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

Eiji Munetsuna, Yasushi Hojo, Minoru Hattori, Hirotaka Ishii, Suguru Kawato, Atsuhiko Ishida, Shiro A. J. Kominami, and Takeshi Yamazaki

Laboratory of Molecular Brain Science (E.M., A.I., S.A.J.K., T.Y.) and Department of Behavioral Sciences (M.H.), Graduate School of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan; and Department of Biophysics and Life Sciences (Y.H., H.I., S.K.), Graduate School of Arts and Sciences, University of Tokyo at Komaba, Core Research for Evolutional Science and Technology Project of Japan Science and Technology Agency, University of Tokyo, Meguro, Tokyo 153-8902, Japan

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.

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.

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 non-treated 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 $\pm$ 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.

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).

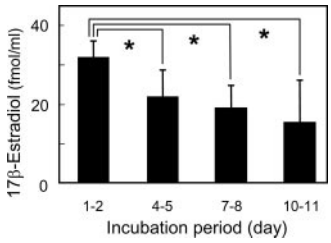

FIG. 1. 17β-Estradiol content in medium from hippocampal slice cultures. Hippocampal slices were precultured with serum-containing medium for 24 h and then cultured with serum-free medium. The medium was changed daily, and medium collected on d 1 and 2, 4 and 5, 7 and 8, and 10 and 11 were combined. 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	$0.14 \times 10^6 \pm 0.04 \times 10^6$

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) $\times$ 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) $\times$ 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 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 $\times$ 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 $\times$ retinoid dose interaction were not significant [$F < 1$, all cases] (data not shown).

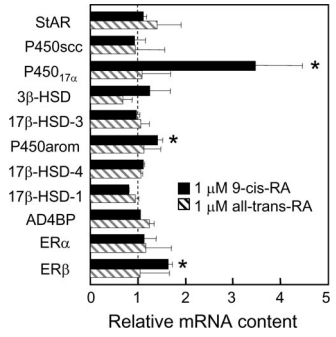

FIG. 2. Relative mRNA contents for steroidogenic proteins and ERs in cultured hippocampal slices after treatment with 1 μM retinoic acid. Slices were incubated with or without 1 μM 9-*cis*-retinoic acid (*black bar*) or 1 μM all-*trans*-retinoic acid (*hatched bar*) for 48 h. The amounts of mRNAs for 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.

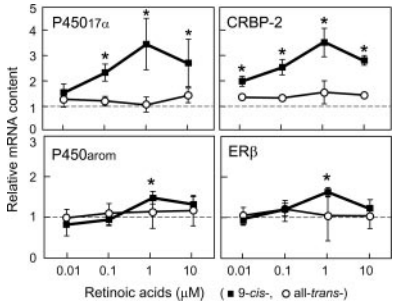

FIG. 3. Retinoic acid dose dependence of mRNA contents in cultured hippocampal slices. Slices were incubated with or without 0.01–10 μM 9-*cis*-retinoic acid (*filled square*) or all-*trans*-retinoic acid (*open circle*) for 48 h. The amounts of mRNA were determined by real-time RT-PCR as described in *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.

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

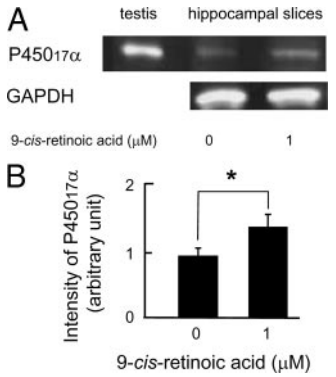

FIG. 4. Western blotting and protein contents of $P450_{17\alpha}$ in cultured hippocampal slices. Slices were incubated with or without 1 μM 9-*cis*-retinoic acid for 48 h. The homogenates of rat testis (2.5 μg protein) and cultured slices (50 μg protein) were subjected to Western blotting analysis 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.

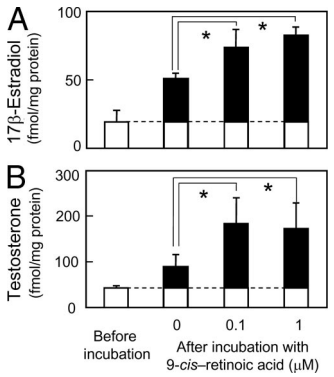

FIG. 5. 17β-Estradiol and testosterone contents in cultured hippocampal slices. Steroids were extracted from slices after 24 h preculture (before incubation) and from slices and medium after 48 h incubation with 0, 0.1, or 1 μM 9-*cis*-retinoic acid. The levels of extracted 17β-estradiol (A) and testosterone (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

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 $\pm$ 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 $\pm$ 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

(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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Address all correspondence and requests for reprints to: Takeshi Yamazaki, Graduate School of Integrated Arts and Sciences, Hiroshima University, 1-7-1 Kagamiyama, Higashi-Hiroshima 739-8521, Japan. E-mail: takey@hiroshima-u.ac.jp.

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Present address for E.M.: Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan.

Present address for H.I.: Department of Physiology, Nippon Medical School, Tokyo 113-8602, Japan.

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