[23] Histological and Metabolism Analysis of P450 Expression in the Brain

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Introduction

Neurosteroids, synthesized *de novo* in the brain, are promising neuromodulators that influence learning and memory.[1,2] Neurosteroidogenesis has, however, not been well elucidated due to the extremely low levels of steroidogenic cytochrome P450s in the brain.[3] This article demonstrates the sensitive methods for the immunostaining of steroidogenic P450s, as well as the measurement of their steroidogenic activity, particularly in the hippocampus.[4] The hippocampus, which is involved essentially in the learning and memory processes, is a target for the neuromodulatory actions of neurosteroids, which are synthesized locally in hippocampal neurons, in addition to those of steroid hormones produced in the adrenal glands and gonads.[1,4,5] Neurosteroids include pregnenolone (PREG), dehydroepiandrosterone (DHEA), testosterone, and 17β-estradiol and their sulfated esters (PREGS, DHEAS, etc.). Neurosteroids may modulate neurotransmission acutely in an either excitatory or inhibitory manner.[6] The acute actions of neurosteroids are thought to be mediated through ion-gated channel receptors, such as *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, rather than through the nuclear steroid receptors that promote the classic genomic actions of steroid hormones.[1,7–10]

Procedures

Preparation of Rat Hippocampal Slices

Male Wistar rats (1- to 12-week-old) are deeply anesthetized with pentobarbital and perfused transcardially with phosphate-buffered saline (PBS) [0.1 *M*

[1] S. Kawato, M. Yamada, and T. Kimoto, *Adv. Biophys.* **37,** 1 (2001).
[2] E.-E. Baulieu, *Recent Prog. Horm. Res.* **52,** 1 (1997).
[3] A. G. Mensah-Nyagan, J. L. Do-Rego, D. Beaujean, V. Luu-The, G. Pelletier, and H. Vaudry, *Pharmacol. Rev.* **51,** 63 (1999).
[4] T. Kimoto, T. Tsurugizawa, Y. Ohta, J. Makino, H. Tamura, Y. Hojo, N. Takata, and S. Kawato, *Endocrinology* **142,** 3578 (2001).
[5] M. Schumacher, R. Guennoun, P. Robel, and E.-E. Baulieu, *Stress* **2,** 65 (1997).
[6] F. S. Wu, T. T. Gibbs, and D. H. Farb, *Mol. Pharmacol.* **40,** 333 (1991).
[7] M. Joels, *Front. Neuroendocrinol.* **18,** 2 (1997).
[8] F. L. Moore and S. J. Evans, *Brain Behav. Evol.* **54,** 41 (1999).
[9] E. Falkenstein, H. C. Tillmann, M. Christ, M. Feuring, and M. Wehling, *Pharmacol. Rev.* **52,** 513 (2000).
[10] H. Mukai, S. Uchino, and S. Kawato, *Neurosci. Lett.* **282,** 93 (2000).

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phosphate buffer and 0.14 *M* NaCl (pH 7.3)], followed by a fixative solution (4% paraformaldehyde in PBS) at 4°. After dissection from the skulls, hippocampi are postfixed for 24–48 hr in a fixative solution at 4° and cryoprotected in PBS with 30% sucrose. Hippocampi are frozen-sliced coronally at a thickness of 20 μm, with a cryostat at −17°.

Immunohistochemical Staining

Staining of cytochrome P450s (P450scc, P45017α, and P450arom), hydroxysteroid sulfotransferase, and StAR is performed using the avidin–biotin–peroxidase complex (ABC) technique according to the free-floating method. Endogenous peroxidase activity is blocked with 0.3% H_2O_2 (v/v) in methanol. The slices are preincubated in PBS containing 5% (v/v) normal goat serum, 3% (w/v) fat-free skim milk, and 0.5% (v/v) Triton X-100 for 2 hr at room temperature with gentle shaking in order to eliminate nonspecific binding sites for primary antibodies. Then the slices are treated with primary antibodies for 18–24 hr (P45017α and StAR) or 36–48 hr (P450scc, P450arom, and sulfotransferase) at 4° in the presence of 3% skim milk and 0.5% Triton X. Dilution of antibodies is 1 : 200 for anti-rat P450scc IgG (peptide antibody against amino acids 421–441, Chemicon, Temecula, CA),[11] 1 : 1000 for anti-guinea pig P45017α IgG, 1 : 1000 for anti-human P450arom IgG, 1 : 1000 for anti-rat sulfotransferase IgG, and 1 : 500 for anti-mouse StAR IgG (peptide antibody against amino acids 88–98), respectively. Primary antibodies are pretreated with 0.5% liver acetone powder and 3% skim milk for 18 hr at 4°. The slices are then washed three times with a PBS solution containing 0.05% Tween 20. Biotinylated anti-rabbit IgG (Vector Lab, Burlingame, CA), diluted in a PBS solution containing 0.5% skim milk, is then applied for 30 min, followed by incubation for 30 min with the avidin–horseradish peroxidase complex (Vector Lab). Immunoreactivity is 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, immunoreactive cells are examined.

Fluorescence double immunostaining of steroidogenic proteins is carried out in the same manner as ABC staining except that the streptavidin–Oregon Green 488 complex (Molecular Probe, Eugene, OR) is substituted for the avidin–horseradish peroxidase complex. A low dilution of primary antibodies of 1 : 200 is used for P45017α IgG and P450arom IgG because the signal intensity of fluorescence immunostaining is weaker than that of diaminobenzidine-nickel staining. The fluorescence of Oregon Green 488 is measured using a MRC-1024 confocal microscope equipped with an Ar–Kr laser (Bio-Rad, Hercules, CA). The distributions of neurons, astroglial cells, and oligodendroglial cells are visualized by immunostaining with monoclonal antibodies to NeuN (1 : 100), GFAP (1 : 3,000),

[11] K. F. Roby, D. Larsen, S. Deb, and M. J. Soares, *Mol. Cell. Endocrinol.* **79,** 13 (1991).

and MBP (1 : 10), respectively (all of these antibodies are from Chemicon). The detection of neuronal/glial marker proteins is achieved using Cy3-labeled anti-mouse IgG (Jackson Immunoresearch Lab, West Grove, PA) without avidin–biotin amplification.

Preparation of Mitochondria, Microsomes, and Cytosolic Fractions

The hippocampus, testis, ovary, and lung from 1- to 12-week-old male Wistar rats are excised, minced, and homogenized in a glass–Teflon homogenizer at 4° in homogenization buffer [50 m*M* potassium phosphate buffer (pH 7.4), 250 m*M* sucrose, 5 m*M* EDTA, 0.5 m*M* phenylmethylsulfonyl fluoride, 0.1 m*M* leupeptin, and 3 m*M* 2-mercaptoethanol]. After the removal of nuclei by centrifugation at 3000*g* for 10 min, mitochondrial fractions are pelleted by centrifugation at 10,000*g* for 10 min at 4°. Purification is repeated to obtain the final mitochondrial fractions. After the removal of nucleic fractions and mitochondria by centrifugation at 9500*g* for 20 min, microsomal fractions are pelleted by centrifugation at 105,000*g* for 60 min. Purification is repeated to obtain the final microsomal fractions. The supernatant is used as the cytosolic fractions.

Western Immunoblot Analysis

Mitochondria, microsomes, and cytosolic fractions are diluted to 10 mg protein/ml with a Tris buffer composed of 62.5 m*M* Tris–HCl (pH 6.8), 6% sodium dodecylsulfate, 5% sucrose, 5% 2-mercaptoethanol, and 0.01% bromphenol blue. The samples are denaturated for 5 min at 90° and subjected to electrophoresis. Ten percent polyacrylamide gels are employed for P450scc, P45017α, and P450arom. For StAR and the sulfotransferase, 12.5 and 12% polyacrylamide gels are used, respectively. After electrophoresis, proteins are transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA) with the TE70 semidry blotting apparatus (Amersham Pharmacia Biotech, Piscataway, NJ) for 90 min at 2.2 mA/cm^2. Blotted membranes are washed three times with PBS containing 0.05% Tween 20 and are blocked for 15 min in PBS containing 0.05% Tween 20 and 10% fat-free skim milk. Blots are then probed with antibodies against P450scc (1 : 5000), P45017α (1 : 5000), P450arom (1 : 3000), sulfotransferase (1 : 5000), and StAR (1 : 1000) for 12–18 hr at 4° in PBS containing 0.05% Tween 20 and 2% skim milk. After the primary antibody treatment, blots are washed and treated with biotinylated goat anti-rabbit IgG (1 : 3000) for 1 hr. Finally, the membranes are treated with streptavidin–horseradish peroxidase complex (1 : 3000) for 1 hr. Protein bands are detected with ECL (enhanced chemiluminescence) plus Western blotting detection reagents (Amersham Pharmacia Biotech).

Radioimmunoassay of Neurosteroid Synthesis

Male Wistar rats (aged 1 to 12 weeks) are decapitated and trunk blood is collected in heparinized tubes. Blood is centrifuged at 1800*g* for 20 min at 4° to obtain plasma. Just after blood collection, hippocampi are excised, sliced (1 × 1 × 1-mm pieces) using a razor, and transferred into a low Mg^{2+} physiological saline [low Mg^{2+} PSS, composed of 137 m*M* NaCl, 2.5 m*M* $CaCl_2$, 1 m*M* $NaHCO_3$, 0.34 m*M* Na_2HPO_4, 0.44 m*M* KH_2PO_4, 5.7 m*M* KCl, 0.1 m*M* $MgSO_4$, 22 m*M* glucose, and 5 m*M* HEPES (pH 7.2)] into which O_2 gas is bubbled at 4°. To measure the NMDA-inducible synthesis, the hippocampal cubic slices are then incubated at 37° for 30 min in low Mg^{2+} PSS in the presence and absence of NMDA (stimulator) and inhibitors such as MK-801. During a 30-min incubation period, the incubation medium is gassed constantly with 95% O_2 and 5% CO_2. After the steroidogenic reactions are terminated by the addition of 1 *N* NaOH, the slices are homogenized with a glass–Teflon homogenizer. For the measurement of PREG(S), 2 μM trilostane (Mochida, Japan) and 20 μM SU-10603 (Novartis Pharma, Switzerland) are used to inhibit 3β-hydroxysteroid dehydrogenase (3β-HSD) and P45017α, respectively. For the measurement of estradiol, 500 μM metyrapon is used to inhibit P45011β activity.

Extraction and purification of PREG(S) are performed by mixing the homogenates with methanol solution containing 1% acetic acid (1 : 10, v/v), followed by sonication for 10 min.[12] The extraction is performed over 1–2 days, after which the mixture is centrifuged at 10,000*g* for 30 min. The extraction is repeated, the collected supernatant is combined, and the solvents are evaporated under a N_2 stream. Dried residues are reconstituted in methanol–water (40 : 60, v/v) and subjected to solid-phase extraction on C_{18} Amprep minicolumns (500 mg, Amersham Pharmacia Biotech) in order to separate nonconjugated steroids and sulfated steroids. For PREG measurements, unconjugated steroid fractions are used. PREG is purified by System I Celite column chromatography (ICN Biomedicals, Costa Mesa, CA), according to the manufacturer's instructions. PREG is then reconstituted in a radioimmunoassay (RIA) buffer, which consists of 0.15 *M* NaCl, 0.1% gelatin, 0.02% NaN_3, and 0.1 *M* sodium phosphate (pH 7.0). The mass of the PREG is measured by RIA, using a PREG RIA kit of ICN Biomedicals. The average PREG recovery should be 60–70%. For PREGS, the sulfated steroid fraction is used. The solvent is evaporated, and the steroids are reconstituted in ethyl acetate. Sulfuric acid is added (final concentration 2 m*M*), and PREGS is converted to PREG by overnight solvolyzation. After washing once with 1 *N* NaOH and twice with water, the PREG is purified using Celite columns. Finally, the mass of the PREG is measured by RIA. The recovery of PREGS should be about 50%.

[12] P. Liere, Y. Akwa, S. Weill-Engerer, B. Eychenne, A. Pianos, P. Robel, J. Sjovall, M. Schumacher, and E.-E. Baulieu, *J. Chromatogr. B* **739,** 301 (2000).

Extraction of 17β-estradiol is performed by mixing the homogenates with ethyl acetate : hexane (3 : 2) and shaking for 10 min. Extraction is repeated, and the collected organic supernatant is evaporated under a N_2 stream. The dried residue is reconstituted in a HPLC solvent (hexane : 2-propanol : acetic acid = 98 : 2 : 1) and applied to HPLC (JASCO, Japan) with a silica gel column (0.46 × 15 cm, Cosmosil 5SL, Nacalai Tesque, Japan) for purification after filtration with a membrane filter (0.45 μm pore, Ultrafree-MC, Millipore). The amount of estradiol is then measured by RIA using the estradiol RIA kit of ICN. The average estradiol recovery should be about 50%.

HPLC Analysis of Metabolism from PREG to DHEA and Estradiol

The activity of P45017α in the hippocampus is observed by measuring the conversion of [7-^{3}H]pregnenolone (specific activity, 22.5 Ci/mmol, New England Nuclear, Boston, MA) to [^{3}H]DHEA. Hippocampal cubic slices from two rats (the same as those used for RIA) are incubated at 20° for 0, 1, 3, or 5 hr in 2 ml of low Mg^{2+} PSS containing 10^7 cpm of [^{3}H]pregnenolone and 10 μM of trilostane. The incubation medium should be gassed constantly with 95% O_2 and 5% CO_2. After the reaction is terminated by the addition of 2 ml of chloroform, the slices are homogenized. After the water phase is removed, an equal volume of methanol is added in order to extract steroids. The homogenates are stirred for 15 min and centrifuged at 3000g for 5 min at 4°. The organic phase is then collected, and the extraction step is repeated twice. The combined organic extracts are dried, dissolved in 200 μl of an elution solvent, and filtrated through a membrane filter. Steroid metabolites are separated by a HPLC system with a silica gel column using an elution solvent consisting of hexane : 2-propanol : acetic acid (97 : 3 : 1, v/v). The steroids are eluted at a flow rate of 1.0 ml/min for 30 min, and the eluate is fractionated every 0.5 min. Fractions with a retention time of 9–10 min are assigned as DHEA fractions because the retention time of control [^{3}H]DHEA and [^{14}C]DHEA is 9.5 min. The radioactivity of the fractions is measured with a liquid scintillation spectrometer (LSC-701, Aloka, Japan).

The activity of P450arom is evaluated by measuring the conversion of [7-^{3}H]DHEA (specific activity, 60.0 Ci/mmol, New England Nuclear) to [^{3}H]estradiol. Slices from two rats are incubated at 20° for 5 hr in 2 ml of a low Mg^{2+} PSS containing 10^7 cpm of [7-^{3}H]DHEA. Extraction procedures of steroid metabolites are the same as those used for the RIA of estradiol. The dried residue is reconstituted in a HPLC solvent [hexane : 2-propanol : acetic acid = 98 : 2 : 1 (solvent A) or 99 : 1 : 1 (solvent B)] and subjected to HPLC after filtration with a membrane filter. The eluate is fractionated every 1 min from 0 to 60 min for solvent A and every 20 sec from 45 to 70 min for solvent B. The eluate with retention times of 21 and 22 min (for solvent A) or 49–53 min (for solvent B) is assigned to be 17β-estradiol, as judged from the retention time of control [^{14}C]estradiol

(21 min for solvent A and 51 min for solvent B). The radioactivity of the fractions is measured with a liquid scintillation spectrometer.

Results and Discussion

Distributions of Neurosteroidogenic Proteins in the Hippocampus

An intense immunoreaction with antibodies against P450scc, P45017α, P450arom, hydroxysteroid sulfotransferase, and StAR is restricted to pyramidal neurons in the CA1–CA3 regions and granule cells in the dentate gyrus, as indicated by immunostaining with NeuN antibodies (Fig. 1).[1,4] Fluorescence double immunostaining indicates that glial cells (astroglial cells and oligodendroglial cells) do not contain a significant amount of steroidogenic proteins. A good signal-to-noise ratio in immunohistochemical investigations is achieved by considering the following points in order to get rid of nonspecific staining: (a) Rat peptide antibodies, whose epitopes are exposed to water, may be most suitable for specific staining of the rat brain. We, however, do not always have such good rat antibodies.

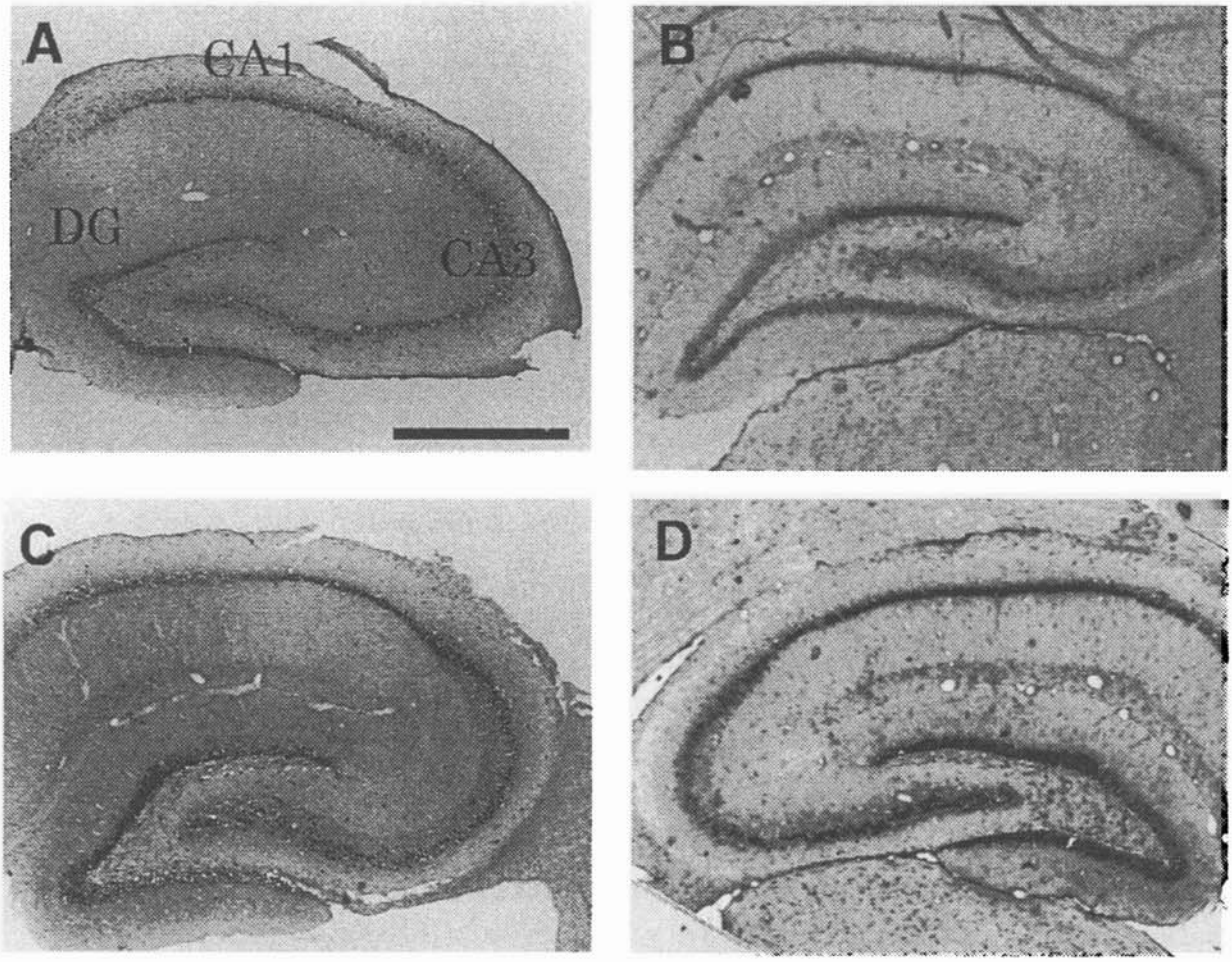

FIG. 1. Immunohistochemical staining of steroidogenic proteins in hippocampal slices from 12-week-old male rats. (A) P450scc. (B) P45017α. (C) P450arom, and (D) StAR. Staining is restricted to pyramidal neurons in the CA1–CA3 regions and granule cells in the dentate gyrus (DG). Immunoreactive cells are visualized by diaminobenzidine-nickel staining. Bar: 800 μm.

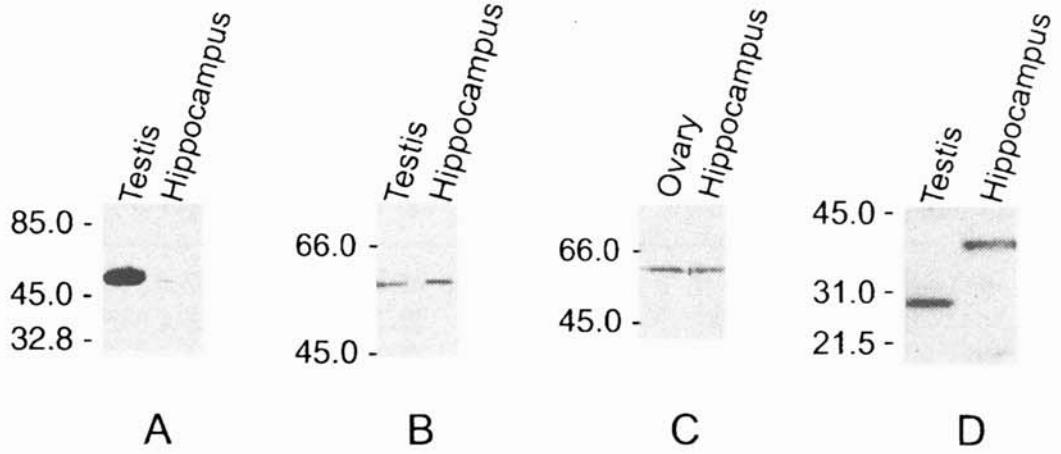

FIG. 2. Western immunoblot analysis of steroidogenic proteins in hippocampal tissues from 12-week-old male rats. (A) P450scc in mitochondria, (B) P45017α in microsomes, (C) P450arom in microsomes, and (D) StAR in mitochondria. For each panel, the left lane indicates a positive control protein band in rat testis (1 μg protein for A and D, 0.5 μg protein for B) or ovary (0.5 μg protein) and the right lane indicates a protein band in the hippocampus (50 μg protein). Numbers along the vertical axis indicate molecular weights. Only the full-length 37-kDa StAR is observed in the hippocampus, while only the truncated 30-kDa StAR is observed in testis. None of these protein bands is observed in the lung used as the negative control.

Trial-and-error processes are then necessary to find second-suitable antibodies. Anti-guinea pig P450 IgG, anti-human P450 IgG, and anti-mouse StAR IgG demonstrated good staining. Antibodies against beef P450scc and beef StAR showed some nonspecific staining in addition to specific staining. (b) When paraffin-embedded sections are used, there may be a considerable loss of antigen reactivity and/or mRNA hybridization reactivity due to the heating used to remove paraffin from slices. Fresh frozen slices should be used.

Western Immunoblot Analysis

Single bands corresponding to steroidogenic proteins are observed when mitochondria (P450scc and StAR), microsomes (P45017α and P450arom), or cytosolic fractions (the sulfotransferase) are subjected to Western blotting (see Fig. 2).[1,4] The electrophoretic mobility of the steroidogenic protein bands for the hippocampus is almost identical to that of purified steroidogenic proteins from testis and ovary. The concentration of these steroidogenic proteins in the hippocampus was around 10^{-2} of those in peripheral organs, whereas extremely low-level expression was reported for mRNAs. For example, mRNAs of P450scc and P45017α in whole brain homogenates were only 10^{-4}–10^{-5} of that in the adrenal gland.[13–15]

[13] S. H. Mellon and C. F. Deschepper, *Brain Res.* **629,** 283 (1993).
[14] J. L. Sanne and K. E. Krueger, *Gene* **165,** 327 (1995).
[15] A. Furukawa, A. Miyatake, T. Ohnishi, and Y. Ichikawa, *J. Neurochem.* **71,** 2231 (1998).

TABLE I
CONCENTRATIONS OF NEUROSTEROIDS BEFORE AND AFTER NMDA STIMULATION[a]

Source	Incubation time (min)	NMDA	MK801	PREG (fmol/mg wet weight)[b]	PREGS (fmol/mg wet weight)	17β-Estradiol (fmol/mg wet weight or μl)
Hippocampus	0	−	−	15.8 ± 1.2	28.2 ± 1.5	0.67 ± 0.05
	30	−	−	16.9 ± 0.5	29.7 ± 4.5	0.68 ± 0.09
	30	+	−	33.3 ± 3.4	57.2 ± 3.8	1.35 ± 0.18
	30	+	+	16.0 ± 1.4	30.3 ± 3.5	
Plasma	0	−	−	2.0 ± 0.1	4.0 ± 0.3	0.098 ± 0.039

[a] In the hippocampus and plasma from 12-week-old rats.
[b] Ten milligrams wet weight of the hippocampal tissue contained 0.96 mg of protein.

Neurosteroidogenic Activity Measured with RIA

The basal concentrations of PREG, PREGS, and estradiol are six- to eightfold higher in the hippocampus than those in the plasma (Table I). On stimulation with 100 μ*M* NMDA for 30 min, the levels of PREG(S) and estradiol *increase* roughly twice. Stimulation of PREG(S) and estradiol production with NMDA is completely suppressed by the application of blockers of NMDA receptors or by the depletion of extracellular Ca^{2+}, indicating that NMDA-induced steroid production is mediated by the influx of Ca^{2+} through NMDA receptors. Aminoglutethimide (inhibitor of P450scc; 1 m*M*) is observed to completely block the PREG production induced by NMDA stimulation.

Neurosteroidogenic Activity Measured with HPLC

The catalytic activity of P45017α of the hippocampus from a 12-week-old rat is demonstrated as the conversion of [^{3}H]PREG to [^{3}H]DHEA using a HPLC system. The eluted radioactive peak of [^{3}H]DHEA increases in a time-dependent manner from 1 to 5 hr. The presence of bifonazole or SU10603 (Novartis Pharmaceutical), specific inhibitors of P45017α, abolishes the appearance of the [^{3}H]DHEA peak, even after 5 hr of incubation. The conversion of [^{3}H]DHEA to [^{3}H]estradiol by 3β-HSD and P450arom is demonstrated as the appearance of significant amounts of [^{3}H]estradiol and [^{3}H]testosterone in the hippocampus from a 12-week-old male rat.

In conclusion, hippocampal neurons are equipped with the machinery that synthesizes PREG(S), DHEA, and estradiol. Neurosteroidogenesis may be acutely performed on stimulation of neurons with glutamate via a NMDA receptor-mediated Ca^{2+} influx. Because neurosteroids such as PREGS and estradiol modulate acutely the Ca^{2+} conductivity of NMDA receptors, neurosteroids should be paracrine modulators of neuronal activity. The methods of investigation used for

the hippocampus are also applicable to other brain regions, such as the cerebellum, cortex, hypothalamus, and olfactory bulb.[3,16,17]

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[16] K. Tsutsui, K. Ukena, M. Usui, H. Sakamoto, and M. Takase, *Neurosci. Res.* **36,** 261 (2000).
[17] P. Guarneri, R. Guarneri, C. Cascio, P. Pavasant, F. Piccoli, and V. Papadopoulos, *J. Neurochem.* **63,** 86 (1994).

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