Neurosteroid Synthesis by Cytochrome P450-Containing Systems Localized in the Rat Brain Hippocampal Neurons: *N*-Methyl-*D*-Aspartate and Calcium-Dependent Synthesis

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Neurosteroidogenesis has not been well elucidated due to the very low level of steroidogenic proteins in the brain. Here we report the first demonstration of the neuronal localization of neurosteroidogenic systems as well as the regulation of neurosteroidogenic activity in the adult rat hippocampus. Significant localization of cytochrome P450scc was observed in pyramidal neurons and granule neurons by means of immunohistochemical staining of slices. We also observed the colocalization, in hippocampal neurons, of P450scc with redox partners, hydroxysteroid sulfotransferase and steroidogenic acute regulatory protein. The distributions of astroglial cells and oligodendroglial cells showed very different patterns from that of the P450scc-containing cells. The expression of P450scc, redox partners, the sulfotransferase, and steroidogenic acute regulatory protein was also confirmed by Western blot analysis.

The process of active neurosteroidogenesis was stimulated by exposing neurons to *N*-methyl-*D*-aspartate. Upon stimulation with *N*-methyl-*D*-aspartate, Ca^{2+} influx through the *N*-methyl-*D*-aspartate subtype of glutamate receptors occurred, and significant net production of pregnenolone and pregnenolone sulfate was observed in the hippocampus. This neurosteroid production was considerably suppressed by the addition of antagonists of *N*-methyl-*D*-aspartate receptors, by Ca^{2+} depletion, or by the addition of an inhibitor of P450scc. Upon stimulation with *N*-methyl-*D*-aspartate, the processing of full-length steroidogenic acute regulatory protein (37-kDa) to the truncated 30-kDa steroidogenic acute regulatory protein was observed.

Taken together, these observations imply that hippocampal neurons synthesize neurosteroids. This synthesis may be stimulated and regulated by glutamate-mediated synaptic communication. (*Endocrinology* 142: 0000–0000, 2001)

THE HIPPOCAMPUS, which is involved essentially in learning and memory processes, is known to be a target for the neuromodulatory actions of the steroid hormones produced in the adrenal glands and gonads. Extensive studies have been performed on the role of glucocorticosteroids in modulating hippocampal plasticity and functions, because of the ability of glucocorticosteroids to reach the brain through the blood circulation after crossing the blood-brain barrier (1). In addition to hormones derived from the endocrine glands, the active *de novo* synthesis of neurosteroids suggests that hippocampal neurons may be exposed to locally synthesized neurosteroids, such as pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulfated esters (PREGS and DHEAS). Recent studies reported the

presence of significant amounts of neurosteroids such as PREG(S), DHEA(S), and other derivatives in the rodent cerebrum, cerebellum, and retina (2–4). Moreover, adrenalectomy did not decrease the concentrations of PREG(S) and DHEA(S) in the rodent brain (5).

In contrast to hormones derived from the circulation, there is increasing evidence that neurosteroids can modulate neurotransmission acutely in an excitatory or inhibitory manner (6). The acute actions of neurosteroids are thought to be mediated through ion-gated channel receptors rather than through the nuclear steroid receptors that promote the classic genomic actions of adrenal steroid hormones. In particular, PREGS potentiates the Ca^{2+} conductivity of the *N*-methyl-*D*-aspartate (NMDA) receptors (7–9) and suppresses the Cl^- conductivity of the γ-aminobutyric acid receptors in cultured rat hippocampal neurons (10). In combination, these actions of PREGS could facilitate excitation of the postsynaptic neurons (11).

It is plausible to assume that neurosteroid synthesis in brain cells might be partially similar to the synthesis of peripheral steroids [*i.e.* initiates with the transport of cholesterol into the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR) and follows with the conversion of cholesterol to PREG by cytochrome P450scc

(CYP11A1)]. Cytochrome P450scc catalyzes the side-chain cleavage reaction of cholesterol, which is promoted by electron transfer from NADPH to P450scc through NADPH-adrenodoxin reductase (ADR) and adrenodoxin (ADX). PREG would then be converted to neuroactive PREGS by hydroxysteroid sulfotransferase (12).

Many studies have reported the low level expression of mRNAs, encoding for steroidogenic enzymes, in brain homogenates prepared from whole cerebrum and cerebellum. For example, the level of P450scc mRNA present in the brain homogenates was only a small fraction (*i.e.* 10^{-4}–10^{-5}) of that observed in the adrenal gland (13). However, the identity of the cell types that contained the steroidogenic enzymes was not clearly determined and has remained controversial, not only in the hippocampus but also in other regions of the brain. To enable understanding of the mode of neurosteroid action, it is clear that the cells producing neurosteroids should be determined (*e.g.* whether the neurosteroids are synthesized in neurons or glial cells). Although the cellular location of mRNAs encoding for several steroidogenic enzymes, such as StAR, 3β-hydroxysteroid dehydrogenase (3βHSD), and P450scc, was shown to be the pyramidal cells and the granule cells of the hippocampus, the clarity of P450scc mRNA hybridization in these studies was very low (14, 15).

As a discrepancy has been reported between the mRNA level and the protein level for P450scc (13), a direct investigation of the protein level should be performed for the various steroidogenic enzymes (*i.e.* the amounts and distribution). A fully successful direct examination of the steroidogenic proteins using immunostaining and Western blot has not yet been reported for the hippocampal cells. In addition, the net neurosteroidogenesis of PREGS from domestic cholesterol through PREG has not been demonstrated in the hippocampus, although the analysis of such activity and its regulation is essential for determining whether neurosteroids contribute significantly to the modulation of neuronal activities. Several reports along this line of investigation have appeared for the cerebellar Purkinje cells (4) and the retina (16).

In the present study to clarify the molecular mechanisms of neurosteroidogenesis in the hippocampal cells, 1) we performed a direct demonstration of cell-specific colocalization of a complete set of neurosteroidogenic enzymes responsible for PREG and PREGS production, and 2) we examined the regulatory mechanisms of neurosteroidogenesis by stimulating neurons with NMDA-receptor mediated Ca^{2+} influx. Because PREGS is known to potentiate NMDA receptor-mediated Ca^{2+} currents, we also discuss the possibility that PREGS may act as a paracrine modulator of NMDA receptors.

Materials and Methods

Materials

Bovine liver acetone powder, phenylmethylsulfonylfluoride, (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclo-hepten-5,10-imine hydrogen malate (MK-801), 2-amino-5-phosphonopentanoic acid (AP5), leupeptin, aminoglutethimide (AMG) and cremophor EL were purchased from Sigma (St. Louis, MO). NMDA was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Normal goat serum,

biotinylated goat antirabbit IgG, and avidin-horseradish peroxidase complex were obtained from Vector Laboratories, Inc. (Burlingame, CA). Cy3-labeled antimouse IgG was purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA). Streptavidin-Oregon Green 488 complex and fura-2/AM were obtained from Molecular Probes, Inc. (Eugene, OR). Streptavidin-horseradish peroxidase complex, C_{18} Amprep minicolumns, and ECL plus Western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Antirat P450scc antibodies, raised against a peptide sequence (amino acids 421–441) of rat P450scc (17), were obtained from Chemicon (Temecula, CA). Antibodies against neuronal nuclear antigen (NeuN), glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) were also purchased from Chemicon. [7-^{3}H]PREG (23.5 Ci/mmol) and [7-^{3}H]DHEAS (16.0 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA). The PREG ^{3}H RIA kit and System I Celite columns were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Anti-StAR polyclonal antibodies raised against a peptide sequence (amino acids 88–98) of mouse StAR (18) were donated by Dr. Stocco. Anti-bovine ADR and anti-bovine ADX antibodies were donated by Dr. Hara. Purified P450scc, ADR, and ADX were obtained from Drs. Kominami and Hara. Trilostane and SU-10603 were purchased from Mochida Pharmaceutical (Japan) and Novartis (), respectively. All other chemicals used were of the highest purity commercially available.

Preparation of rat hippocampal slices for immunohistochemistry

Adult male Wistar rats, aged 3 months (from SLC Japan Co., Japan), were deeply anesthetized with pentobarbital and perfused transcardially with PBS comprised of 0.1 M phosphate buffer and 0.14 M NaCl (pH 7.3), followed by a fixative solution (4% paraformaldehyde in PBS) at 4 C. After dissection from the skulls, the hippocampi were postfixed for 24–48 h in the fixative solution at 4 C and then cryoprotected in PBS with 30% sucrose. The hippocampi were frozen-sliced coronally at 20-μm thickness with a cryostat (CM1510, Leica Corp., Germany) at −17 C. All experiments using animals were conducted according to the institutional guidelines.

Immunohistochemical staining of hippocampal slices

Staining of cytochrome P450scc, hydroxysteroid sulfotransferase, and StAR was performed by the avidin-biotin-peroxidase complex technique according to the free floating method. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. After blocking with a PBS solution containing 5% normal goat serum, 3% fat-free skim milk, and 0.5% Triton X-100 for 2 h, the slices were treated with antirat P450scc antibodies (1:200), anti-sulfotransferase IgG (1:1000) (19), or anti-StAR IgG (1:500) for 36–48 h at 4 C in the presence of 3% skim milk and 0.5% Triton X. Anti-P450scc antibodies were pretreated with 0.5% liver acetone powder and 3% skim milk for 18 h at 4 C. The slices were then washed three times for 5 min each time with a PBS solution containing 0.05% Tween 20. Biotinylated antirabbit IgG diluted in a PBS solution containing 0.5% skim milk was then applied for 30 min, followed by incubation for 30 min with the avidin-horseradish peroxidase complex. Immunoreactivity was detected by immersing the slices for 2 min in a detection solution [0.1 M Tris-HCl (pH 7.2) containing 0.05% diaminobenzidine, 0.1% H_2O_2, and 0.3% ammonium nickel sulfate]. After dehydration and embedding, the immunoreactive cells were examined.

Fluorescence immunohistochemistry of P450scc and the sulfotransferase was carried out in the same manner as avidin-biotin-peroxidase complex staining, except that the avidin-horseradish peroxidase complex was substituted with streptavidin-Oregon Green 488 complex. The distributions of neurons, astroglial cells, and oligodendroglial cells were visualized by immunostaining with monoclonal antibodies to NeuN (1:100), GFAP (1:10,000), and MBP (1:100), respectively. Detection of neuronal/glial marker proteins was achieved with Cy3-labeled antimouse IgG without avidin-biotin amplification. Fluorescence signals were observed by an MRC-1024 confocal microscope equipped with an argon-kryton laser (Bio-Rad Laboratories, Inc., UK). Oregon Green 488 was excited at 488 nm, and fluorescence above 522 nm was selected. Cy3 was excited at 563 nm, and emission above 605 nm was observed.

The immunoreactions of ADR (1:100,000 IgG dilution) and ADX (1:50,000 IgG dilution) were visualized by the tyramide signal amplifi-

cation method (TSA-Indirect Kit, NEN Life Science Products). For pre-absorption of antibodies with antigens, 30 μg/ml purified antigen proteins were used.

Preparation of mitochondrial fractions and cytosolic fractions

The hippocampus, cerebellum, testis, and lung excised from 3-month-old male Wistar rats were minced and homogenized in a glass-Teflon homogenizer (40 strokes) at 4 C in the homogenization buffer [50 mM potassium phosphate buffer (pH 7.4), 250 mM sucrose, 5 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.1 mM leupeptin, and 3 mM 2-mercaptoethanol]. After the removal of nuclei and debris by centrifugation at 3,000 $\times$ g for 10 min, the mitochondrial fractions were pelleted by centrifugation at 10,000 $\times$ g for 10 min (20). Purification was repeated, and the mitochondrial fractions were suspended in a 10 mM potassium phosphate buffer (pH 7.4) containing 250 mM sucrose, 5 mM MgCl$_2$, and 20 mM KCl.

For the cytosolic fractions, homogenates were centrifuged for 90 min at 105,000 $\times$ g (19). The protein concentration was measured with a bicinchoninic acid protein determination kit (Pierce Chemical Co., Rockford, IL) using BSA as a standard.

Western immunoblot analysis

The mitochondrial fractions and cytosolic fractions were diluted to 10 mg protein/ml with a sample buffer composed of 62.5 mM Tris-HCl (pH 6.8), 6% SDS, 5% sucrose, 5% 2-mercaptoethanol, and 0.01% bromophenol blue. Samples were denatured for 5 min at 90 C and subjected to electrophoresis. Ten percent polyacrylamide gels were employed for P450scc and ADR. For ADX, the sulfotransferase, and StAR, 15%, 12%, and 12.5% polyacrylamide gels were used, respectively. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA) with a TE70 semi-dry blotting apparatus (Amersham Pharmacia Biotech) for 90 min at 2.2 mA/cm^2. Blotted membranes were washed three times with PBST (PBS containing 0.05% Tween 20) and blocked for 15 min in a PBST solution containing 10% fat-free skim milk. Blots were then probed with antibodies against P450scc (1:5,000), ADR (1:5,000), ADX (1:20,000), sulfotransferase (1:5,000), and StAR (1:1,000) for 12–18 h at 4 C in PBST containing 2% skim milk. After primary antibody treatment, the blots were washed and treated with biotinylated goat antirabbit IgG (1:2,000) for 1 h. Finally, the membranes were treated with streptavidin-horseradish peroxidase complex (1:3,000) for 1 h. Biotinylated SDS-PAGE standards (Bio-Rad Laboratories, Inc.) were used as a mol wt marker. The protein bands were detected with Amersham Pharmacia Biotech ECL Plus Western blotting detection reagents. For quantitative analysis, images of the blots were captured with a scanner, and densitometric analysis was performed using NIH Image 1.61 software.

RIA of neurosteroids

Adult male Wistar rats, aged 3 months, were decapitated, and trunk blood was collected in heparinized tubes. Blood was centrifuged at 1,800 $\times$ g for 20 min at 4 C to obtain plasma. Just after the blood collection, the hippocampi were excised and transferred into low Mg^{2+} physiological saline [low Mg^{2+} PSS, composed of 137 mM NaCl, 2.5 mM CaCl$_2$, 1 mM NaHCO$_3$, 0.34 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 5.7 mM KCl, 0.1 mM MgSO$_4$, 22 mM glucose and 5 mM HEPES (pH 7.2)] into which O$_2$ gas was bubbled at 4 C. The hippocampi were rinsed once and sliced into 400-μm cubes. The hippocampal cubes were then incubated at 37 C in a low Mg^{2+} PSS solution containing 2 μM trilostane and 20 μM SU-10603 in the presence and absence of NMDA and inhibitors such as MK-801, AP5, and AMG. Trilostane and SU-10603 were used to inhibit the further conversion of PREG to other steroids. A 30-min incubation period was used in most experiments, except for the time-course measurements. For the measurements of basal neurosteroid concentrations, incubation was omitted. For the Ca^{2+} depletion assay, CaCl$_2$ was substituted by 2.7 mM EGTA. The steroidogenic reaction was terminated by adding 1 N NaOH. The hippocampal cubes were then homogenized. A trace amount of [7-^{3}H]PREG (500 cpm) was added to monitor the recovery of PREG through the extraction and chromatographic proce-

dures. For monitoring the recovery of PREGS through the extraction and solvolysis, [7-^{3}H]DHEAS (500 cpm) was employed (21).

Extraction and purification of PREG(S) were performed according to Liere *et al.* (22) with a slight modification. Briefly, homogenates were mixed with methanol solution containing 1% acetic acid (1:10, vol/vol) and sonicated for 10 min with a sonifier (XL2020, Heat Systems). The extraction was performed overnight, after which the mixture was centrifuged at 10,000 $\times$ g for 30 min. The extraction was then repeated, the collected supernatant was combined, and the solvents were evaporated under an N$_2$ stream. Dried residues were reconstituted in methanol-water (40:60, vol/vol) and subjected to the solid phase extraction on Amersham Pharmacia Biotech C$_{18}$ Amprep minicolumns (500 mg) to separate nonconjugated steroids and sulfated steroids.

For the PREG measurements, the unconjugated steroid fractions were used. PREG was purified by Celite column chromatography (system I), according to the manufacturer's instructions. PREG was then reconstituted in RIA buffer consisting of 0.15 M NaCl, 0.1% gelatin, 0.02% NaN$_3$, and 0.1 M sodium phosphate (pH 7.0). The average PREG recovery was 65.5%. The mass of the PREG was measured by RIA using a PREG RIA kit (ICN Biomedicals, Inc.). The lower limit of PREG detection was 0.025 ng/sample. The intra- and interassay coefficients of variation were 5–8% and 11–17%, respectively. The cross-reactivity of the anti-PREG antibody with other steroids, such as deoxycorticosterone, progesterone, aldosterone, testosterone, 17β-estradiol, corticosterone, and DHEA, was less than 0.03%, as determined by the RIA kit manufacturer.

For PREGS, the sulfated steroid fraction was used. The solvent was evaporated, and the steroids were reconstituted in ethyl acetate. Sulfuric acid was added (final concentration, 2 mM), and PREGS was converted to PREG by solvolization. After washing once with 1 N NaOH and twice with water, PREG was purified by Celite columns. Finally, the mass of PREG was measured by RIA. Recovery of PREG through Celite chromatography was monitored using [7-^{3}H]PREG (300 cpm). The average final recovery of PREGS was 48.0%.

Measurement of Ca^{2+} signals with digital fluorescence microscopy

Intact slices of the hippocampus (400-μm thickness) were prepared from 3-month-old male Wistar rats with a Vibratome (Dosaka, Japan) for both Ca^{2+} measurements and electrophysiological measurements. The intracellular Ca^{2+} concentration was measured using fura-2 (23). Slices were loaded for 30 min at 37 C with 10 μM fura-2/AM in low Mg^{2+} PSS in the presence of 0.03% cremophor EL.

The slices were stimulated by perfusion with an O$_2$-bubbled low Mg^{2+} PSS solution containing 100 μM NMDA at 37 C at a rate of 1.5 ml/min. We used a digital fluorescence microscope system that consisted of an inverted microscope (TMD-300, Nikon, Japan) equipped with a CCD camera (Hamamatsu Photonics C2400–77, Japan). The excitation wavelength (340 and 380 nm) was changed every 1.15 sec with a step motor, and fluorescence above 520 nm was measured. The intracellular Ca^{2+} concentration was expressed as F340/F380, which is the ratio of the fura-2 fluorescence intensity at 340 nm excitation (F340) to that at 380 nm excitation (F380). Data in each area of 10 $\times$ 10 pixels were averaged with a 2.3-sec time resolution. The image analysis was performed with an ARGUS-50 system (Hamamatsu Photonics).

Electrophysiological measurements of hippocampal slices

We used a field electrophysiology apparatus. Electrical stimulation was regulated with a Master 8 controller (Axon Instruments, Foster City, CA), and signal processing and analysis were performed with a personal computer using Clamp 8 software (Axon Instruments). Intact hippocampal slices were perfused with O$_2$-bubbled low Mg^{2+} PSS at 37 C at a rate of 1.5 ml/min.

Field excitatory postsynaptic potentials (EPSPs) were recorded from the stratum radiatum of CA1 with a tungsten electrode. In each case the Schaffer collateral was stimulated by a test stimulus (0.05 Hz) at an intensity adjusted to evoke a response that was 50% of the maximum EPSPs.

Data analysis

Results of RIA and the electrophysiological experiments are expressed as the mean ± SEM. Statistical significance was evaluated using Student's *t* test for pairwise comparison.

Results

Distributions of neurosteroidogenic proteins in the hippocampus

To determine the neurosteroidogenic cells in the hippocampus, we investigated the localization of cytochrome

P450scc by immunohistochemical staining of the hippocampus of adult male rats (Fig. 1). An intense immunoreaction with antirat P450scc IgG was restricted to pyramidal neurons in the CA1–CA3 regions as well as granule cells in the dentate gyrus. Neuronal nuclear antigen staining with anti-NeuN IgG was used as a reference to determine the distribution of neurons. The somata layer of pyramidal neurons was characterized as an elongated C-shaped long curved layer throughout the CA1–CA3 regions of the hippocampus. Granule cells in the dentate gyrus showed a characteristic arrow-
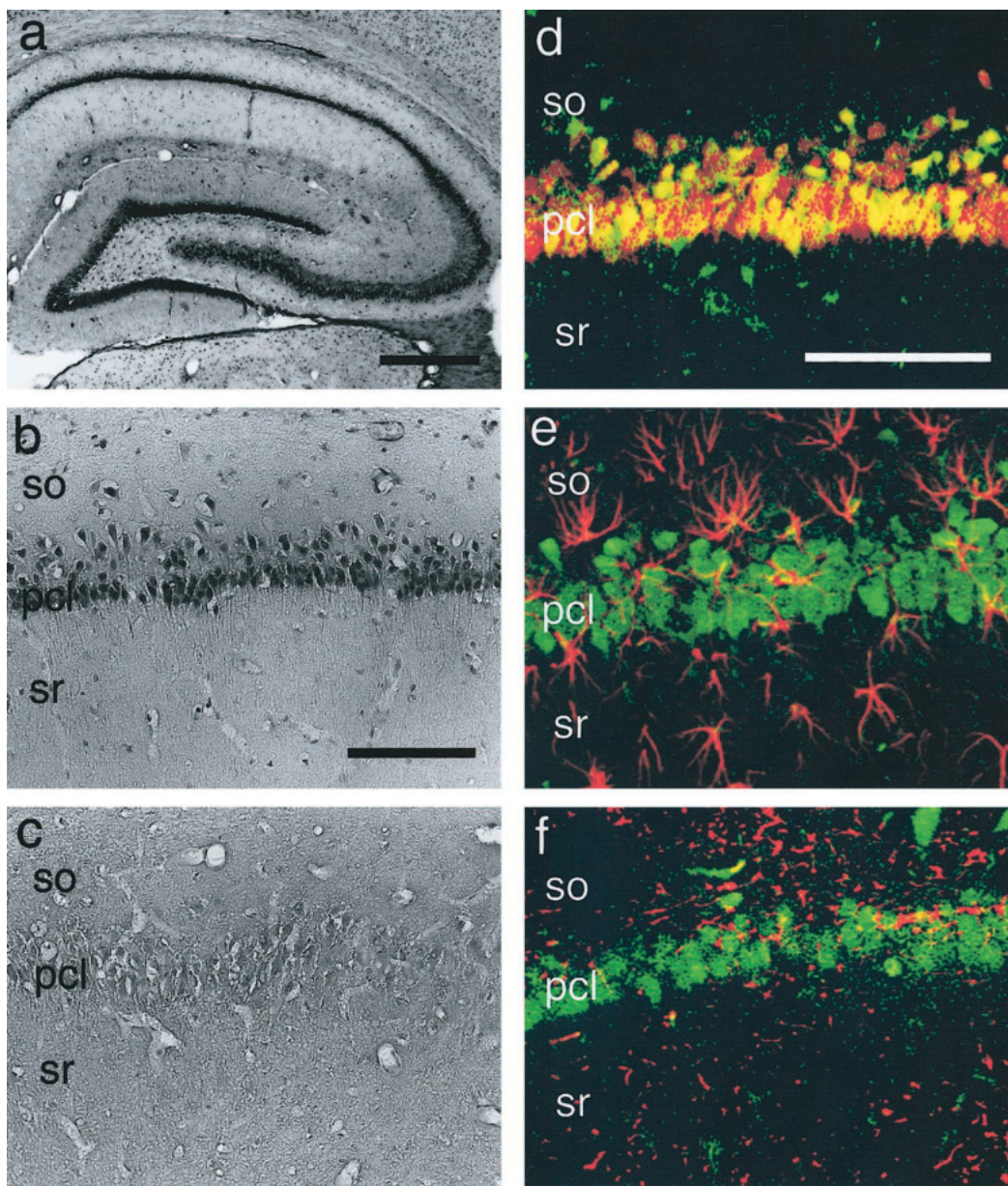

FIG. 1. Immunohistochemical staining of cytochrome P450scc in hippocampal slices of an adult male rat. A, Low magnification image of the whole hippocampus, stained with antibodies against rat cytochrome P450scc. B, The hippocampal CA1 region, stained with antibodies against rat P450scc. C, Staining with anti-P450scc IgG, preincubated with a saturated concentration of purified P450scc in the CA1 region. D, Fluorescence dual staining of P450scc (*green*) and NeuN (*red*). E, Fluorescence dual staining of P450scc (*green*) and GFAP (*red*). F, Fluorescence dual staining of P450scc (*green*) and MBP (*red*). A superimposed region of green and red fluorescence is represented in *yellow*. B and C, and D–E are at the same magnification. so, Stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. A–C, Immunoreactive cells were visualized by diaminobenzidine-nickel staining. *Scale bar*, 800 μm (A), 120 μm (B and C), and 100 μm (D–F). Immunohistochemical experiments were performed for several different animals. In each case the same results were obtained.

head-like distribution. The colocalization of immunoreactivity against P450scc and NeuN was demonstrated with fluorescence dual labeling procedures, showing that P450scc was present in pyramidal neurons. Preabsorption of the antibody with an excess of purified bovine P450scc antigen (30 μg/ml) resulted in the complete absence of P450scc immunoreactivity in all of the positively stained cells in the hippocampus (Fig. 1) due to cross-reaction of the antirat P450scc antibodies that we used (17). Nonimmunized serum did not

cause any positive staining of the hippocampus. The localization of hydroxysteroid sulfotransferase was examined with antirat sulfotransferase IgG. The immunoreaction was restricted to pyramidal neurons and granule cells in the hippocampus, as shown in Fig. 2 using fluorescence double labeling. Preabsorption of IgG with purified rat liver hydroxysteroid sulfotransferase resulted in the complete disappearance of immunoreactive cells.

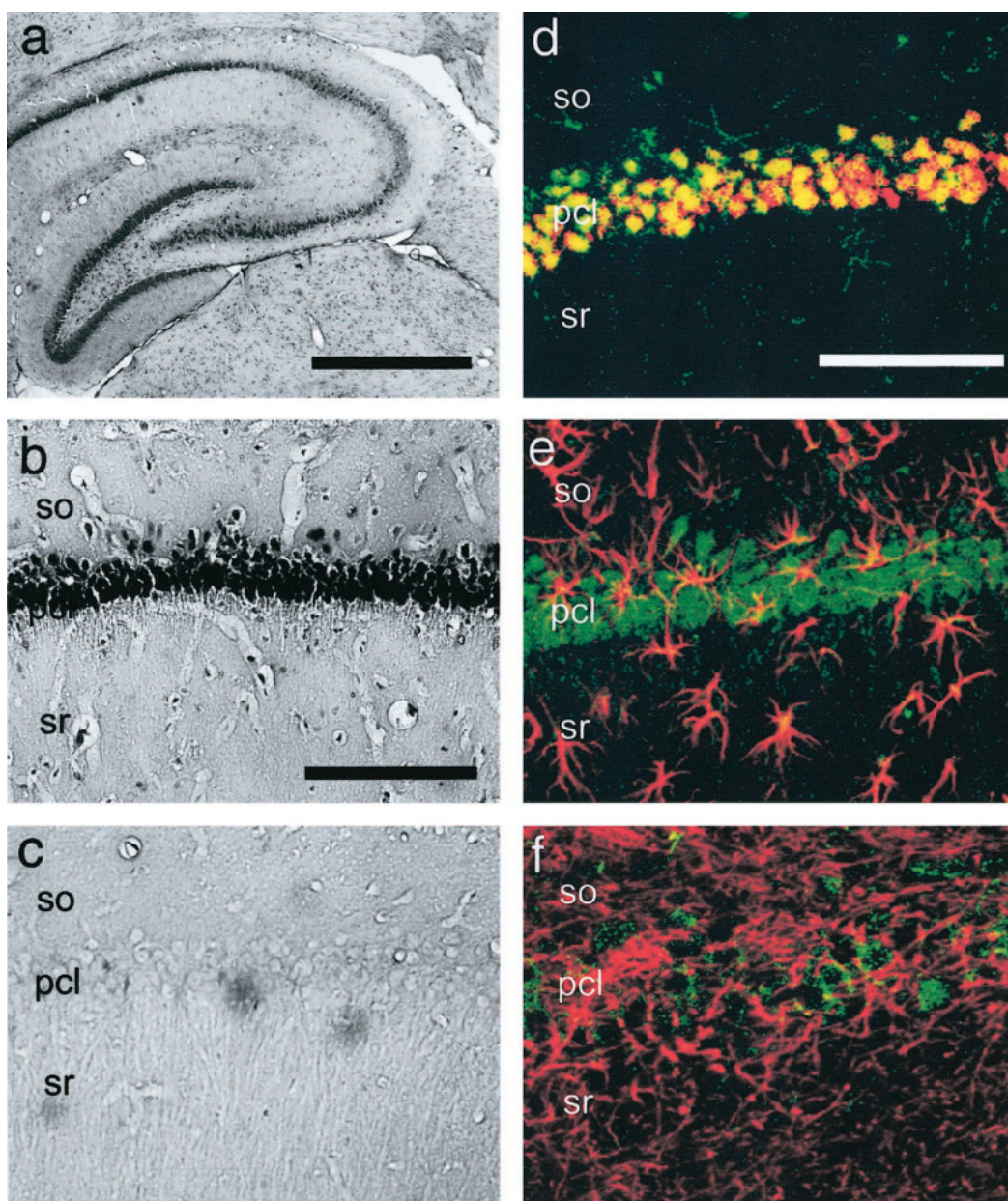Distribution of glial cells was investigated by immuno-

FIG. 2. Immunohistochemical staining of the hydroxysteroid sulfotransferase in hippocampal slices of an adult male rat. A, Low magnification image of the whole hippocampus, stained with antibodies against rat hydroxysteroid sulfotransferase. B, The hippocampal CA1 region, stained with antibodies against rat sulfotransferase. C, Staining with antisulfotransferase IgG, preincubated with a saturating concentration of purified hydroxysteroid sulfotransferase in the CA1 region. D, Fluorescence dual staining of the sulfotransferase (*green*) and NeuN (*red*). E, Fluorescence dual staining of the sulfotransferase (*green*) and GFAP (*red*). F, Fluorescence dual staining of the sulfotransferase (*green*) and MBP (*red*). A superimposed region of green and red fluorescence is represented in *yellow*. B and C, and D–F are at the same magnification. so, Stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. A–C, Immunoreactive cells were visualized by diaminobenzidine-nickel staining. *Scale bar*, 800 μm (A), 120 μm (B and C), and 100 μm (D–F).

staining of marker proteins. Antibodies against GFAP, a marker protein of astroglial cells, stained astro-shaped cells in the stratum radiatum and stratum oriens in the hippocampus. The antibodies against MBP, a marker protein of oligodendroglial cells, stained many long fibril cells in the hippocampus. Almost all cells that were immunoreactive to anti-GFAP antibodies and anti-MBP antibodies were also lacking in immunoreactivity to anti-P450scc antibodies and antisulfotransferase antibodies, indicating that most of the P450scc- and sulfotransferase-containing cells are neither astroglial cells nor oligodendroglial cells (see Figs. 1 and 2).

We also investigated the presence of redox partners of P450scc in pyramidal neurons. Immunolabeling was performed for ADR and ADX, which transfer electrons to P450scc. Antibodies against ADR and ADX stained pyramidal neurons and granule cells (Fig. 3). These results support the hypothesis that pyramidal neurons contain a complete neurosteroidogenic system that catalyzes the conversion of cholesterol to PREG and is driven by electron transport from NADPH to P450scc through ADR and ADX. The localization of StAR protein was investigated with anti-StAR IgG. The immunoreaction was also restricted to pyramidal neurons and granule cells in the hippocampus (see Fig. 4).

Western immunoblot analysis

To demonstrate the presence of steroidogenic enzymes biochemically, we performed a Western blot analysis. The single bands for P450scc, ADR, ADX, and hydroxysteroid sulfotransferase were observed when mitochondrial fractions (for P450scc, ADR, and ADX) or cytosolic fractions (for the sulfotransferase) derived from the hippocampus were subjected to Western blotting, as illustrated in Fig. 5. The electrophoretic mobility of the P450scc band for the hippocampus was almost identical to that of the purified bovine adrenocortical P450scc, whose M_r was approximately 54 kDa. The M_r of ADR, ADX and the sulfotransferase were approximately 54, 12, and 30 kDa, respectively, which were close to those of bovine adrenal proteins (ADR and ADX) or rat liver proteins (hydroxysteroid sulfotransferase).

To analyze the presence of StAR in the hippocampus, mitochondria were subjected to Western blotting (see Fig. 6). A dense band with a M_r of approximately 37 kDa was observed in the mitochondria. For the mitochondria of bovine adrenal cortex and rat testis, a single band was observed at 30 kDa. When we subjected the mitochondria that were treated with 100 μM NMDA to Western blotting for 30 min,
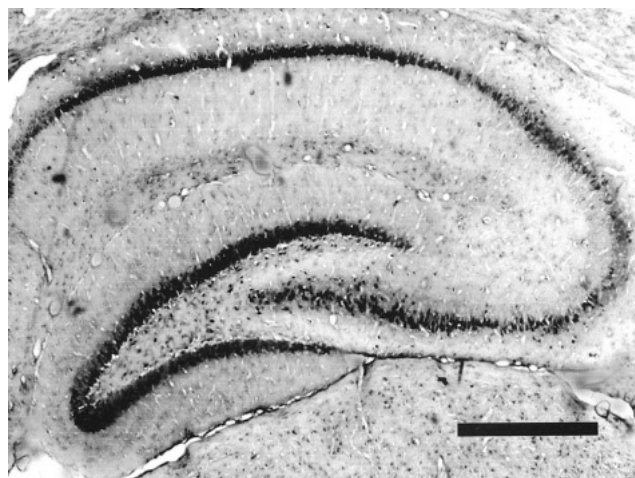

FIG. 4. Immunohistochemical staining of StAR in the hippocampal slice of an adult male rat. Immunoreactive cells were visualized by diaminobenzidine-nickel staining. *Scale bar*, 800 μm.
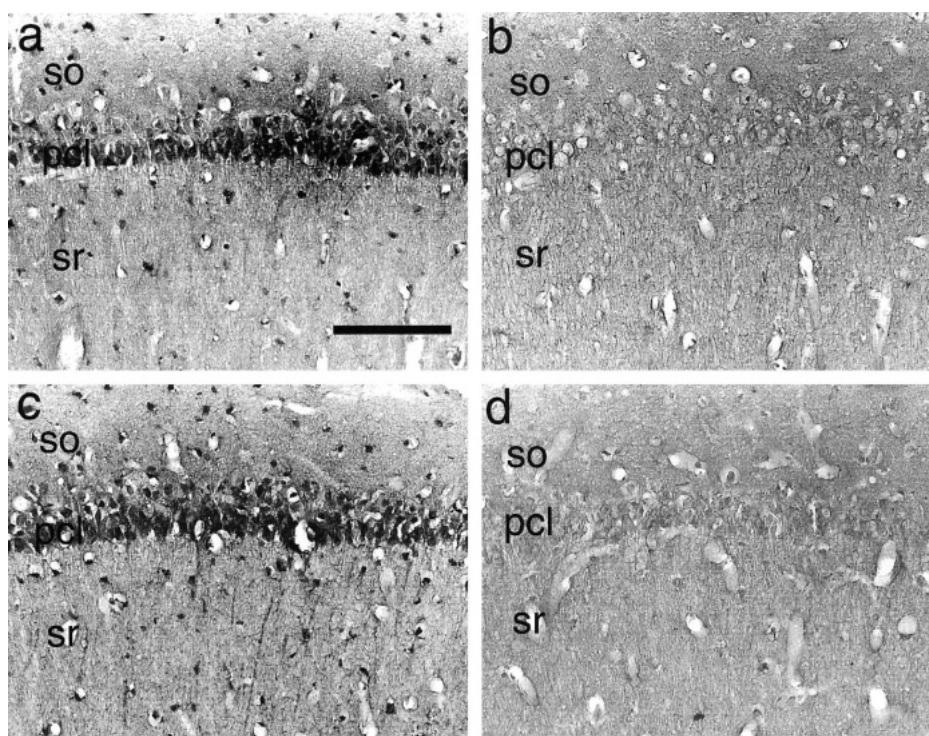

FIG. 3. Immunohistochemical staining with antibodies against ADR and ADX in the hippocampal CA1 region of an adult male rat. A, Staining with anti-ADR IgG. B, Staining with anti-ADR IgG, preabsorbed with purified ADR. C, Staining with anti-ADX IgG. D, Staining with anti-ADX IgG, preabsorbed with purified ADX. so, Stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. Immunoreactivity was visualized by diaminobenzidine-nickel staining. *Scale bar*, 120 μm.
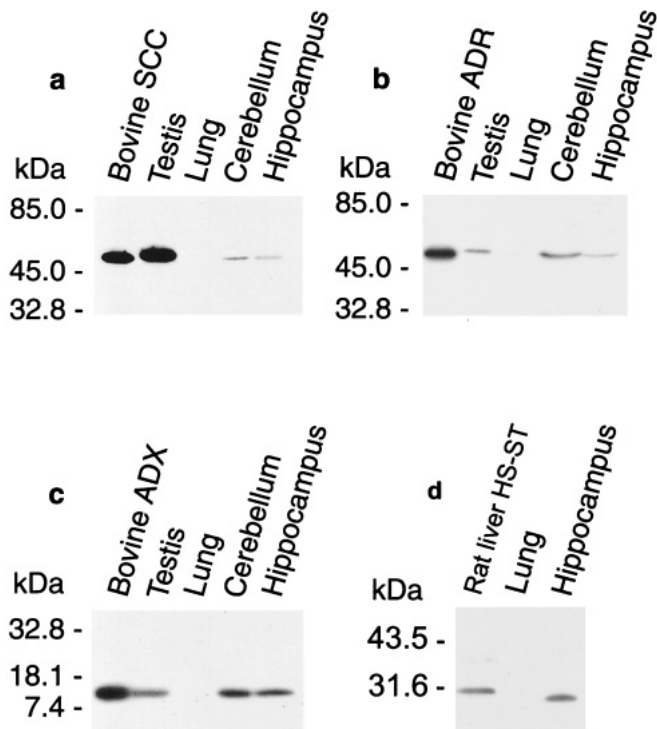
FIG. 5. Western immunoblot analysis of P450scc, ADR, and ADX in mitochondria and that of hydroxysteroid sulfotransferase in cytosol. A, P450scc; B, ADR; C, ADX; D, the sulfotransferase. From *left* to *right* (A–C), the purified bovine proteins (P450scc in A, ADR in B, and ADX in C), mitochondria from rat testis, lung, cerebellum, and hippocampus. Rat lung mitochondria were used as a negative control. Purified proteins (1 ng protein each), testis mitochondria, and cerebellum mitochondria (1 μg protein each) were used as positive controls. Each lane of mitochondria from lung, cerebellum, and hippocampus contained 50 μg protein. From *left* to *right* (D), rat liver hydroxysteroid sulfotransferase (1 ng protein) and cytosol from lung and hippocampus (50 μg protein).

the density of the 37-kDa band decreased, and the density of the 30-kDa band increased. These observations imply that the full-length 37-kDa StAR was converted to 30-kDa StAR upon NMDA stimulation.

Neurosteroidogenic activity in the rat hippocampus

We examined the activity of the neurosteroidogenic system in the adult male rat hippocampus. First, the basal concentrations of PREG and PREGS were measured in the hippocampus, cerebellum, and plasma (see Table 1). Significant concentrations of PREG were observed in the hippocampus (0.165 pmol/mg protein) and cerebellum (0.172 pmol/mg protein). The concentration of PREGS was 0.294 pmol/mg protein in the hippocampus. The concentrations of PREG and PREGS in the plasma were considerably lower than those in the hippocampus (see Table 1). The basal concentrations of PREG and PREGS in the hippocampus, cerebellum, and plasma were close to previously reported values (4, 22, 24).

Next, the NMDA-stimulated production of neurosteroids was investigated in hippocampal tissues. Incubation of hippocampal cubes for 30 min with various concentrations of NMDA (10–500 μM) resulted in a dose-dependent increase in the concentration of PREG (Fig. 7). The NMDA dosage that
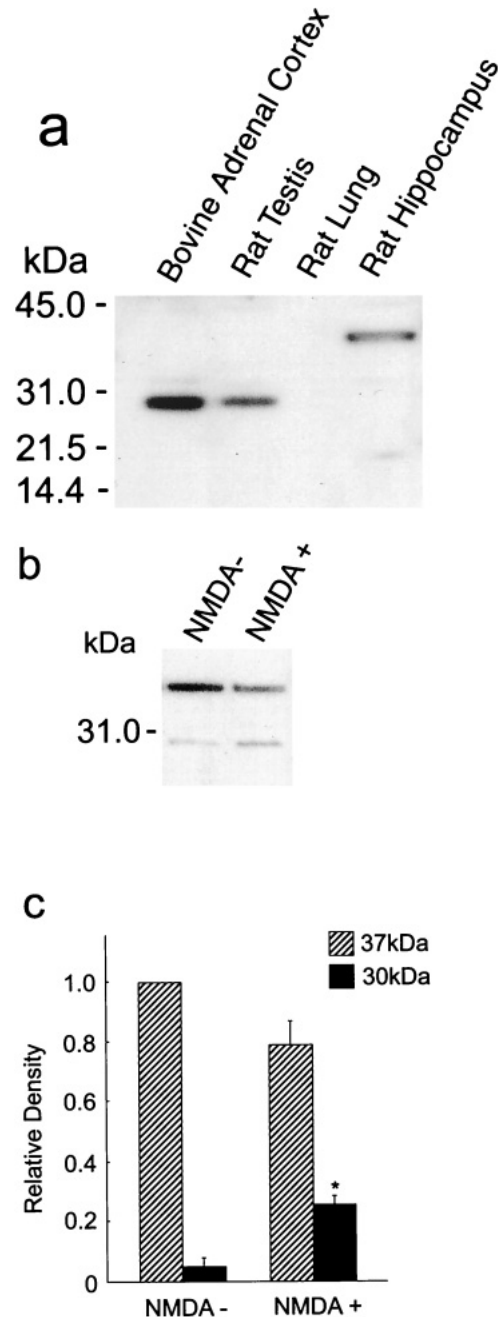

FIG. 6. Western immunoblot analysis of StAR in mitochondria. A, From *left* to *right*, mitochondria from bovine adrenal cortex (1 μg protein), rat testis (1 μg protein), rat lung (50 μg protein), and rat hippocampus (50 μg protein). Rat lung mitochondria were used as a negative control. Mitochondria from bovine adrenal cortex and rat testis were used as a positive control. B, Effect of NMDA stimulation on StAR in hippocampal mitochondria. *Left lane*, Mitochondria incubated without NMDA for 30 min; *right lane*, mitochondria stimulated with 100 μM NMDA for 30 min. *Upper bands* and *lower bands* correspond to 37 and 30 kDa, respectively. Each lane contained 50 μg protein. C, Quantitative comparison for immunoblots of StAR in mitochondria derived from the hippocampus. Data are the mean ± SEM from three independent experiments and are expressed as a relative density compared with that of 37-kDa StAR bands in the mitochondria without stimulation by NMDA. *, $P < 0.05$ compared with the density of 30-kDa StAR in the mitochondria from the hippocampus incubated without NMDA.
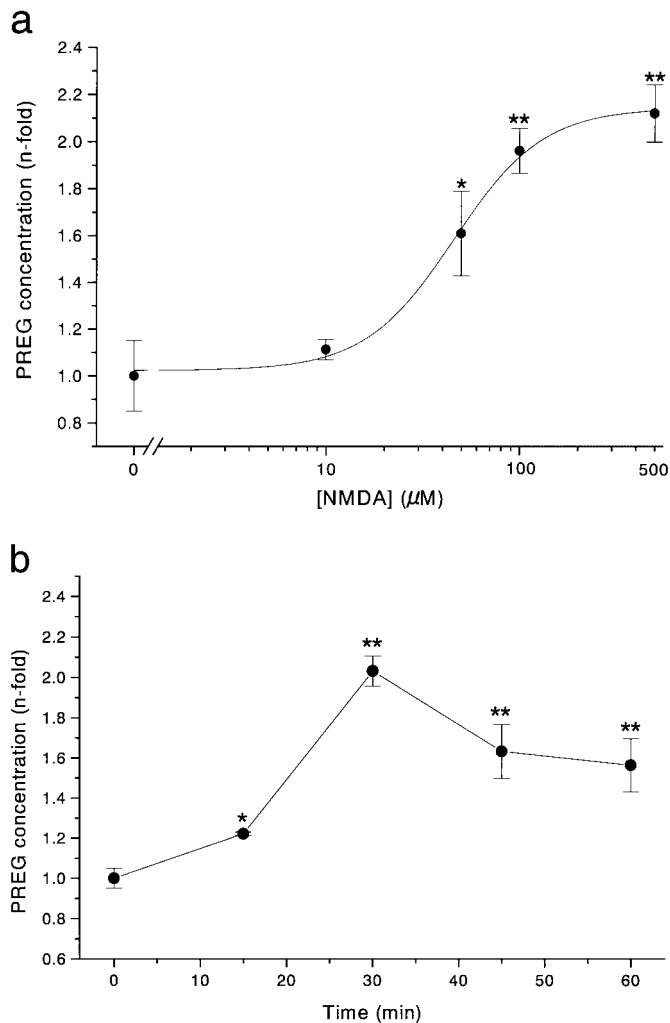
a

b

FIG. 7. A, Dependence of the PREG production on NMDA concentrations. Hippocampal cubes were incubated with various concentrations of NMDA for 30 min. The vertical scale is the relative PREG concentration, normalized by the PREG concentration in the hippocampus, incubated for 30 min without NMDA (0.177 pmol PREG/mg protein). *, $P < 0.05$; **, $P < 0.01$ [compared with the PREG concentration, with the 30-min incubation, without NMDA stimulation]. B, Time course of PREG production after 100 μM NMDA stimulation in the hippocampal cubes. The vertical scale is the relative PREG concentration normalized by the basal PREG concentration at time zero (0.165 pmol PREG/mg protein). *, $P < 0.05$; **, $P < 0.01$ (compared with the PREG concentration at time zero). PREG was measured with specific RIA as described in *Materials and Methods*. Data are the mean ± SEM of four to seven independent measurements, each analyzed in duplicate.

yielded half-maximum net production of PREG (EC_{50}) was estimated to be 45 μM by calculation using a logistic equation. We also investigated the time course of PREG production in the hippocampal cubes that were incubated in the presence of 100 μM NMDA. The PREG concentration reached a maximal value at 30 min after the NMDA application and decreased gradually thereafter (Fig. 7). This time course of NMDA-induced PREG production resembled that which has been observed in the retina (3). Upon stimulation with 100 μM NMDA for 30 min, the hippocampal concentration of neurosteroids increased to 0.347 pmol for PREG and 0.596

pmol for PREGS/mg protein, respectively, which is roughly twice the basal concentration. Stimulation of PREG production with NMDA was completely suppressed by the application of 50 μM MK-801 or 110 μM AP5, both of which are specific blockers of NMDA receptors (Fig. 8). NMDA-stimulated PREG production was also inhibited by the depletion of extracellular Ca^{2+}, suggesting that NMDA-induced PREG production was mediated by the influx of Ca^{2+} through NMDA receptors. AMG (a specific inhibitor of cytochrome P450scc; 1 mM) completely blocked the PREG production induced by 100 μM NMDA stimulation, indicating that NMDA-induced PREG production in the hippocampus is solely due to the P450scc enzyme. NMDA-induced PREGS production was also inhibited by 50 μM MK-801 (see Fig. 8).

NMDA-induced Ca^{2+} signals in the hippocampal slices

To investigate the NMDA-induced Ca^{2+} influx that was occurring in the hippocampal cubic slices used for measurements of neurosteroid production, we examined the characteristics of NMDA-induced Ca^{2+} signals in the fura-2-loaded hippocampal slices. Transient Ca^{2+} elevation was observed upon stimulation with 100 μM NMDA solution. The time course of the Ca^{2+} transients consisted of a rapidly rising phase of 20–30 sec, followed by a slow decay phase, which reached the basal level after 10–15 min. The application of 50 μM MK-801 abolished the NMDA-induced Ca^{2+} signals. The peak amplitude of Ca^{2+} elevation, indicated as the increase in F340/F380 from the resting level [Δ(F340/F380)], was 0.098 ± 0.002 (mean ± SD; n = 3). These results suggest that a significant NMDA-induced Ca^{2+} influx may occur in the first 10–15 min of the 30-min incubation employed for the steroid production analysis.

Electrophysiological investigations of neuronal excitability

We investigated the vulnerability of neurons in the hippocampal slices under the incubation conditions used for NMDA-induced neurosteroid production by observing the EPSP of the CA1 pyramidal neurons in response to a test stimulus (50 μsec) at 0.05 Hz applied to the Schaffer collaterals. Before the application of NMDA, the mean magnitude of the EPSP peak was 0.494 ± 0.060 mV, and the mean left slope was 0.512 ± 0.103 mV/msec (n = 3). After a 30-min exposure to 100 μM NMDA, both the magnitude of the EPSP peak and the mean left slope increased significantly (by 184 ± 31% and 297 ± 47%, respectively; n = 3; $P < 0.05$). The mean right slope was not significantly affected, within experimental error, by the 30-min exposure to 100 μM NMDA. These results suggest that the 30-min exposure to NMDA did not considerably induce neuronal degeneration in the hippocampal slices. In a separate experiment, perfusion with 100 μM exogenous PREGS for 30 min did not attenuate, but, rather, significantly enhanced, both the mean amplitude of the EPSP peak and the mean left slope (by 158 ± 27% and 120 ± 12%, respectively; n = 3; $P < 0.05$). The mean right slope was again not significantly changed by the application of PREGS. Based on these results, a consideration of the excitotoxicity of PREGS and NMDA was excluded for the analysis of neurosteroid synthesis. A 100-μM PREGS solution was used, which should be a saturating concentration for the

a

Inc Time (min)	0	30	30	30	30	30	30
NMDA	−	−	+	+	+	+	+
Calcium	+	+	+	−	+	+	+
MK-801	−	−	−	−	+	−	−
AP5	−	−	−	−	−	+	−
AMG	−	−	−	−	−	−	+

b

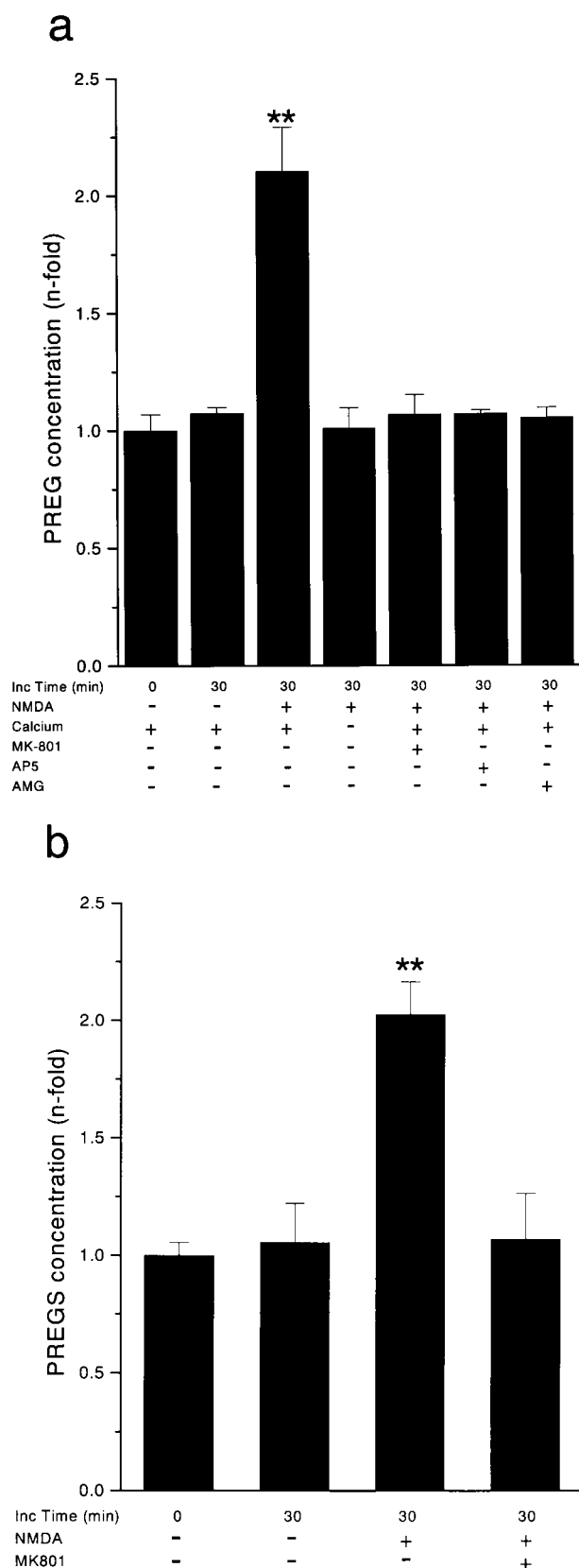| Inc Time (min)	0	30	30	30
NMDA	−	−	+	+
MK801	−	−	−	+

FIG. 8. Effect of inhibitors and Ca^{2+} on the synthesis of PREG and PREGS in the hippocampal cubes. A, PREG concentration. From *left* to *right*, basal PREG (without incubation), PREG after incubation in the absence of NMDA and inhibitors, PREG after incubation with 100 μM NMDA, PREG after incubation with 100 μM NMDA in the Ca^{2+}-depleted medium, PREG after incubation with 100 μM NMDA in the presence of 50 μM MK-801, PREG after incubation with 100 μM NMDA in the presence of 110 μM AP5, and PREG after incubation with 100 μM NMDA and 1 mM AMG. B, PREGS concentration. From *left* to *right*, basal PREGS (without incubation), PREGS after incubation in the absence of NMDA and inhibitors, PREGS after incubation with 100 μM NMDA, and PREGS after incubation with 100 μM NMDA in the presence of 50 μM MK-801. All incubations were performed for 30 min at 37 C. The vertical scale in each panel is the relative PREG or PREGS concentration normalized by the basal values (0.165 pmol/mg protein for PREG and 0.294 pmol/mg protein for PREGS). Each *column* is the mean ± SEM of four to seven independent determinations, each analyzed in duplicate. **, $P < 0.01$ compared with the PREG or PREGS concentration after 30-min incubation without NMDA stimulation. PREG and PREGS were measured as described in *Materials and Methods*.

modulation of NMDA receptors, as judged from its EC_{50} of approximately 25–30 μM on NMDA receptors (9, 25).

Discussion

In the present study we have demonstrated in the hippocampal neurons the neuronal localization of a complete neurosteroidogenic system, composed of P450scc, ADR, ADX, StAR, and hydroxysteroid sulfotransferase, with the immunohistochemical staining. We also observed significant neurosteroidogenic activity, which was dependent on the NMDA receptor-mediated Ca^{2+} signaling. We discuss here the possibility that this neuronal steroidogenic system functions as an NMDA- and Ca^{2+}-dependent machinery whose products serve to modulate neuron-neuron communication as paracrine modulators.

Distribution of steroidogenic proteins and mRNAs in the hippocampus

The present study is the first demonstration of a complete neuronal steroidogenic system for PREG(S) synthesis. To date, glial cells have been considered to play a major role in neurosteroid production, because anti-P450scc antibodies were absorbed by the white matter throughout the rat brain (26, 27) and because many reports indicated the presence of P450scc (albeit at low concentration) in astrocytes and oligodendrocytes (26, 28, 29). In this study the main immunohistochemical staining for P450scc and other neurosteroidogenic proteins was observed in neurons. Therefore, the neurons are likely to play a major role in neurosteroidogenesis in the hippocampus. The possibility of glial neurosteroidogenesis was not completely excluded, because we observed a weak staining of P450scc in some glia-like cells. Previous results for the immunostaining of P450scc in the white matter are likely to be an artifact due to the nonspecific adsorption of the bovine antibodies (15). We achieved good staining by choosing suitable rat antibodies that reduced nonspecific binding in the rat hippocampus, because the concentration of P450scc was extremely low (13, 30). Specificity of the antirat P450scc antibodies used in this study was satisfactory, as judged from the Western blot analysis.

The distribution in the hippocampus of mRNAs, encoding for several steroidogenic proteins, has recently been investigated by means of *in situ* hybridization. Both StAR mRNA

and 3βHSD mRNA (each of approximately 10^{-2}–10^{-3} of the amount in the adrenal gland) was shown to be localized along the pyramidal cell layer in the CA1–CA3 regions and the granule cell layer in the dentate gyrus (14, 15). Our results of the distribution of StAR protein were in good agreement with the reported mRNA distribution of StAR (15). Concerning the P450scc protein, its neuronal distribution in the hippocampus has previously not been well elucidated. Although the *in situ* hybridization analysis indicated the presence of P450scc mRNA along the pyramidal cell layer and the granule cell layer, the quality of the hybridization was much lower than that of StAR (15). To obtain detectable signals, exposure of the hybridized samples to x-ray films had to be performed for nearly 3 months. Several RT-PCR-based analyses indicated that mRNA encoding for P450scc was expressed in the brain homogenates at an extremely low level (of approximately 10^{-4}–10^{-5} of that in the adrenal gland) (13, 30). The topological localization of the sulfotransferase protein was qualitatively demonstrated in the dentate gyrus of the hippocampus (12). Our finding on the sulfotransferase protein has strongly supported their previous observation by clearly demonstrating neuronal localization in the pyramidal cell layer and the granule cell layer, and a single protein band in the Western blot analysis.

Our study demonstrated the neuronal localization of a significant amount of P450scc protein as well as other neurosteroidogenic proteins in the hippocampus. From our Western blot analysis, the concentration of P450scc protein in the hippocampus was estimated to be as high as approximately 5×10^{-2} of that present in the testis and the adrenal cortex. This appears to be in agreement with the results of Mellon and Deschepper (13), which reported that the P450scc protein in the brain was roughly 10^{-2} of that in the adrenal gland, although the amount of P450scc mRNA in the brain was only roughly 10^{-4}–10^{-5} of that in the adrenal gland. This discrepancy between the mRNA and protein concentrations may be due to a slower turnover rate for the P450scc protein in the brain than in the peripheral steroidogenic tissues (13).

Neuronal expression of the P450scc protein and the synthesis of PREG(S) has been reported in several other regions of the rat nervous system, such as retinal ganglion neurons and cerebellar Purkinje neurons (3, 4, 31). In the cerebellar Purkinje neurons and granule cells, we also observed the immunoreactivity of P450scc, StAR, and the sulfotransferase (data not shown).

Does Ca^{2+} influx promote neurosteroid production?

We observed that NMDA stimulated the production of PREG(S) by approximately 2-fold in the hippocampus. Because the application of NMDA also induced the influx of Ca^{2+} through NMDA receptors in hippocampal neurons, it is likely that Ca^{2+} signaling drives the neuronal steroidogenic reactions. In fact, the promotive effect of NMDA on PREG production was considerably attenuated by preventing the Ca^{2+} influx by means of the NMDA receptor blockers, MK-801 and AP5, and also by the direct depletion of extracellular Ca^{2+}. We also obtained essentially the same Ca^{2+} dependency for PREGS synthesis. These observations suggest that NMDA-induced PREG and PREGS production in

the hippocampus was mediated by the influx of Ca^{2+} through NMDA receptors. The influx of Ca^{2+} has also been reported to play a significant role in the regulation of NMDA-induced steroidogenesis in retinal tissues (3).

Judging from the many investigations reported on peripheral steroidogenesis, one of the key Ca^{2+}-dependent processes may be cholesterol transfer, catalyzed by the StAR protein (32). We found the presence of full-length StAR (*i.e.* 37 kDa) in the mitochondria of the hippocampal neurons. Upon NMDA stimulation, we observed that the amount of full-length StAR decreased and the 30-kDa StAR increased in the mitochondria. This suggests that the processing of StAR may coincide with the cholesterol transfer from the outer to the inner membranes of the mitochondria. Inhibition of Ca^{2+} influx through NMDA receptors by MK-801 also suppressed the processing of StAR (33). These observations lead to the interpretation of a Ca^{2+}-driven neurosteroidogenesis. It is likely that the increase in the 30-kDa StAR in the NMDA-treated hippocampus is due to some proteolytic conversion from 37-kDa StAR to 30-kDa StAR, because these phenomena appeared within 30 min of NMDA stimulation, which should be too fast for transcriptional regulation. The conversion from 37-kDa StAR to 30-kDa StAR could be catalyzed by a Ca^{2+}-dependent proteases, although experimental evidence has not been reported to date. Even in the peripheral steroidogenic tissues, the protease responsible for the processing of StAR has not been determined, and the Ca^{2+}-dependent and StAR-mediating cholesterol transport mechanisms have not yet been fully elucidated.

Ca^{2+} signaling may also be the second messenger of steroidogenesis in peripheral glands. The crucial role of Ca^{2+} signaling in the regulation of angiotensin II-induced steroidogenesis has been demonstrated in adrenal glomerulosa cells (34, 35). In addition, in adrenal fasciculata cells a physiological concentration of ACTH (1 pM) was observed to promote PREG production by inducing Ca^{2+} signals without a cAMP increase (36, 37). It should be noted that many reports have supported the conclusion that cAMP is another second messenger in ACTH-induced steroidogenesis in adrenal fasciculata cells (37, 38). The involvement of cAMP in the neuronal steroidogenesis awaits further investigation.

Concentrations of PREG and PREGS

Are the concentrations of PREGS and PREG sufficiently high so that they may act as local mediators? To examine the significance of the concentration of PREGS, we attempted to convert the dimensions from picomoles per mg protein to molar concentrations (moles per liter). We determined that 10 mg wet weight of the hippocampal tissue contained 0.96 ± 0.02 mg protein. We assumed that tissue having 1 mg wet weight has an approximate volume of 1 μl, as 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). The basal concentration of PREGS in the hippocampus is then estimated to be approximately 28 nM, and the bulk concentration of PREGS, after NMDA stimulation, is estimated to be approximately 57 nM. Because P450scc and hydroxysteroid sulfotransferase were highly localized in

hippocampal neurons, the local concentration of PREGS around the neurons may be 10- to 20-fold greater than the bulk concentration (57 nM) observed after NMDA stimulation. This is due to the relatively small volume of the neurons that contained P450scc systems in the whole hippocampus, as judged from the immunostaining pattern. These considerations suggest that the local concentration of PREGS may be sufficiently high to act as a local mediator that modulates NMDA receptors (7, 8). The present basal neurosteroid concentration was close to the reported values for rodents (4, 22, 24). On the other hand, the concentration of PREGS in plasma was 4 nM. These data indicate that concentrations of PREG and PREGS in the hippocampus were 7- to 8-fold higher than those in plasma. A significant net synthesis of PREG(S) induced by the NMDA stimulation would explain the much higher concentration of hippocampal PREG(S). However, we cannot exclude the possibility that this high concentration might in part be caused by the accumulation and good retention of PREG(S) in the brain due to high levels of brain lipids, as suggested by previous studies (24, 39). It should be noted that to show the net synthesis of PREG and PREGS induced by NMDA stimulation, trilostane (3βHSD inhibitor) and SU-10603 (P450c17 inhibitor) were employed. Without these inhibitors, the concentrations of PREG and PREGS are probably lower than the observed values due to a possible further conversion to progesterone and/or DHEA.

Possible physiological role of neurosteroids

The present results may shed light on the novel physiological role of neurosteroids as local mediators, in terms of their site of synthesis and where and how they act. PREGS may facilitate excitation of the postsynaptic neurons. PREGS enhances glutamate actions via NMDA receptors by increasing the opening probability in hippocampal neurons (7, 8, 26). In this study the concentrations of PREG and PREGS in the hippocampus were enhanced by approximately 2-fold upon stimulation with NMDA, suggesting the possibility of positive feedback between NMDA receptor activation and PREGS production. Our experimental knowledge, taken together, leads to the hypothesis that PREGS may cause postsynaptic signal amplification in the following manner: the NMDA-gating Ca^{2+} influx increases cholesterol transfer to the mitochondrial inner membrane by activating StAR; this is followed by the conversion of cholesterol to PREG and PREGS with P450scc systems, which increases the production of PREG and PREGS; this, in turn, potentiates the NMDA receptor-mediated Ca^{2+} influx.

It should be noted that a high amount of PREGS, produced by long exposure of neurons to NMDA or glutamate, may induce the excitotoxicity of neurons and result in hippocampal neuronal cell death, as has been demonstrated in the retina (3, 40). In fact, the exposure of hippocampal cubes to NMDA for longer than 30 min resulted in a decreased PREG concentration, which may have been due to the degeneration of cells. PREGS has also been shown to be excitotoxic in hippocampal cell cultures (33, 41). These results can be interpreted as the result of an enhanced Ca^{2+} influx induced by the PREGS through NMDA receptors (7–9). Excess Ca^{2+} influx was observed to cause neuronal cell death, which was

also induced by an excessive exposure to glutamate, in terms of either concentration or duration (42). PREGS may function as a physiological potentiator for neuronal communication. When its concentration is too high, however, PREGS may function as an excitotoxin, leading to the cell death. This is also the case for glutamate, a reasonable concentration of which is required for normal neuronal communication. Stimulation with 100 μM NMDA for 30 min in low Mg^{2+} medium did not considerably attenuate the hippocampal neuronal activity, as judged from electrophysiological measurements, which supports the conclusion that many neurons were still intact under our experimental conditions.

The present experimental evidence supports the hypothesis of local neuronal synthesis of neurosteroids in the hippocampus. Although further investigations are needed, we should now consider the possibility that the neurosteroid PREGS is a local mediator that contributes to glutamate-dependent neuronal excitability in the hippocampus.

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Table 1. Basal concentrations of PREG and PREGS in the hippocampus, cerebellum and plasma.

	PREG	PREGS
(pmol/mg protein)		
hippocampus	0.165 ± 0.013	0.294 ± 0.016
cerebellum	0.172 ± 0.034	0.208 ± 0.021
(pmol/mg tissue weight or μL)		
hippocampus	0.0158 ± 0.0012[**]	0.0282 ± 0.0015[**]
cerebellum	0.0165 ± 0.0033[**]	0.0199 ± 0.0020[**]
plasma	0.0020 ± 0.0001	0.0040 ± 0.0003

Basal concentrations of PREG and PREGS were measured with specific RIA as described in *Materials and Methods*. PREG and PREGS concentrations are represented as pmol/mg protein and pmol/mg tissue weight (for hippocampus and cerebellum) or pmol/μL (for plasma). Data represent the mean±SEM of four to seven independent measurements, each analyzed in duplicate. **P<0.01 compared with the PREG or PREGS concentration in the plasma.