Endogenous Synthesis of Corticosteroids in the Hippocampus

Shimpei Higo1,7, Yasushi Hojo1,2,3, Hirotaka Ishii1, Yoshimasa Komatsuzaki1,4, Yuuki Ooishi1, Gen Murakami1,3, Hideo Mukai1,2,3, Takeshi Yamazaki5, Daiichiro Nakahara6, Anna Barron1, Tetsuya Kimoto1, Suguru Kawato1,2,3,4,5,6

1 Department of Biophysics and Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Meguro, Tokyo, Japan, 2 Core Research for Evolutional Science and Technology Project of Japan Science and Technology Agency, The University of Tokyo, Meguro, Tokyo, Japan, 3 Bioinformatics Project of Japan Science and Technology Agency, The University of Tokyo, Meguro, Tokyo, Japan, 4 Department of Physics, College of Science and Technology, Nihon University, Chiyoda, Tokyo, Japan, 5 Laboratory of Molecular Brain Science, Graduate School of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima, Japan, 6 Department of Psychology, Hamamatsu University School of Medicine, Hamamatsu, Japan

Abstract

Background: Brain synthesis of steroids including sex-steroids is attracting much attention. The endogenous synthesis of corticosteroids in the hippocampus, however, has been doubted because of the inability to detect deoxycorticosterone (DOC) synthase, cytochrome P450(c21).

Methodology/Principal Findings: The expression of P450(c21) was demonstrated using mRNA analysis and immunogold electron microscopic analysis in the adult male rat hippocampus. DOC production from progesterone (PROG) was demonstrated by metabolism analysis of 3H-steroids. All the enzymes required for corticosteroid synthesis including P450(c21), P450(2D4), P450(11b) and 3β-hydroxysteroid dehydrogenase (3β-HSD) were localized in the hippocampal principal neurons as shown via in situ hybridization and immunoelectron microscopic analysis. Accurate corticosteroid concentrations in rat hippocampus were determined by liquid chromatography-tandem mass spectrometry. In adrenalectomized rats, net hippocampus-synthesized corticosterone (CORT) and DOC were determined to 6.9 and 5.8 nM, respectively. Enhanced spinogenesis was observed in the hippocampus following application of low nanomolar (10 nM) doses of CORT for 1 h.

Conclusions/Significance: These results imply the complete pathway of corticosteroid synthesis of ‘pregnenolone →PROG→DOC→CORT’ in the hippocampal neurons. Both P450(c21) and P450(2D4) can catalyze conversion of PROG to DOC. The low nanomolar level of CORT synthesized in hippocampal neurons may play a role in modulation of synaptic plasticity, in contrast to the stress effects by micromolar CORT from adrenal glands.

Introduction

The hippocampus is a target of corticosterone (CORT) modulatory actions, with high levels of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) expression [1,2]. Traditionally, CORT had been thought to be synthesized exclusively in the adrenal cortex, reaching the brain via blood circulation. De novo synthesis of CORT from PROG in the brain has been doubted partly because brain CORT disappears after adrenalectomy (ADX) in rats [3]. However, recent evidence shows that the hippocampus expresses steroidogenic enzymes required for corticosteroid synthesis, including cytochromes P450c1b, P450(11b1), P450(11b2), and 3β-hydroxysteroid dehydrogenase (3β-HSD) [4-10]. Some of these enzymes are also necessary for sex-steroid synthesis [11-14]. Yet, the complete corticosteroid synthesis of ‘pregnenolone (PREG) → progesterone (PROG)→ deoxycorticosterone (DOC)→CORT or aldosterone (ALDO)’ in the hippocampus has not been proven (see Fig. S1). Although previous studies have shown parts of the corticosteroid synthesis pathway in brain, including, PREG→PROG and DOC→CORT and DOC→ALDO[5,6,10], the conversion of PROG→DOC to be demonstrated. Cytochrome P450(c21) (DOC synthase), a key enzyme catalyzing the conversion of PROG to DOC, has not been detected in the hippocampus, although mRNA expression has been demonstrated in other brain regions including the hypothalamus, cortex, cerebellum and striatum [15,16].

In the current study, we demonstrated expression and activity of P450(c21) and the complete metabolism of PROG→DOC→
CORT in rat hippocampus. In addition to P450(c21), expression of cytochrome P450(2D4), another candidate for DOC synthase [16,17], was also observed. Further, the net hippocampus-derived CORT content was determined in ADX rats by mass-spectrometric analysis. We further investigated the effect of low dose CORT on spinogenesis of hippocampal neurons in order to show a possible role of hippocampus-derived CORT.

**Results**

**Molecular biological analysis of corticosteroid synthesizing enzymes**

Cellular localization and expression of enzymes responsible for CORT synthesis including P450(c21), P450(2D4), P450(11β1), P450(11β2), and 11β-HSD (types 1 and 2) were examined. Typical RT-PCR patterns of mRNA transcripts are shown in Fig. 1. Because the expression level of P450(c21) is extremely low in the hippocampus, we performed careful primer design using minimization of Gibbs free energy (ΔG) upon hybridization of primers with target sequences. We also choose primers with ΔG for 5 bases in 3'-side of the primer to be larger than average ΔG for improved specificity, because the specificity of primer-target recognition is mainly governed by 3'-side sequence of the primer. In the current study, ΔG for 5 bases in 3'-side of the P450(c21) primer was –6 kcal/mol, which was larger than the average ΔG for the P450(c21) primer (–8 kcal/mol) (Fig. S2). As a result, we succeeded the detection of P450(c21) mRNA.

![Figure 1. RT-PCR analysis of enzymes required for corticosteroid synthesis in the adult (12-week old) male brain.](https://www.plosone.org/figure/1)
Relative number of transcripts, expressed in the hippocampus of adult male rats, was in the order of 1/20,000 of that in the adrenal gland for P450(2D4), almost same level of that in the liver for P450(2D4), in the order of 1/10,000 of that in the adrenal gland for P450(11β), in the order of 1/5,000 of that in the adrenal gland for P450(11β2), approximately 1/30 of that in the liver for 11β-HSD (type 1), and approximately 1/300 of that in the kidney for 11β-HSD (type 2) (Fig. 1). The mRNA expression levels of P450(2D4), P450(11β1), P450(11β2) and 11β-HSD (type 2) in the hippocampus were lower than those in the hypothalamus. The expression level of P450(2D4) in the hippocampus was almost equal of that in the hypothalamus. The expression level of 11β-HSD (type 2) was higher than that in the hypothalamus.

The cellular localization of P450(2D4), P450(11β1) and P450(2C1) was identified using in situ hybridization and immuno-histochemical staining. Significant expressions of both P450(2D4) and P450(11β1) were observed in pyramidal neurons (CA1, CA3) and granule neurons (DG) and these enzymes were weakly expressed in glial cells (Fig. 2 and S3). The weak expression of P450(2C1) was observed in pyramidal and granule neurons (Fig. 2C). Because the expression level of mRNA for P450(2C1) was very low, the Tyramide signal amplification system was used to obtain sufficient sensitivity.

Cellular and Subcellular localization of enzymes for corticosteroid synthesis

Immunoelectron microscopic analysis using postembedding immunogold was performed in order to determine the localization of enzymes for corticosteroid synthesis (P450(2D4), P450(2D4), P450(11β1) and 3β-HSD) in the hippocampus. This method is particularly useful to detect enzymes with extremely low expression level such as P450(2C1). All enzymes were mainly localized in principal neurons including pyramidal neurons of CA1 and CA3 regions as well as granule neurons of the DG. Weak expression was observed in some glial cells. P450(2C1) and P450(2D4) were localized not only in the endoplasmic reticulum but also in both the axon terminals and dendritic spines of principal neurons (Fig. 3A, 3B). P450(11β1) was localized in both the mitochondria and synapses of principal neurons (Fig. 3C). We also observed 3β-HSD in the synapses in addition to the endoplasmic reticulum (Fig. 3D).

The subcellular localization of these enzymes was confirmed by western blot with purified fractions of postsynaptic density, endoplasmic reticulum and mitochondria (see Fig. S4 and Text S1).

Mass-spectrometric analysis of corticosteroids in the hippocampus

The concentration of CORT and DOC was determined in steroid extracts from adult male rat hippocampus using a chromatogram analysis of the fragmented ions (Fig. 4). After selection of mother ions, fragmentation and detection were performed via MS/MS procedures. Chromatographic profiles for the fragmented ions of CORT (m/z = 121) showed a clear peak with the retention time of 3.19 min which was the same as that of the fragmented ion of the standard CORT, indicating a good specificity of the analysis (Fig. 4A). In the chromatographic profiles of the fragmented ion of DOC (m/z = 97), a single peak was observed at 3.73 min (Fig. 4B).

In order to determine the net corticosteroid synthesis in the hippocampus, we used ADX rats to eliminate adrenal-derived steroids (CORT and DOC) via the blood circulation. Results are summarized in Table 1. Two weeks after ADX, the concentrations of CORT in the hippocampus and plasma were 2.4 ng/g wet weight (≈6.9 nM) and 0.6 ng/ml (2.3 nM), respectively. The DOC concentration was 1.9 ng/g wet weight (5.9 nM) in the hippocampus, and 0.5 ng/ml (1.4 nM) in plasma, respectively.

Even at 4 weeks after the ADX, the level of CORT did not decrease further from the level at two weeks within experimental error (±2.1 nM, SEM). These results imply that the circulation-derived CORT in the hippocampus was cleaned up already at two weeks after ADX, therefore 6.9 nM is solely hippocampus-synthesized CORT.

Validation of mass-spectrometric analysis

To confirm the assay accuracy, the hippocampal homogenate spiked with known amounts of the steroids was prepared and its concentration of steroid was determined (Table S1). Satisfactory accuracy was obtained, supporting the accuracy of determined hippocampal steroid content in Table 1. The limits of quantifications (LOQs) were defined in Table S2 as the lowest value with an acceptable accuracy (91.6–107.1 %) and precision (i.e. RSD <11 %). The results of intra- and inter-assay were shown in Table S2. The RSD for intra- and inter-assay was less than 10.2 % and 10.9 %, respectively. These results indicate that the present method is highly reproducible and accurate.

In addition, we observed approximately 0.68 nM of 11-deoxycortisol (roughly 1/10 of DOC) in the hippocampus. Because P450(17α) is expressed in the hippocampus, DOC→11-deoxycortisol → cortisol pathway may also work.

Hippocampal corticosteroid metabolism

Analysis of the pathway of corticosteroid metabolism is necessary (Fig. S1), because mass-spectrometric determination shows only the contents of individual steroids. The metabolism of radioactive steroids in hippocampal slices was investigated using normal phase HPLC. Hippocampal slices were incubated with 5 × 10⁶ cpm of 3H-labeled steroid substrate for 5 h. Typical results of HPLC analysis are illustrated in Fig. 5. A significant production of DOC from 3H-PROG was observed (Fig. 5A). The incubation buffer contains 100 nM of finasteride, a specific inhibitor of 5α-redutase, in order to reduce the conversion to 5α-reduced metabolites such as allopregnanolone. Because of the strong activity of 5α-reductase and 3α-HSD [18], some 5α-dihydro PROG and TH-PROG (allopregnanolone) were produced even in the presence of finasteride. In the absence of finasteride, the production of DOG and CORT were very weak to observe. When 3H-DOC was used as a substrate, CORT from DOC was produced (Fig. 5B). The conversion of CORT to other steroids was very weak (Fig. 5C), indicating that CORT is stably present once it is produced.

Low dose effect of CORT on spinogenesis of hippocampal neurons

To determine the potential physiological significance of nanomolar concentrations of hippocampal CORT, we investigated CORT effects on dendritic spine density and morphology in hippocampal ‘acute’ slices. It should be noted that CORT levels were depleted in control ‘acute’ slices, containing only 1.9 nM CORT following 2 h recovery incubation in ACSF. Treatment with exogenous 10 nM CORT for 1 h significantly increased the total spine density (1.18 spines/μm) compared with control slices
(0.98 spines/μm, with no exogenous CORT) (Fig. 6). On the other hand, treatment with 100 nM CORT only slightly increased the total spine density.

Morphological changes in spine head diameter induced by 1 h CORT administration were also assessed. Spines were classified into three categories based on head diameters: small-head spines (0.2–0.4 μm); middle-head spines (0.4–0.5 μm); and large-head spines (0.5–1.0 μm). Morphological categorization of spines into three subclasses enabled complex responses in spine subpopulations upon CORT application to be distinguished. Treatment with

Figure 2. In situ hybridization analysis of P450(2D4) (A), P450(11β1) (B) and P450(c21) (C). Reaction time for color development is 12 h. (A1–2) Coronal sections of the whole hippocampus hybridized with antisense (A1) or sense (A2) probes for P450(2D4). (A3–4) The magnified images of CA3 (A3) and DG (A4) in the hippocampus hybridized with antisense probes for P450(2D4). (B1–2) Coronal sections of the whole hippocampus hybridized with antisense (B1) or sense (B2) probes for P450(11β1). (B3–4) The magnified images of CA1 (B3) and CA3 (B4) in the hippocampus hybridized with antisense probes for P450(11β1). (C1–2) Coronal sections of the whole hippocampus hybridized with antisense (C1) or sense (C2) probes for P450(c21). P450(2D4), P450(11β1) and P450(c21) are expressed in pyramidal neurons in CA1–CA3 region and granule cells in DG. Their expression in glial cells is weak. Scale bar, 800 μm for (A1–2), (B1–2) and (C1–2); 120 μm for (A3), (A4), (B3) and (B4). Abbreviations: gcl, granule cell layer; hl, hilus; pcl, pyramidal cell layer; so, stratum oriens; sr, stratum radiatum.
doi:10.1371/journal.pone.0021631.g002
10 nM CORT significantly increased the density of small-head spines from 0.58 (control) to 0.81 spines/μm, while the density of middle-head spines (approx. 0.28 spines/μm) and large-head spines (approx. 0.12 spines/μm) was not significantly altered (Fig. 6). Morphological changes induced by 100 nM CORT were smaller than those induced by 10 nM CORT. The majority of spines (>95%) had a distinct head and neck, therefore these analysis cover major populations of spines.
In the current study, we clarified the complete pathway for corticosteroid synthesis ‘PREG→PROG→DOC→CORT’ in hippocampal neurons. We demonstrated for the first time the expression, neuronal localization and activity of P450(c21) in the hippocampus. In addition, the localization of P450(2D4), another enzyme participating in DOC synthesis, was demonstrated. Previous investigations of brain corticosteroids have focused on PROG conversion and P450(11b) expression. The expression of P450(11b) in the hippocampus has been described in several studies and neuronal localization of P450(11b) has been demonstrated using immunohistochemistry [6,10,21]. We observed the localization of P450(11b) in cell bodies of pyramidal and basal dendrites in the hippocampus. However, the role of P450(11b) in hippocampal corticosteroid synthesis remains to be elucidated.

Table 1. Mass spectrometric analysis of the concentration of steroids in the hippocampus and plasma of adult ADX male rats.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Hippocampus (ng/g wet weight or mL)</th>
<th>Plasma (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CORT</td>
<td>2.4±0.6 (n=11)</td>
<td>0.8±0.3 (n=11)</td>
</tr>
<tr>
<td>DOC</td>
<td>1.9±0.5 (n=23)</td>
<td>0.5±0.1 (n=23)</td>
</tr>
</tbody>
</table>

aData are expressed as mean ± SEM.

Discussion

In the current study, we clarified the complete pathway for corticosteroid synthesis ‘PREG→PROG→DOC→CORT’ in hippocampal neurons. We demonstrated for the first time the expression, neuronal localization and activity of P450(c21) in the hippocampus. In addition, the localization of P450(2D4), another enzyme participating in DOC synthesis, was demonstrated.

Previous investigations of brain corticosteroids have focused on PROG conversion and P450(11b) expression. To date, direct evidence of DOC synthesis in the adult brain had not been reported. No expression of P450(c21) in the rat/mouse hippocampus has been reported, although a few studies have reported weak expression of P450(c21) mRNA in some regions of the rat brain such as hypothalamus, striatum, cerebellum [15,16,19]. As an exception, weak expression of P450(c21) mRNA was observed in the human hippocampus, with levels 1/20,000 of that of the adrenal gland [20]. Thus far, it has been believed that P450(c21) is not physiologically active in the hippocampus, and all CORT in the hippocampus is derived from the adrenal glands via the blood circulation. Although P450(2D4) has been known as the drug metabolizing P450, recently, it has been demonstrated that P450(2D4) can also convert PROG to DOC in hippocampus [16]. Therefore, both P450(c21) and P450(2D4) could contribute to DOC production. We observed the evidence of conversion of PROG to DOC, confirming de novo synthesis of DOC.

**DOC→CORT conversion and P450(11b) expression.** The expression of P450(11b) in the hippocampus has been described in several studies and neuronal localization of P450(11b) has been demonstrated using immunohistochemistry [6,10,21]. We observed the localization of P450(11b) in cell bodies of pyramidal and basal dendrites in the hippocampus. However, the role of P450(11b) in hippocampal corticosteroid synthesis remains to be elucidated.
granule neurons at mRNA and protein level. Gomez-Sanchez and co-workers observe a weak CORT production from 3H-DOC, roughly 0.4 pmol/mg/3 h in the hippocampus [6]. However, they doubted de novo synthesis of CORT from PROG since CORT levels in whole brain were negligible after adrenalectomy (ADX) of rats [3]. According to their report, CORT in the whole brain of five ADX rats was below the detection limit, but four ADX rats had measurable CORT in the brain (0.6 ng/g = 1.8 nM). Therefore, their results do not completely eliminate a possibility of the presence of brain-synthesized CORT. It should be noted that the expression level of P450(11\(^{b}\)) in the hippocampus does not change upon ADX [21]. In the hippocampus of ADX rats we observed the direct conversion of DOC to CORT (Fig. 5) as well as low, but physiologically significant level of CORT. The expression of P450(11\(^{b}\)) (both mRNA and protein) in neurons supports endogenous synthesis. Interestingly, the expression level of P450(11\(^{b}\)) is roughly 10-fold more in the cortex than in the hippocampus.

**Functional significance of hippocampus-synthesized corticosteroids**

The low nanomolar level of CORT, synthesized in the hippocampal neurons, may play an essential role in enhancement of synaptic plasticity, in contrast to the deleterious effects (e.g., neuronal cell death or shrinkage of dendrites) elicited by micromolar plasma CORT secreted from the adrenal gland under stressful conditions [22,23]. In the current study, low dose CORT (10 nM) enhanced spinogenesis, particularly increasing the density of small-head spines (Fig. 6). It has been previously demonstrated that nanomolar doses of CORT (10 nM) drives the Erk MAP kinase pathway, increasing both the expression and phosphorylation of MAP kinase [24]. Taken together, hippocampus-synthesized CORT (approx. 7 nM) might induce spinogenesis via activation of the Erk MAP kinase pathway.

Earlier studies have also shown a range of beneficial effects of nanomolar doses of CORT including strengthened signaling, neuroprotection as well as a potential role in development [25,26].
In vivo, low plasma levels of CORT increased the amplitude of population spike, as did application of low concentration (5–50 nM) of CORT to CORT-depleted slices [26]. Low doses of CORT may also have neuroprotective effects. For example, low dose CORT attenuates dexamethasone-induced apoptosis in dentate gyrus via MR signaling pathway [27]. Nanomolar low dose of CORT may also play a role in neuronal development, with 5–10 nM CORT found to enhance differentiation of neural stem cells by inducing the expression of astrocyte marker GFAP (J Kanno, K Igarashi, K Tanemura, H Asano, K Nakashima., 14th Int Cong of Endocrinol, Kyoto, 2010, P8-3-1).

Importantly, all the 7 nM of hippocampal CORT could participate in modulation of neuronal functions such as spineogenesis, because corticosteroid binding globulin (CBG) and serum albumin are not present in the hippocampus, therefore all the hippocampal synthesized CORT should be biologically active. The persistence of a significant concentration of corticosterone in the plasma (2 nM) even after ADX may be derived from local synthesis in other peripheral tissue such as adipocytes. Although we have a significant concentration of CORT in the plasma, the majority (95–98%) of plasma CORT may be biologically inactive in basal conditions, with the majority of plasma CORT (90%) bound to CBG and a large proportion of the remaining CORT bound to serum albumin [28]. Although basal plasma CORT levels range between 100 and 300 nM, biologically active free CORT comprises only 2–5% of the total plasma CORT levels [29].

Moderate CORT level is kept by weak CORT production in the hippocampus

A trace amount of endogenous DOC and CORT had been very difficult to detect by $^3$H-steroid metabolism analysis, because many different pathways exist other than PROG→DOC→CORT. Therefore, we employed mass-spectrometric assay for detection of a trace amount of these steroids in the hippocampus of ADX rats. We determined the concentrations of DOC and CORT to be 5.8 nM and 6.9 nM, respectively. These values are comparable to hippocampus-synthesized sex steroids such as estradiol (roughly 7 nM), testosterone (3 nM) and dihydrotestosterone (0.6 nM) [18]. Because the volume of hippocampus is very small (nearly 0.1 mL for one whole hippocampus), the calculated concentrations were
relatively high in nM range for DOC and CORT. The absolute contents of DOC and CORT were only around 0.27 ng and 0.34 ng in one hippocampus with a weight of 0.14 g, respectively (Table 1). Although steroid production capacity is strong in the adrenal gland, circulation levels of steroids are diluted due to approx. 20 mL of blood (200-fold of the hippocampal volume). Though the hippocampal expression levels of enzymes (such as P450(c21) and P450(11b1)) are roughly 1/20,000 and 1/10,000 in dilution, from adrenal gland, kidney and liver.

RT-PCR and Southern hybridization

The procedures were the same as described elsewhere [31]. Rats were deeply anesthetized with ethyl ether and decapitated. The brains were then removed. Total RNAs including mRNA were isolated from adult rat tissues such as the hippocampus, hypothalamus, adrenal gland, kidney and liver, using a total RNA Purification Kit (Nippongene, Japan). The purified RNAs were quantified on the basis of the absorbance at 260/280 nm, and treated with RNase-free DNase to eliminate the possibility of genomic DNA contamination. The purified RNAs were reverse-transcribed, using a M-MLV Reverse Transcriptase (Promega, USA). The oligonucleotides for PCR amplification were designed as illustrated in Table S3. The PCR protocols comprised application of a 30 sec denaturation period at 95°C, a 2 sec annealing period at individual temperature for each enzyme, and a 30 sec extension at 72°C for individual number of cycles for each enzyme (Table S3). For semiquantitative analysis, the RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide, and analyzed with a fluorescence gel scanner (Asto, Japan) and Image J software, in comparison with standard curves obtained from PCR of diluted RT products (between 1/100 and 1/10,000 in dilution), from adrenal gland, kidney and liver.

To confirm the expression, Southern hybridization was performed. The amplified RT-PCR products of steroidogenic enzymes were directly cloned into TA-cloning vector (Promega, USA), and sequenced. The resulting sequence was identical to the reported cDNA sequences of these enzymes. These cloned probes were used as the template of DNA probes for Southern hybridization. After transfer of the RT-PCR products from agarose gels to nylon membrane (Hybond N+, Amersham, USA), Southern hybridization was performed with 32P-labeled cRNA probes for these enzymes. The Southern hybridization signals were then measured using a BAS-1000 Image analyzer (Fuji film, Japan).

In situ hybridization of hippocampal slices

Rats were deeply anesthetized with ethyl ether and decapitated. The brains were removed and the hippocampus was dissected. Preparation of adult hippocampal slices fixed with 4% paraformaldehyde, was performed essentially as described in elsewhere [8,9,12]. The postfixed brain tissue was frozen-sliced coronally at 15 μm thickness with a cryostat (Leica, Germany) and slices were immediately mounted on the slide glass at ~18°C. Digoxigenin (DIG)-labeled sense and antisense cRNA probes were in vitro transcribed from PCR products of P450(11b1), P450(2D4) and P450(c21) by using T7 or Sp6 promoters inherent in pGEM-T-Easy vector. The hippocampal slices were treated with 10 μg/mL of Proteinase K (Wako, Japan) for ~20 minutes, and then postfixed with fixative solution for 10 minutes. After acetylation with acetic anhydride and dehydration, the mRNA in hippocampal slices were hybridized with 0.5 μg/mL of DIG-labeled sense or antisense cRNA probes. In order to digest and wash out the excess cRNA probes, the slices were treated with RNase A (Wako, Japan) and stringent washes after hybridization. For P450(11b1) and P450(2D4), the slices were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnosis, USA) (1/1000)
for 30 min. After washing the hippocampal slices twice, target mRNAs were visualized by color development with 0.43 mg/mL of nitro blue tetrazolium chloride (NBT) and 0.175 mg/mL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche Diagnostics, USA) for 12 h. For P450(c21), the sections were incubated with horseradish peroxidase-conjugated anti-DIG antibody (Roche, USA) (1/1000) for 30 min. To amplify signals of in situ hybridization, the slides were incubated with dinitrophenyl labeled amplification reagent using the TSA Plus DNP (AP) System (Perkin Elmer, USA) and stained with NBT/BCIP for 12 h.

**Post-embedding immunogold method for electron microscopy**

Rat hippocampal slices were prepared in essentially the same manner as described elsewhere [32], except that slicing was performed at 4°C using a vibratome instead of frozen slicing. Brains from five animals were used and from each brain, a single representative coronal section including the dorsal hippocampus was processed for ultrathin sectioning. Freeze substitution and low-temperature embedding of the specimens was performed as described previously [32]. Briefly, slices were plunged into liquid propane. The samples were immersed in uranyl acetate in anhydrous methanol (−90 °C), then infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences, USA); polymerization was performed with ultraviolet light. Ultrathin sections were cut using a Reichert-Jung ultramicrotome. For immunolabeling, sections were incubated with primary antibody for β-HSD(type1) (1:500) [33], P450(c21) (1:2000) [34], P450(2D4) (1:500) [16] or P450(1β) (1:1000) [35] in the above diluent overnight, then incubated with secondary gold-tagged (10 nm) Fab fragment in Tris-buffered saline (TBS). Sections were counter-stained with 1% uranyl acetate, and viewed on a JEOL 1200EX electron microscope (Japan). Images were captured using the CCD camera (Advanced Microscopy Techniques, USA). Controls omitting the primary antibody were performed and no immunogold labeling was observed. Controls of preabsorption with purified primary antibody were performed and no immunogold labeling was observed.

**Mass-spectrometric assay of steroids**

Detailed procedures are described elsewhere [18] (Text S1).

**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 elsewhere [12]. 1H-steroids (20,000 cpm each) were added to homogenates as internal standards. The steroid extracts were applied to a C18 Amprep solid phase column (Amersham Biosciences, USA) to remove contaminating fats. Then the steroids were separated into fractions of CORT, DOC and PROG using a normal phase HPLC system (Jasco, Japan). The steroids were separated into fractions of CORT, DOC and PROG using LC-MS/MS. For the determination of the concentration for CORT, DOC and PROG using LC-MS/MS, the hippocampus from one rat was sliced into 400 μm thickness with a vibrator and incubated with 5×10⁶ cpm of [3H]-steroids at 30°C for 5 h in 4 mL of physiological saline containing 1.2 mM Mg²⁺. The incubation medium was gassed with 95% O₂ and 5% CO₂ during the incubation in order to keep the activity of hippocampal neurons. After termination of the reaction, the slices were homogenized. A portion of the purified radioactive metabolites (total of 10⁶ cpm)
was analyzed using an HPLC system. Procedures used for the analysis of steroid metabolites from hippocampal homogenates with normal phase HPLC were the same as described in Slupski of Mass-spectrometric assay. Fraction radioactivity was measured using a liquid scintillation spectrometer LS6500 (Beckman, USA). The rate of steroid production was normalized as products (cpm) / g wet weight / 5 h.

Imaging and analysis of dendritic spine density and morphology

Slice preparation. Twelve weeks male rats were deeply anesthetized with ethyl ether and decapitated. Immediately after decapitation, the brain was removed from the skull and placed in ice-cold oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 22 NaHCO₃, and 10 D-glucose (all from Wako); pH was set at 7.4. Hippocampal slices, 400 μm thick, were prepared with a vibratome (Dosaka, Japan). These slices were ‘freshly prepared’ slices without ACSF incubation. Slices were then incubated in oxygenated ACSF for 2 h (slice recovery processes) in order to obtain conventional ‘acute’ slices. These acute slices were then incubated at room temperature with CORT for 1 h. Then, slices were prefixed with 4% paraformaldehyde at 4°C overnight.

Spine imaging and analysis by confocal microscopy was performed as described previously [38–40].

Current injection of neurons by Lucifer Yellow. Neurons within slices were visualized by an injection of Lucifer Yellow under a Nikon E600FN microscope (Japan) equipped with a C2400–79H infrared camera (Hamamatsu Photonics, Japan) and with a 40 x water immersion lens (Nikon). Dye was injected with a glass electrode filled with 5% Lucifer Yellow for 15 min, using Axopatch 200B (Axon Instruments, USA). Approximately five neurons within a 100–200 μm depth from the surface of a slice were injected (Duan H et al., 2002). After labeling, slices were fixed again with 4% paraformaldehyde at 4°C overnight.

Confocal laser microscopy and morphological analysis. Imaging was performed from sequential z-series scans with LSM5 PASCAL confocal microscope (Zeiss, Germany). For analysis of spines, three-dimensional images were constructed from approximately 40 sequential z-series sections of neurons scanned every 0.45 μm with a 63 x water immersion lens, NA 1.2 (Zeiss). The excitation and emission wavelengths were 488 nm and 515 nm, respectively. The applied zoom factor (3.0) was sufficient to allow the determination of the density of thorns or spines. Confocal images were then deconvoluted using AUTODEBLUR software (AutoQuant, USA). The density of spines as well as the head diameter was analyzed with Spiso-3D (automated software calculating geometrical parameters of spines) developed by Kawato’s group of Bioinformatics Project [41]. Spiso-3D has an equivalent capacity with NeuroLucida (MicroBrightField, USA), furthermore, Spiso-3D considerably reduces human errors and experimenter labor. We analyzed the secondary dendrites in the stratum radiatum, lying between 100 and 250 μm from the soma. The spine density was calculated from the number of spines having a total length of 50–80 μm. Spine shapes were classified into three categories as follows. (1) A small-head spine whose head diameter is 0.2–0.4 μm. (2) A middle-head spine whose head diameter is 0.4–0.5 μm. (3) A large-head spine whose head diameter is 0.5–1.0 μm. These three categories were useful to distinguish complex responses upon CORT application.

Because the majority of spines (>95%) had a distinct head and neck, and stubby spines and filopodium did not contribute much to overall changes, we analyzed spines having a distinct head. While counting the spines in the reconstructed images, the position and verification of spines was aided by rotation of three-dimensional reconstructions and by observation of the images in consecutive single planes.

Statistical analysis

Data were expressed as mean ± SEM. For Table 1, an unpaired, two-tailed t-test, under the assumption of unequal variances, was utilized 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. For analysis of spinogenesis, we used Tukey–Kramer post hoc multiple comparisons test when one way ANOVA tests yielded P<0.05.

Supporting Information

Text S1 Supporting information for materials and methods, results, and discussions. (DOC)

Figure S1 Pathways of corticosteroid synthesis in the hippocampus. The abbreviated names of steroid (underline) and enzyme (italic) involved in each reaction are indicated. (TIF)

Figure S2 Gibbs free energy (ΔG) of P450c21 primer pairs used in the current study. The vertical axis indicates ΔG of the primer and template DNA. The dotted line indicates the average of ΔG (ΔGav). We design these high sensitive primer pairs which have higher ΔG for the 3’-side primer than the 5’-side primer. The last five bases of the primers with the higher ΔG than ΔGav avoid the non-specific amplification (closed bar). (TIF)

Figure S3 Immunohistochemical staining for P450 (11b1) in the hippocampus. The coronal section of the whole hippocampus is used. P450(11B1) is expressed in pyramidal neurons in CA1-CA3 region and granule cells in DG. The expression of P450(11B1) in glial cells is weak. Scale bar, 800 μm. (TIF)

Figure S4 Western immunoblot analysis of P450c21 (A), P450(2D4) (B), P450(11b1) (C), and 3b-HSD (type 1) (D) in subcellular fractions of male rat hippocampus. From left to right, postsynaptic membrane-rich fraction (Post), presynaptic membrane-rich fraction (Pre), postsynaptic density fraction (PSD), microsome (MS) and positive control. Adrenal gland (Ad) for (A) and (C), Liver (Li) for (B); and ovary (Ov) for (D) were used as positive control samples. The amount of protein applied to the gels is 20 μg for each hippocampal fraction, 0.5 μg for Ad, and 1 μg for ovary or liver. (TIF)

Figure S5 Calibration curves for LC-MS/MS using standard steroids dissolved in ethanol. Horizontal (x) axis indicates the concentration of added standard steroid. Vertical (y) axis indicates the relative intensity obtained from the chromatogram. (A) Calibration curve for CORT. Linearity is observed between 2 pg/mL to 4000 pg/mL (in this figure only until 1000 pg/mL is shown). (B) Calibration curve for DOC. Linearity is observed between 1 pg/mL to 1000 pg/mL. (TIF)
**Table S1** The accuracy of steroid determination for hippocampal tissue spiked with exogenous steroids. (DOC)

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

**Table S3** The sequence of primer oligonucleotides for PCR amplification. (DOC)

**Acknowledgments**

Dr. Seijiro Honma (Aska Pharma Medical) is acknowledged for collaboration of mass-spectrometric analysis. Prof. John Morrison (Mount Sinai School of Medicine) is acknowledged for immunoelectron microscopic analysis. Prof. J. Ian Mason (University of Edinburgh), Dr. Fumiko Furuse (Osaka City University) are acknowledged for their kind gifts of 3β-HSD antibody, P450(1β) antibody, and P450(2D4) antibody, respectively.

**Author Contributions**

Conceived and designed the experiments: SH YH SK. Performed the experiments: SH YH HI YK YO GM HM TK. Analyzed the data: SH YH YK. Contributed reagents/materials/analysis tools: SK TY DN. Wrote the paper: SK SH YB.

**References**


9. Hojo Y, Higo S, Ishii H, Miura M, Mitani (Keio University) and Prof. Yoshihiko Funae (Osaka City University) collaboration of mass-spectrometric analysis. Prof. John Morrison (University of Edinburgh), Dr. Fumiko Furuse (Osaka City University) are acknowledged for their kind gifts of 3β-HSD antibody, P450(1β) antibody, and P450(2D4) antibody, respectively.


Synthesis of Corticosteroids in the Hippocampus