP11A1, cytochrome P450 having steroid C20-C22 side-chain cleavage activity; RAR, retinoic acid receptor; RXR, retinoid X receptor; StAR, steroidogenic acute regulatory protein.

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aptic density (7, 8). These data indicate the essential role of sex steroids in the hippocampus.

Brain 17β-estradiol and testosterone can be derived from peripheral steroidogenic organs via the blood stream and *de novo* synthesis at specific brain regions, from steroid precursor(s), or from cholesterol as neurosteroids (9). Brain-synthesized steroid hormones act as an autocrine and paracrine modulators (10–12). The adult rat hippocampus possesses active steroidogenic enzymes for the *de novo* synthesis of 17β-estradiol and testosterone, such as cytochrome P450 having steroid C20-C22 side-chain cleavage activity (P450scc), 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD), cytochrome P450 having steroid 17α-hydroxylation and C17-C20 side-chain cleavage activity ($P450_{17\alpha}$), steroid 17β-hydroxysteroid dehydrogenase (17β-HSD) types 1 and 3, and cytochrome P450 having steroid aromatization activity (P450arom) (12–16). Estradiol synthesis has been demonstrated in cultured rat hippocampal slices and dispersed cells (15, 17). A significant amount of 17β-estradiol must be synthesized in the organ because the estradiol content in the hippocampus is six times higher than that in plasma (15). The *de novo* synthesized 17β-estradiol plays important roles in the hippocampus. Treatment of cultured rat hippocampal neurons with letrozole, a specific inhibitor of P450arom, induced decreases in estradiol synthesis and resulted in decreases in the density of spine synapses and the number of presynaptic boutons (17). Letrozole treatment induced apoptosis of hippocampal neurons, suggesting that 17β-estradiol might be involved in neuroprotection (18, 19). Although significant roles of brain-derived steroids have been demonstrated, the mechanism underlying the regulation of steroid hormone biosynthesis in brain has not been fully elucidated.

Retinoids, which are vitamin A derivatives, play important roles in the central nervous system during development and maintenance in adulthood as well as in other tissues (20–24). Retinoid signaling is involved in the mature brain via its involvement in cellular and synaptic plasticity processes (20). Most retinoid functions are performed by retinoic acids (24). All-*trans*-retinoic acid is the most abundant retinoic acid species. 9-*cis*-Isomer has been claimed to be involved in retinoid function, although its biological role remains controversial (24). The retinoic acids work as gene regulators via ligand-activated transcription factors, namely the retinoic acid receptors (RARs)-α, -β, and -γ, and the retinoid X receptors (RXRs)-α, -β, and -γ (25, 26). All-*trans*-retinoic acid activates RARs, and 9-*cis*-isomer activates both the RARs and RXRs. mRNAs for all of these receptors except RARγ have been detected in the brains of adult mice and rats, including the hippocampus (27). A functional role for retinoid signaling in hippocampal plasticity and spatial memory has been demonstrated in rodents by knockout of RARβ or RXRβ/γ, vitamin A deprivation, and retinoic acid administration (28, 29). Retinoic acids are neuroprotective in the rat hippocampus (30).

Although the functions of the retinoic acids and sex steroids in the adult hippocampus are similar, cross talk between their signaling pathways has not been focused on.

Steroid hormone synthesis is stimulated by retinoid in peripheral steroidogenic organs. In mouse Leydig cells, retinoid stimulate steroidogenic acute regulatory protein (StAR) gene expression and promoter function as well as steroidogenesis (31). The level of the mRNA for $P450_{17\alpha}$ is enhanced by all-*trans*-retinoic acid in the K9 mouse Leydig cell line (32). Gene expression of StAR, $P450_{17\alpha}$, and P450scc and production of testosterone and dehydroepiandrosterone are stimulated by all-*trans*- and/or 9-*cis*-retinoic acid in human ovarian thecal cells (33).

Based on the findings described above, we hypothesized that retinoic acid modifies sex steroid biosynthesis in the hippocampus. To test this hypothesis, we used cultured hippocampal slices from 10- to 12-d-old rats. Because the steroidogenic activity in cultured slices decreases with time, we used the slices after only 24 h preculture. After the treatment of these slices with 9-*cis*-retionoic acid for 48 h, the mRNA contents for $P450_{17\alpha}$ and P450arom, protein content of $P450_{17\alpha}$, and biosynthesis of 17β-estradiol and testosterone, were increased significantly. The increase was concomitant with an increase in the level of mRNA for cellular retinoid binding protein type 2 (CRBP-2), transcription of which is activated by RXR signaling. This stimulation did not affect the level of adrenal 4 binding protein/steroidogenic factor-1 (NR5A1), which is a major transcriptional activator for steroidogenic enzymes in peripheral organs.

Materials and Methods

Quantitative RT-PCR

Hippocampi were quickly isolated from 10-d-old male Wistar rats (SLC, Shizuoka, Japan). The hippocampus was homogenized in 1 ml of buffer RLT (RNeasy minikit; QIAGEN, GmbH, Hilden, Germany) by vigorous agitation with a zirconia ball on a Micro Smash MA-100 mixer-mill disruptor (TOMY, Tokyo, Japan) for two periods of 30 sec. The protein content of the homogenate was determined using protein assay CBB solution (Nacalai Tesque, Kyoto, Japan) with bovine γ-globulin as a standard. Total RNA was extracted from the homogenate using an RNeasy minikit (QIAGEN) in accordance with the manufacturer's protocol. After the RNA was incubated with deoxyribonuclease I (ribonuclease free; Takara, Otsu, Japan), single-stranded hippocampal cDNA was prepared from 5 μg of total RNA following the ReverTra Ace protocol (Toyobo, Osaka, Japan) with a random primer (9-mer; Takara). Rat ovarian cDNA was also prepared from the ovaries of 6-month-old female Wistar rats using the same method.

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The transcript levels for steroidogenic enzymes and related proteins, as well as for receptors, were determined by real-time PCR. The primers for the PCR were designed using the online Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). The sequences of the designed primers, the sizes of the PCR products, and the annealing temperatures for PCR can be found in the supplemental data, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Real-time PCR was performed using a LightCycler (Roche Diagnostics, Basel, Switzerland) in a total reaction mixture volume of 20 μl containing 10 μl of Sybr Green Real-time PCR master mix (Toyobo), 100–600 ng hippocampal cDNA and 2.5–10 pmol of each of the primers. In some tubes, serially diluted PCR products were used as the quantification standard instead of hippocampal cDNA. Detailed methods for synthesis of the quantification standard and for real-time PCR are available in the supplemental data. The amount of mRNA in the rat hippocampus is given as the number of mRNA molecules per milligram of protein in the hippocampus homogenate.

Rat hippocampal organotypic slice cultures

Rat hippocampal slices were prepared from 10- to 12-d-old male Wistar rats and cultured as described (34). Approximately 12–15 slices of 0.3 mm thickness were obtained from one hippocampus. One or two slices were randomly placed on each of 12 culture membranes (Millicell; Millipore, Temecula, CA). Finally, about 15 slices from the 12 hippocampi of six rats were placed on each membrane. After 24 h of preculture with serum-containing medium, the medium was changed to serum-free medium consisting of 75% MEM and 25% Hanks' balanced salt solution, and slices were incubated for an additional 48 h with or without 9-*cis*- or all-*trans*-retinoic acid (Sigma-Aldrich, St. Louis, MO). The serum-free medium containing 0.1–10 μM 9-*cis*- or all-*trans*-retinoic acid was changed every 12 h. In some experiments, the slices were cultured for 11 d in serum-free medium. The cell viability was determined by the propidium iodide uptake method after a 48-h incubation, as described (35). The viability of the slices was not altered by incubation with or without retinoic acids.

After the incubation, the slices on one Millicell membrane were scraped and homogenized in 1 ml of buffer RLT for mRNA analysis, as described above. The mRNA content of each enzyme in retinoic acid-treated slices was compared with that in nontreated slices. In some experiments, slices were homogenized for quantification of $P450_{17\alpha}$ protein or sex steroid levels, as described below.

The experimental protocol was in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University (Higashi-Hiroshima, Japan) and was approved.

Western blotting of cultured rat hippocampal slices

After the hippocampal slices were incubated for 48 h with or without 1 μM 9-*cis*-retinoic acid, the slices on six membranes were scraped and homogenized in 1 ml of 0.02% sodium dodecyl sulfate. Rat testis was also homogenized in 0.02% sodium dodecyl sulfate. The protein contents of the hippocampus and testis homogenates were determined using a BCA protein assay kit (Pierce, Rockford, IL) with BSA as a standard. Fifty micrograms of solubilized hippocampus and 2.5 μg of testis protein were separated by SDS-PAGE on a 10% polyacrylamide gel. Western blotting was conducted as described previously using specific antibodies against glyceraldehyde 3-phosphate dehydrogenase (Millipore) or guinea pig $P450_{17\alpha}$ (36, 37). The immunoreactive bands were visualized using ECL+ chemiluminescence detection reagent (GE Healthcare UK, Amersham Place, UK). Using the antibody for $P450_{17\alpha}$, one specific band was detected in rat hippocampal microsome fractions and bovine adrenal homogenates by Western blotting (15, 36). The intensities of the chemiluminescence of specific bands for $P450_{17\alpha}$ were digitized using a FAS-1000 image analyzer (Toyobo).

Estradiol and testosterone content in the slices and cultured medium

After collecting the cultured medium, the hippocampal slices on four membranes were scraped and put into 2-ml homogenization tubes (Assist, Tokyo, Japan). The slices were homogenized with 1.1 ml of lysis solution, consisting of 150 mM NaCl and 0.2% Tween 20, by vigorous agitation with a zirconia ball on a Micro Smash MA-100 (TOMY) for 30 sec. The protein contents of the homogenates were determined using a BCA protein assay kit (Pierce). To the homogenates and culture media from four membranes, 2000 cpm of [2, 4, 6, 7, 16, 17-^{3}H]17β-estradiol or [1, 2, 6, 7-^{3}H]testosterone (GE Healthcare) were added to determine the recovery levels. Steroids were extracted and partially purified by C18 minicolumn as described (15). For 17β-estradiol analysis, the eluate from the C18 column was further purified by normal-phase HPLC and analyzed by RIA as described (15). Contaminating lipids were removed during HPLC. The recovery level was 50–70%.

For testosterone analysis, the eluate from the C18 column was reconstituted in EIA buffer (Cayman, Ann Arbor, MI) and analyzed using a testosterone EIA kit (Cayman) according to the manufacturer's protocol. The specificities of this kit were as follows: 19-nortestosterone 140%, testosterone 100%, 5α-dihydrotestosterone 27%, androstenedione 3.7%, and 17β-estradiol less than 0.01%. The detection limit was 2 fmol per sample. The recovery level was 55–75%.

Statistical analysis

Each experiment was conducted independently at least three times with different set of rats. The statistical significance of differences in the levels of mRNAs for steroidogenic proteins and estrogen receptors (ERs) induced by retinoid were analyzed by two-way ANOVA for the data from each Millicell membrane. Incubation was repeated four times and performed in triplicate membranes. The levels of estradiol and testosterone were analyzed by one-way ANOVA for four sets of data. If differences were found to be significant by these ANOVAs, the analyses were followed by *post hoc t* test with Bonferroni correction. The differences in the levels of $P450_{17\alpha}$ protein were analyzed by the Student's *t* test for three sets of data. The criterion for significance was $P < 0.05$. All results are expressed as means ± SD.

Results

Transcript levels of StAR, steroidogenic enzymes, and retinoid receptors in rat hippocampus

To determine *de novo* steroidogenic activity, we used hippocampal slices from 10- to 12-d-old rats in this study.

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We first quantified the transcript levels of StAR and steroidogenic enzymes in freshly isolated hippocampi from 10-d-old rats. StAR protein stimulates cholesterol transport into the mitochondrial inner membrane, in which P450scc converts cholesterol into pregnenolone (37). Pregnenolone is further metabolized to 17β-estradiol by the activities of $P450_{17\alpha}$, 3β-HSD, 17β-HSD type 1, 17β-HSD type 3, and P450arom (11). 17β-HSD type 4 catalyzes the reverse reactions to those catalyzed by 17β-HSD type 1, namely 17β-estradiol to estrone or androstenediol to dehydroepiandrosterone (11). The mRNA levels for each enzyme were variable: 110 molecules per milligram of protein for P450scc to 28 million molecules per milligram of protein for 17β-HSD type 3 (Table 1). No signals were observed by real-time PCR using hippocampal cDNA and the primers for $P450_{C21}$, 5′-CAGAATACCGACCTTTGG-3′ and 5′-CCGTAGTTAGAGAATTAAGGA-3′, or those for 17β-HSD type 2, 5′-GCTGGGGTCTTGCACTTTCC-3′ and 5′-TAAAGTCTACCACCGGCGG-3′. $P450_{C21}$ catalyzes the reactions from progesterone to 11-deoxycorticosterone or 17α-hydroxyprogesterone to 11-deoxycortisol (37). 17β-HSD type 2 catalyzes the reverse reaction to that catalyzed by 17β-HSD type 3, namely the conversion of testosterone into androstenedione (11). The amount of P450scc mRNA was lowest in the mRNAs listed in Table 1, the activity of P450scc may be the rate-limiting step for steroidogenesis from cholesterol. Alternatively, hippocampal sex steroids may be synthesized from precursors other than cholesterol, such as pregnenolone or progesterone, which can be derived from the blood stream.

The expression levels of retinoid receptors in the rat hippocampus were analyzed by real-time PCR. The hippocampi of 10- to 12-d-old rats expressed significant levels of mRNAs for the retinoid receptors RXRα, -β, and -γ and RARα, -β, and -γ (Table 1). The levels of mRNA for these receptors were similar, about 10^6 molecules per milligram of protein. The mRNA contents for these receptors were higher than those for ERβ (Table 1).

After hippocampal slices from 10-d-old rats had been cultured for 24 h, the total RNA content decreased to about one third of the level in freshly isolated hippocampus and then remained stable during the following 2 wk of culture. The relative contents of mRNAs for steroidogenic enzymes and receptors in cultured slices were not significantly altered from those in fresh hippocampus (data not shown).

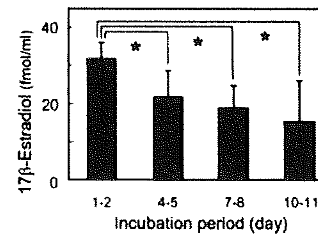

FIG. 1. 17β-Estradiol content in medium from hippocampal slice cultures. Hippocampal slices were precultured with serum-containing medium for 24 h and then cultured with serum-free medium. The medium was changed daily, and medium collected on d 1 and 2, 4 and 5, 7 and 8, and 10 and 11 were combined. The 17β-estradiol content of the combined medium from six membranes was determined by RIA as described in *Materials and Methods*. The values are means for three independent cultures. *Error bars*, SD. *, $P < 0.05$, *post hoc t* test with Bonferroni correction, compared with d 1 and 2.

Estradiol synthesis in cultured hippocampal slices

To measure *de novo* synthesis of sex steroids, hippocampal slices must be cultured in serum-free medium to avoid any supply of steroids from the serum. The secretion of 17β-estradiol from hippocampal slices from 10- to 12-d-old rats was determined during 11 d of culture in serum-free medium after 24 h preculture in serum-containing medium. The 17β-estradiol contents in serum-free medium collected on d 1 and 2, 4 and 5, 7 and 8, and 10 and 11 were determined by RIA (Fig. 1). One-way ANOVA revealed a main effect of the incubation period [$F(3,11) = 7.76$, $P < 0.01$]. The amount of secreted 17β-estradiol in the serum-free medium was highest on d 1 and 2 and then decreased gradually. Because hippocampal slices retain the ability for neuronal development and the formation of new synaptic connections after 2–3 wk in culture, many studies have been performed using hippocampal slices after a few weeks of preculture (38). However, the maximum level of estradiol synthesis could be obtained in slices after a short preincubation period. The slices were hardly attached to the culture membrane without serum during

TABLE 1. Amounts of mRNAs for StAR protein, steroidogenic enzymes, and receptors in freshly isolated rat hippocampus (molecules per milligram of protein)

mRNA	Content (molecules/milligram protein)	mRNA	Content (molecules/milligram protein)
StAR	$220 \times 10^3 \pm 60 \times 10^3$	RXRα	$1.2 \times 10^6 \pm 0.2 \times 10^6$
P450scc	110 ± 0.15	RXRβ	$1.9 \times 10^6 \pm 0.2 \times 10^6$
$P450_{17\alpha}$	$57 \times 10^3 \pm 1.8 \times 10^3$	RXRγ	$0.59 \times 10^6 \pm 0.5 \times 10^6$
3β-HSD-1	$9.7 \times 10^3 \pm 3.9 \times 10^3$	RARα	$1.6 \times 10^6 \pm 0.9 \times 10^6$
17β-HSD-1	$49 \times 10^3 \pm 11 \times 10^3$	RARβ	$0.91 \times 10^6 \pm 0.1 \times 10^6$
17β-HSD-3	$28 \times 10^6 \pm 3.7 \times 10^6$	RARγ	$0.28 \times 10^6 \pm 0.05 \times 10^6$
17β-HSD-4	$2.9 \times 10^6 \pm 0.4 \times 10^6$	ERα	$0.84 \times 10^6 \pm 0.16 \times 10^6$
P450arom	$42 \times 10^3 \pm 3.1 \times 10^3$	ERβ	$0.14 \times 10^6 \pm 0.04 \times 10^6$

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the first 24 h, and the total RNA content of the slices decreased steeply to one third of the level in onset of the culture in the first 24 h. Therefore, we used the slices after a 24-h preculture in serum-containing medium and then incubated them in serum-free medium for 48 h.

Effect of retinoic acids on the levels of transcripts for steroidogenic enzymes and ERs

After the hippocampal slices of 10- to 12-d-old rats were incubated with or without 0.01–10 μM 9-*cis*- or all-*trans*-retinoic acid for 48 h, the mRNA contents for steroidogenic enzymes and receptors were determined. The effects of retinoic acids on each mRNA level were analyzed by two-way ANOVA of treatment (without retinoid, 1 μM 9-*cis*-retinoic acid, 1 μM all-*trans*-retinoic acid) × each mRNA level. ANOVA revealed an interaction between mRNA levels and treatment [$F(20,146) = 5.74$, $P < 0.01$] and a significant main effect of retinoid [$F(2,146) = 9.44$, $P < 0.05$] and each mRNA [$F(10,146) = 6.45$, $P < 0.05$]. *Post hoc* analysis clarified that transcription of $P450_{17\alpha}$, P450arom, and ERβ increased significantly as a result of incubation with 9-*cis*-retinoic acid compared with transcription levels in nontreated slices but not with all-*trans*-retinoic acid (Fig. 2). The retinoic acid dose dependence of the relative mRNA levels of $P450_{17\alpha}$, P450arom, and ERβ is shown in Fig. 3. Treatment (all-*trans*-retinoic acid, 9-*cis*-retinoic acid) × retinoid dose (0, 0.01, 0.1, 1, 10 μM) two-way ANOVA revealed interactions between treatment and retinoid dose for $P450_{17\alpha}$, P450arom, and ERβ mRNA levels [$F(4,45) = 3.39$, $F(4,62) = 3.22$, $F(4,41) = 2.82$, respectively, $P < 0.05$]. There were significant main effects of retinoid for $P450_{17\alpha}$ mRNA level [$F(1,45) = 25.2$, $P < 0.05$] and dose for $P450_{17\alpha}$ and ERβ mRNA

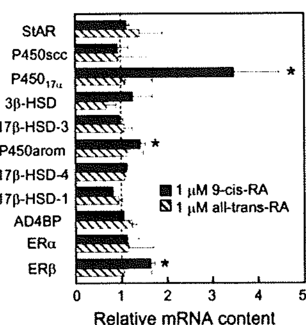

FIG. 2. Relative mRNA contents for steroidogenic proteins and ERs in cultured hippocampal slices after treatment with 1 μM retinoic acid. Slices were incubated with or without 1 μM 9-*cis*-retinoic acid (*black bar*) or 1 μM all-*trans*-retinoic acid (*hatched bar*) for 48 h. The amounts of mRNAs for steroidogenic proteins and ERs were determined by real-time RT-PCR as described in *Materials and Methods*. The mRNA contents in retinoic acid-treated slices are given as relative values to those in nontreated slices (*dashed line*). The values are means of the results from three or four separate experiments, each performed in triplicate. *Error bars*, SD. *, $P < 0.05$, *post hoc t* test with Bonferroni correction, compared with nontreated slices.

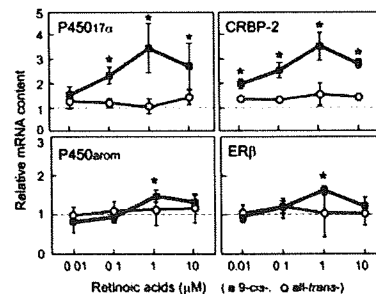

FIG. 3. Retinoic acid dose dependence of mRNA contents in cultured hippocampal slices. Slices were incubated with or without 0.01–10 μM 9-*cis*-retinoic acid (*filled square*) or all-*trans*-retinoic acid (*open circle*) for 48 h. The amounts of mRNA were determined by real-time RT-PCR as described in *Materials and Methods*. The mRNA contents are given as values relative to those in nontreated slices (*dashed line*). Some data are replicated from Fig. 2. The values are means of the results from four separate experiments, each performed in triplicate. *Error bars*, SD. *, $P < 0.05$, *post hoc t* test with Bonferroni correction, compared with nontreated slices.

levels [$F(4,45) = 3.85$, $F(4,41) = 4.99$, respectively, $P < 0.05$]. *Post hoc* analysis clarified that the level of $P450_{17\alpha}$ mRNA was increased by 0.1–10 μM concentrations of 9-*cis*-retinoic acid compared with the levels in nontreated slices (Fig. 3). The maximal activation was 3-fold at 1 μM. The mRNA contents for P450arom and ERβ were less sensitive: significant increases were observed only in the presence of 1 μM 9-*cis*-retinoic acid (Fig. 3). Treatment with 0.01–10 μM all-*trans*-retinoic acid did not affect the mRNA levels for $P450_{17\alpha}$, P450arom, or ERβ (Fig. 3). Treatment × retinoid dose two-way ANOVA was also conducted for StAR, P450scc, 3β-HSD, 17β-HSD type 3, 17β-HSD type 1, 17β-HSD type 4, ERα, and Ad4 binding protein/steroidogenic factor-1, but the main effects of retinoid treatment, retinoid dose, and treatment × retinoid dose interaction were not significant [$F < 1$, all cases] (data not shown).

Because transcription of $P450_{17\alpha}$, P450arom, and ERβ is activated by 9-*cis*-retinoic acid, we examined whether the RXR signaling pathway exists in the hippocampus. The CRBP-2 gene possesses a specific RXR-specific response element in the promoter region, which can be activated by 9-*cis*-retinoic acid via RXR signaling (39). The level of CRBP-2 mRNA in the cultured slices was determined after a 48-h incubation with or without 0.01–10 μM 9-*cis*- or all-*trans*-retinoic acid. Two-way ANOVA revealed an interaction between retinoid and dose [$F(4,58) = 2.72$, $P < 0.05$] and a significant main effect of retinoid and dose [$F(1,48) = 17.4$, $F(4,48) = 6.93$, respectively, $P < 0.05$]. As shown in Fig. 3, the level of CRBP-2 mRNA in the cultured slices was significantly increased after incubation with 0.01–10 μM 9-*cis*-retinoic acid compared with the levels in nontreated slices with an inverted U-shaped dose response. Treatment with 0.01–10 μM all-*trans*-retinoic acid did not affect the

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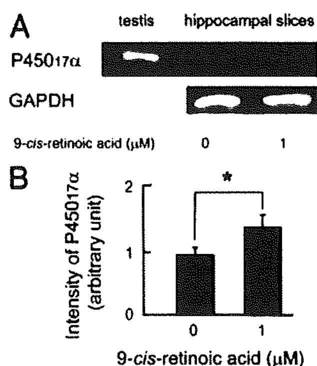

FIG. 4. Western blotting and protein contents of $P450_{17\alpha}$ in cultured hippocampal slices. Slices were incubated with or without 1 μM 9-*cis*-retinoic acid for 48 h. The homogenates of rat testis (2.5 μg protein) and cultured slices (50 μg protein) were subjected to Western blotting analysis using antibodies for $P450_{17\alpha}$ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described in *Materials and Methods* (A). Band intensities for $P450_{17\alpha}$ in the slice homogenates were digitized and represented as relative intensities (B). The values are means of the results from three separate experiments. *Error bars*, SD. *, $P < 0.05$, Student's *t* test, compared with nontreated slices.

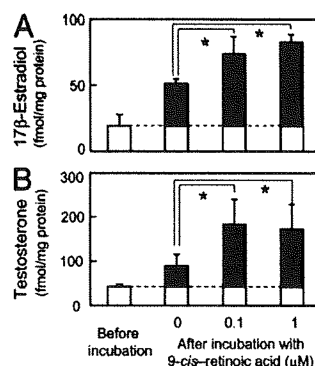

FIG. 5. 17β-Estradiol and testosterone contents in cultured hippocampal slices. Steroids were extracted from slices after 24 h preculture (before incubation) and from slices and medium after 48 h incubation with 0, 0.1, or 1 μM 9-*cis*-retinoic acid. The levels of extracted 17β-estradiol (A) and testosterone (B) were quantified as described in *Materials and Methods*. The values are means of the results from four separate experiments. *Error bars*, SD. *, $P < 0.05$, *post hoc t* test with Bonferroni correction, compared with nontreated slices and media.

CRBP-2 mRNA level ($P > 0.05$). The magnitude of stimulation and the shape of the dose-response curve for CRBP-2 mRNA were quite similar to those for $P450_{17\alpha}$ mRNA (Fig. 3). These data suggest that transcription of the $P450_{17\alpha}$ gene was activated by 9-*cis*-retinoic acid via RXR signaling.

Effect of 9-*cis*-retinoic acid on the level of $P450_{17\alpha}$ protein

The level of $P450_{17\alpha}$ protein in the cultured slices was analyzed by Western blotting. One single band immunoreactive for $P450_{17\alpha}$ was present in lanes containing hippocampal slice homogenates at a molecular weight of 58,000, which is the same migration position as $P450_{17\alpha}$ in the testis, as previously observed in adult rat hippocampus (15). After a 48-h incubation with 1 μM 9-*cis*-retinoic acid, the $P450_{17\alpha}$ protein level in the slices increased about 1.7-fold (Fig. 4). The band intensity for glyceraldehyde 3-phosphate dehydrogenase, a housekeeping enzyme, was not changed by the treatment.

17β-Estradiol and testosterone contents in rat hippocampal slices after incubation with 9-*cis*-retinoic acid

The rat hippocampal slices contained 19.0 ± 7.1 fmol of 17β-estradiol per milligram of protein after the 24-h preculture. The estradiol contents were increased after a 48-h incubation with serum-free medium containing 0, 0.1, and 1 μM 9-*cis*-retinoic acid to 51.8 ± 4.7, 74.2 ± 13.6, and 83.1 ± 6.7 fmol per milligram of protein, respectively (n = 4) (Fig. 5A). The 17β-estradiol level in serum-free medium was below the detection limit of 1 fmol per milliliter before the incubation. The 17β-estradiol contents after 48 h incubation with 0.1 or 1 μM 9-*cis*-retinoic acid were significantly higher in 9-*cis*-retinoic acid-treated slices than in nontreated slices [one-way ANOVA, $F(2,8) = 14.1$, $P < 0.01$]. The level of *de novo* estradiol synthesis during the 48-h incubation was estimated by subtracting the estradiol content before the incubation from that after the incubation (Fig. 5A, *black bar*). The increments were 32, 55, and 64 fmol/mg of protein after incubations with 0, 0.1, and 1 μM 9-*cis*-retinoic acid, respectively. *de novo* estradiol synthesis was increased about 2-fold by incubations with 0.1 and 1 μM 9-*cis*-retinoic acid.

The testosterone content in rat hippocampal slices was 43.7 ± 2.9 fmol/mg of protein after the 24-h preculture. After the 48-h incubations with serum-free medium containing 0, 0.1, and 1 μM 9-*cis*-retinoic acid, the testosterone contents were increased to 92.6 ± 29, 190 ± 60, and 178 ± 60 fmol/mg of protein, respectively (n = 4) (Fig. 5B). The testosterone content in serum-free medium was below the detection limit of 0.5 fmol/ml before the incubation. The testosterone contents were significantly higher in 9-*cis*-retinoic acid-treated slices than in nontreated slices [one-way ANOVA, $F(2,11) = 6.18$, $P < 0.05$]. The increment of testosterone during the 48-h incubation with 0, 0.1, and 1 μM 9-*cis*-retinoic acid was 49, 146, and 134 fmol/mg of protein, respectively (Fig. 5B, *black bar*). The *de novo* testosterone synthesis was increased 2- to 3-fold by treatment with 0.1 and 1 μM 9-*cis*-retinoic acid.

Discussion

Hippocampal functions are modified by several endogenous modulators, such as retinoid and brain-derived sex

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steroids. Several lines of evidence indicate that both retinoid and steroid signaling enhance cellular and synaptic plasticity processes sustaining learning and memory capabilities in the mature brain (2, 8, 21, 22). Significant localization of the estradiol synthetic enzymes P450scc, $P450_{17\alpha}$, and P450arom proteins and mRNAs for StAR and 3β-HSD was observed in pyramidal neurons in the CA1-CA3 regions of the hippocampus and granule cells in the dentate gyrus (12). mRNA for RARα1 was detected in rat CA1-CA2 regions and the dentate gyrus, and that for RXRβ was detected in CA1-CA3 regions and the dentate gyrus (27). Given the colocalization of steroidogenic enzymes and retinoid receptors in the hippocampus, sex steroid synthesis seems to be modulated by retinoic acid. Here we observed that 17β-estradiol and testosterone synthesis in hippocampal slices were significantly stimulated by 9-*cis*-retinoic acid. To our knowledge, this is the first report of cross talk between retinoid signals and steroid hormone synthesis in the nervous system.

Treatment of slices with 1 μM 9-*cis*-retinoic acid induced an increase in the gene expression levels of $P450_{17\alpha}$ and P450arom and also in the level of $P450_{17\alpha}$ protein. 17β-Estradiol and testosterone synthesis in the hippocampus was stimulated about 2-fold by the same treatment, consistent with the 1.7-fold increase in the level of $P450_{17\alpha}$ protein. The enzymatic activity of $P450_{17\alpha}$ produces dehydroepiandrosterone and androstenedione, both of which are precursors for 17β-estradiol and testosterone. These results indicate that the stimulation of 17β-estradiol and testosterone synthesis is induced by an increase in transcriptional activation of $P450_{17\alpha}$ in the hippocampus. The mRNA content for P450arom was slightly increased by 1 μM 9-*cis*-retinoic acid. This increase might contribute to the stimulation of estradiol synthesis. On the other hand, estradiol synthesis was stimulated by 0.1 μM 9-*cis*-retinoic acid without an increase in the P450arom mRNA level. Taken together, these findings suggest that the increase in the level of transcription of P450arom was not essential for stimulation of estradiol synthesis.

In this experiment, sex steroid synthesis was stimulated by 9-*cis*-retinoic acid without any increase in the level of StAR mRNA. In the gonads and adrenals, the expression of StAR protein is the regulatory step for overall steroidogenesis (40). It has been reported that pregnenolone synthesis in the cultured hippocampus is stimulated by 100 μM N-methyl-D-aspartate (NMDA) without an increase in the level of transcription of StAR mRNA (41). NMDA treatment induces processing of full-length StAR protein to the truncated form, which may be involved in the stimulation of steroidogenesis. These data suggest that hippocampal steroid synthesis can be stimulated without an increase in the transcription of StAR mRNA.

The level of mRNA for $P450_{17\alpha}$ was the most sensitive to stimulation by 9-*cis*-reteinoic acid among the mRNAs we assessed in hippocampal slice cultures. In cultured Leydig cells and ovarian theca cells, transcription of $P450_{17\alpha}$ is also most sensitive to retinoid signals (32, 33). Several putative consensus retinoic acid response elements have been found in the promoter of the $P450_{17\alpha}$ gene (33). In our experiment, the magnitude of stimulation and shape of dose-response curve of the mRNA for $P450_{17\alpha}$ were quite similar to those for CRBP-2 mRNA. The CRBP-2 gene possesses typical directly repeated RG(G/T)TCA motifs upstream of the coding region, which are binding sites for RXR homo- and heterodimers (39, 42). RARs can bind and be activated by both all-*trans*- and 9-*cis*-retinoic acids, whereas RXRs can be activated only by 9-*cis*-retinoic acid (43). In our study, the mRNA for $P450_{17\alpha}$ was increased by treatment with 9-*cis*-retinoic acid but not by all-*trans* isomer. It is strongly suggested that the stimulation of steroidogenesis in the hippocampus is mediated by RXR homodimer or heterodimers of RXR and nuclear receptors other than RAR.

The mRNA levels for P450arom and ERβ in the cultured slices were increased by incubation with 1 μM 9-*cis*-retinoic acid. Transcription of these enzyme and receptor genes might be activated by this retinoic acid. On the other hand, steroids also transactivate these genes. The P450arom gene contains estrogen- and androgen-responsive elements, and the mRNA level for P450arom was up-regulated or down-regulated by 17β-estradiol or testosterone (44). The level of ERβ mRNA was also up-regulated by 17β-estradiol (45). Therefore, transcription of P450arom and ERβ might be activated by an increase in 17β-estradiol and/or testosterone in the slices after treatment with 9-*cis*-retinoic acid. Alternatively, these transcriptions might be regulated by both retinoic acid and sex hormones.

To ensure that we were detecting chemically distinct 17β-estradiol in the RIA, we measured 17β-estradiol contents by liquid chromatography-tandem mass spectrometry coupled with purification steps with a C18 minicolumn and HPLC, as described above, in collaboration with Asuka Pharmamedical Co. Ltd. (Kawasaki, Japan) (46, 47, 54). The 17β-estradiol content in slices and media after a 48-h incubation with 1 μM 9-*cis*-retinoic acid was 64.5 ± 14.5 fmol/mg of protein, as determined by liquid chromatography-tandem mass spectrometry methods ($n = 3$). This value was quite similar to the result with RIA described above, 83.1 ± 6.7 fmol/mg.

In this study, hippocampal slices from 10-d-old rats contained 19 fmol of 17β-estradiol per milligram of protein, which is higher than our previous result in hippocampal slices of adult male rats, namely 6 fmol/mg of protein

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(15). These data appear to indicate that the 17β-estradiol content in the hippocampus is higher in neonates than adults.

17β-Estradiol and testosterone in the hippocampal slices can be synthesized from a steroid precursor(s). Because biosynthesis of these sex steroids is stimulated by an increase in $P450_{17\alpha}$ content, the precursor might be located upstream of $P450_{17\alpha}$ activity, such as pregnenolone, progesterone, and/or cholesterol (11). It has been reported that sufficient pregnenolone for synthesis of 17β-estradiol and testosterone, namely 160–180 fmol/mg of protein, exists in the hippocampi or brains of adult male rats (41, 48). The amount of progesterone in the brains of adult male rats was less than 10% of the amount of pregnenolone (48). More than half of the brain pregnenolone might be derived from the blood stream because the amount of mRNA for P450scc in the hippocampus seems to be too low to support pregnenolone synthesis from cholesterol. On the other hand, we could not eliminate the possibility that some of the steroids in the brain are synthesized from cholesterol. Hippocampal slices from adult rats showed synthesis of pregnenolone from cholesterol (41), although the mRNA content for P450scc in adult rat hippocampus was lower than that in 10-d-old rats (Munetsuna, E., and T. Yamazaki, unpublished data). It has been reported that the level of P450scc mRNA in brain is about $1/10^4$ to 10^5 times that in the adrenal gland, but the level of P450scc protein is $1/10^2$ to $1/10^3$ times that in the adrenal gland. The P450scc protein might be sufficient for a small amount of neurosteroid synthesis (12, 49). Further experiments are required to clarify the substrate(s) for steroid synthesis in the brain.

The mechanism underlying the regulation of steroid synthesis in the hippocampus is not yet fully elucidated. As an acute regulation, 17β-estradiol and pregnenolone synthesis in rat hippocampal slices is stimulated by treatment with 100 μM NMDA for 30 min (15, 41). 17β-Estradiol synthesis in hippocampal slices or dispersed cells was increased by GnRH treatment for 8 d (50). Here we showed that RXR-mediated signaling stimulated 17β-estradiol and testosterone synthesis in male rat hippocampal slices. The high-affinity ligand for RXRs is reported to be 9-*cis*-retinoic acid, which had been considered to be a metabolite of retinal and involved in retinoid signaling (26). However, 9-*cis*-retinoic acid is hardly detected in serum and various organs using sensitive and up-to-date analytical technology (51). Nevertheless, there is a possibility that 9-*cis*-retinoic acid could be present at concentrations that are high enough for biological responses only in localized regions or present transiently (24). If this is the case, then steroidogenesis in brain could be stimulated by retinoid signaling. Otherwise, other ligands of RXRs, such as phytanic acid, eicosanoids, and docosahexanoids, might be responsible for the activation (26). Docosahexanoic acid is particularly enriched in the brain and is essential for normal brain development and function, promoting neurite growth in the hippocampus (52, 53). This polyunsaturated fatty acid is a candidate stimulator of RXR signaling other than 9-*cis*-retinoic acid in the brain.

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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 17β-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 → T → E2 or dehydroepiandrosterone → androstenediol → T → E2 but not androstenedione → E1 → 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 17β-estradiol (17β-E2) as well as expression of cytochromes P450(17α) 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 17β-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; 17β-HSD, 17β-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.

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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-

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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-

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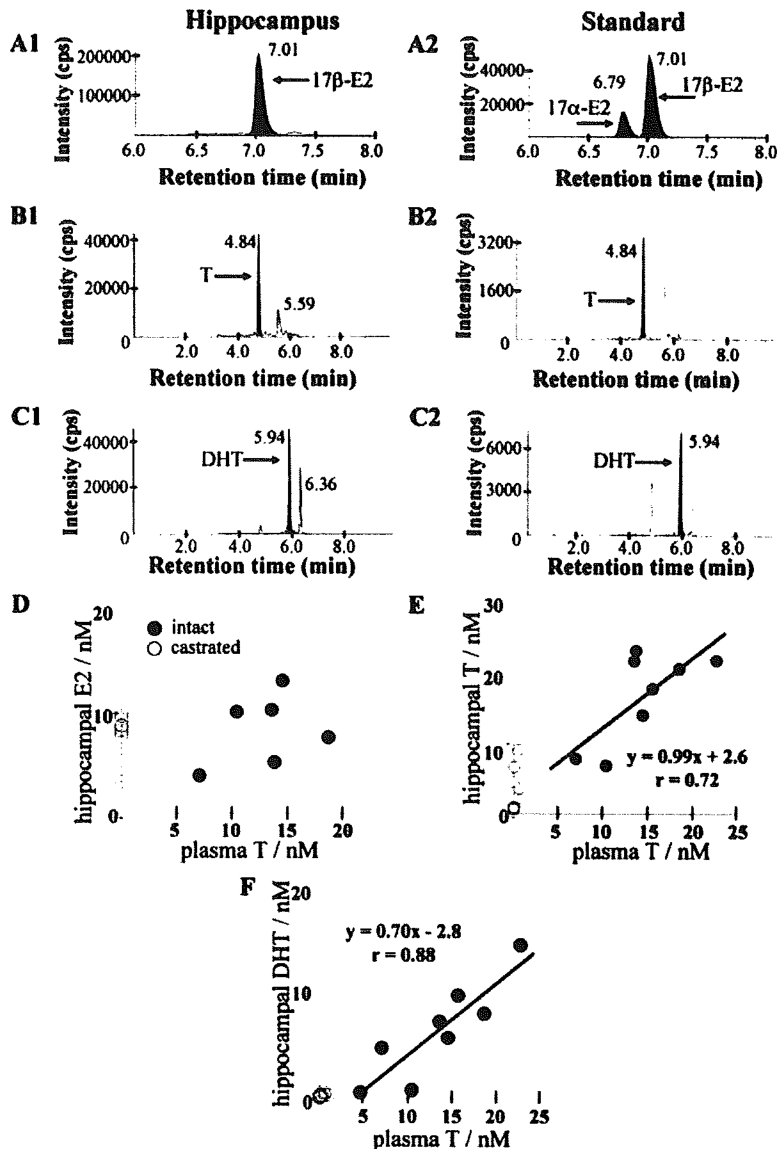

FIG. 1. Mass-spectrometric analysis of hippocampal sex steroids. A–C, LC-MS/MS ion chromatograms of E2 (A), T (B), and DHT (C). A1, B1, and C1 represent the chromatograms of the fragmented ions of each steroid derivative from the hippocampus of adult male rats (12 wk old). Shaded portions indicate the 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 17β-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 (17β-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 17β-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-

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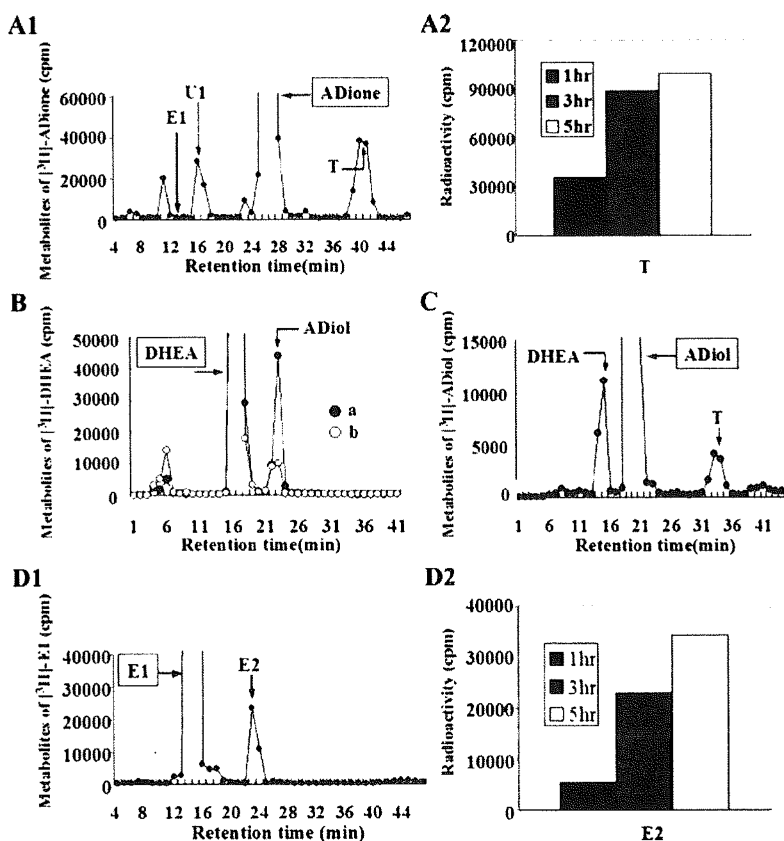

FIG. 2. Normal-phase HPLC analysis of steroid metabolism in hippocampal slices from adult male rat. Hippocampal slices from the adult male rats were incubated with 5×10^6 cpm ^{3}H-labeled steroid substrate for 1, 3, and 5 h. A portion of the purified radioactive metabolites (total of 10^6 cpm) was analyzed 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 μM RM352–26 (28), a specific inhibitor of 17β-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 5α-reductase, 17β-hydroxysteroid dehydrogenase (17β-HSD), 3β-HSD, and 3α-HSD, responsible for sex steroidogenesis, were examined, because this important information had not been fully clarified. The expression level of mRNA for 17β-HSD (types 1 and 3), 3β-HSD type1, 5α-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 5α-reductase and 17β-HSD (type 1), *in situ* hybridization was performed. Significant expression of 5α-reductase (types 1 and 2) as well as 17β-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 17α-E2 exists in the mouse hippocampus and its level elevates upon castration (21), 17α-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

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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 17β-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, 17β-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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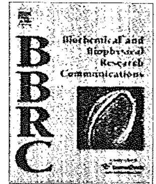

Androgen rapidly increases dendritic thorns of CA3 neurons in male rat hippocampus

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ABSTRACT

Modulation of hippocampal synaptic plasticity by androgen has been attracting much attention. Thorns of thorny excrescences of CA3 hippocampal neurons are post-synaptic regions whose presynaptic partners are mossy fiber terminals. Here we demonstrated rapid effects of dihydrotestosterone (DHT) and testosterone (T) on the density of thorns, by imaging Lucifer Yellow-injected neurons in adult male rat hippocampal slices. The application of 10 nM DHT or T induced rapid increase in the density of thorns within 2 h. The androgen-mediated increase was suppressed by blocking several kinases, such as Erk MAPK, p38 MAPK, PKC, and CaMKII. On the other hand, PKA, PI3K were not involved in the signaling of thorn-genesis. The increase in the thorn density by androgen was also blocked by the inhibitor of classical androgen receptor. Almost no difference was observed between DHT and T in the effect on the thorn density. We observed that the androgen-induced thorn-genesis is opposite to estrogen-induced thorn-degeneration.

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Introduction

The hippocampus is essentially involved in learning and memory processes, and is known to be a target for the modulatory actions of androgen and estrogen from not only the gonads but also the hippocampus [1–6]. Extensive studies have been performed to investigate their role in modulating hippocampal plasticity and function, slowly and genomically (over 1–5 days). The density of dendritic spines of pyramidal neurons in the CA1 region of the hippocampus is modulated *in vivo* by the depletion and replacement of androgens [3]. Compared to the CA1 region (responsible for spatial memory), the effect of androgen on CA3 pyramidal neurons remains almost unknown. The CA3 was considered as a region where control associative memory [7,8]. In the stratum lucidum of the CA3, pyramidal neurons have huge and complex post-synaptic structures, named thorny excrescences. One thorny excrescence consists of multiple heads named thorns with one neck along a dendritic branch [9,10]. One mossy fiber terminal of dentate granule cells contacts multiple thorns of thorny excrescences of CA3 neuron. Thorny excrescences may play essential roles on hippocampal function. Chronic restraint stress has induced retraction of thorny excrescences, which has subsequently been reversed after water maze training. On the other hand, water maze training alone has increased the volume of thorny excrescence as well as the number of thorns per thorny excrescence [11].

As a preceding study, we observed that estradiol (estrogen) induced rapid decrease of thorns in CA3 stratum lucidum within 2 h, and this rapid estradiol modulation of thorny excrescences was mediated by Erk MAPK [4–6,12]. We here demonstrate that androgens rapidly induce the increase of the thorns by driving several kinases. Because testosterone (T) may be partially converted to estradiol by hippocampal endogenous aromatase [4,6,13–18], possible difference of dihydrotestosterone (DHT), non-aromatizable androgen, and T is also investigated.

Materials and methods

Animals. Twelve-week-old adult male Wistar rats were purchased from Saitama Experimental Animal Supply. All experiments using animals in this study were conducted according to the institutional guidelines.

Chemicals. Cyclosporin A, dihydrotestosterone, Lucifer Yellow CH, LY-294,002, SB203580, SP600125, testosterone, and U0126

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were purchased from Sigma (USA). Chelerythrine, KN-93, and Rottlerin were purchased from Calbiochem (Germany). H-89 was purchased from Biomol (USA). Hydroxyflutamide was purchased from Wako Pure Chemicals (Japan).

Current injection of Lucifer Yellow. Adult male rats were anesthetized with ethyl ether and decapitated. The brains were removed and placed in artificial cerebrospinal fluid (ACSF) at 4 °C. The hippocampus was dissected and 400 μm transverse slices to the long axis from the middle third of the hippocampus were cut with a vibratome (Dosaka, Japan). ACSF consisted of (mM): 124 NaCl, 5.0 KCl, 1.25 NaH_2PO_4, 2.0 $MgSO_4$, 2.0 $CaCl_2$, 22 $NaHCO_3$, and 10 glucose, and was equilibrated with 95% O_2/5% CO_2. Hippocampal slices were transferred into an incubating chamber containing ACSF held at 25 °C for 2 h for recovery. Slices were then incubated with 0.1–10 nM DHT or T together with several protein kinase inhibitors. Slices were then fixed with 4% paraformaldehyde in PBS at 4 °C overnight. Neurons within slices were visualized by an injection of Lucifer Yellow (Molecular Probes, USA) under Nikon E600FN microscope (Japan) equipped with a C2400-79H infrared camera (Hamamatsu Photonics, Japan) and with a 40× water immersion lens (Nikon, Japan). Dye injection was performed with glass electrode filled with 5% Lucifer Yellow for 15 min, using Axopatch 200B (Axon Instruments, USA). Approximately five neurons within a depth of 100–200 μm from the surface of a slice were injected with Lucifer Yellow [19].

Confocal laser scan microscopy and analysis. The imaging was performed from sequential z-series scans with confocal laser scan microscope (LSM5; Carl Zeiss, Germany) at high zoom (×3.0) with a 63× water immersion lens, NA 1.2. For Lucifer Yellow, the excitation and emission wavelengths were 488 nm and 515 nm, respectively. Three-dimensional image was reconstructed from approximately 40 sequential *z*-series sections of every 0.45 μm with a 63× water immersion lens, NA 1.2. The applied zoom factor (×3.0) yielded 23 pixels per 1 μm. The *z*-axis resolution was approximately 0.71 μm. The confocal lateral resolution was approximately 0.26 μm. Our resolution limits were regarded to be sufficient to allow the determination of the density of thorns. Confocal images were then deconvoluted using AutoDeblur software (AutoQuant, USA).

In each slice, 2–3 neurons with more than 100 thorns were analyzed, and at least 90 thorns were counted on each frame. In total, N = 4–13 neurons and N = 300–1000 total were analyzed for each drug treatment. The density of thorns was analyzed by tracing neurons with Neurolucida software (MicroBrightField, USA). The single apical dendrite which had thorns was analyzed separately. These dendrites were present within the stratum lucidum, within 100 μm from the soma. The density of thorns was calculated from the number of thorns along both primary and secondary dendrites having a total length of 20–100 μm. While counting the thorns in reconstructed images, the position and verification of thorns were aided by three-dimensional reconstructions and by observation of the images in consecutive single planes.

Statistical analysis. The significance of DHT, T, or drug effect was examined via statistical analysis using Tukey–Kramer post-hoc multiple comparisons test when one-way ANOVA tests yielded $P < 0.05$.

Result

We investigated the effect of DHT or T on the modulation of the thorn density in the hippocampus CA3 stratum lucidum. Lucifer Yellow-injected neurons in hippocampal slices from 12 week-old male rats were imaged using confocal laser scan microscopy (Fig. 1). Thorny excrescences were located on apical dendrites within 100 μm from the soma, on which mossy fiber terminals attached.

Androgens increased the density of thorns in CA3 stratum lucidum

Following a 0.5–4 h treatment with DHT or T, treated dendrites had significantly more thorns than control dendrites (i.e. with no DHT or T). Time dependency was examined by treating slices for

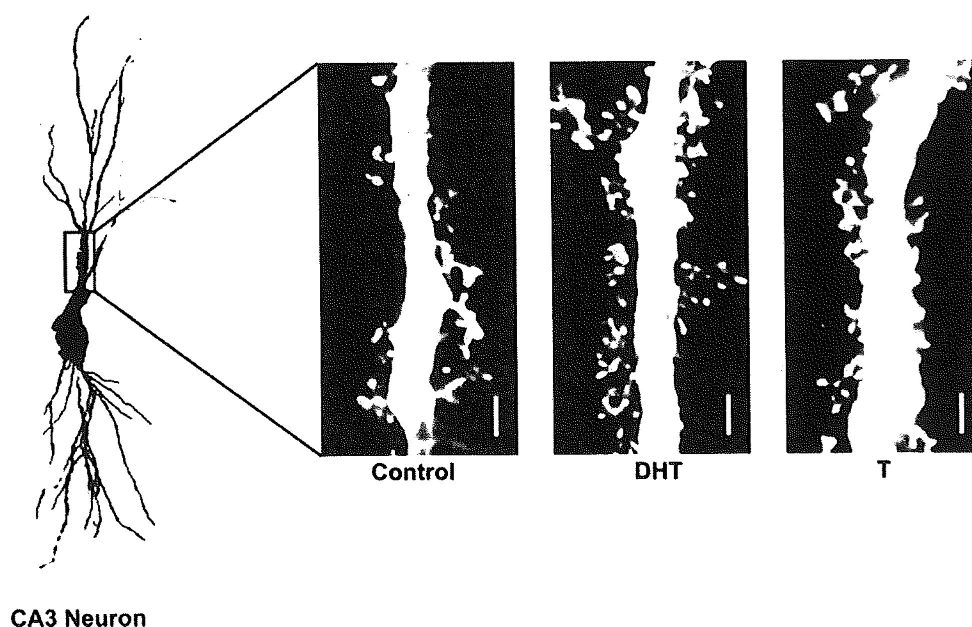

Fig. 1. Changes in the density of thorns by androgens in hippocampal slices. Maximal intensity projections onto *XY* plane from *z*-series confocal micrographs, showing thorns along the primary dendrites of hippocampal CA3 pyramidal neurons. Left image shows a traced whole image of Lucifer Yellow-injected CA3 neuron. Dendritic thorns without drug-treatments (Control), dendritic thorns after 10 nM DHT- (DHT) or T-treatment (T) for 2 h, bar 2 μm.