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Review

Hippocampal cytochrome P450s synthesize brain neurosteroids which are paracrine neuromodulators of synaptic signal transduction

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Abstract

Hippocampal pyramidal neurons and granule neurons of adult male rats are equipped with a complete machinery for the synthesis of pregnenolone, dehydroepiandrosterone, 17β-estradiol and testosterone as well as their sulfate esters. These brain neurosteroids are synthesized by cytochrome P450s (P450scc, P45017α and P450arom) from endogenous cholesterol. Synthesis is acutely dependent on the Ca^{2+} influx attendant upon neuron–neuron communication via *N*-methyl-D-aspartate (NMDA) receptors. Pregnenolone sulfate, estradiol and corticosterone rapidly modulate neuronal signal transduction and the induction of long-term potentiation via NMDA receptors and putative membrane steroid receptors. Brain neurosteroids are therefore promising neuromodulators that may either activate or inactivate neuron–neuron communication, thereby mediating learning and memory in the hippocampus.

Keywords: neurosteroid; P450; hippocampus; brain; LTP; signal transduction

1. Introduction

The aim of this article is to describe brain P450 research that has been focused on the mammalian hippocampus, an attractive new field in neuroscience. Although the purification of steroids from brain tissues, which are very fatty, has been extremely difficult, a number of previous studies have successfully demonstrated the presence and accumulation of several neurosteroids, including pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulfate esters (PREGS and DHEAS) in the mammalian brain [1,2]. In each case, the reported concentration of the brain steroid was an order of magnitude greater than that typical of plasma. Adrenolectomy did not decrease the level of PREG(S) and DHEA(S) in the brain, suggesting the de novo synthesis of these steroids within the brain [1,3]. Active neurosteroidogenesis has, however, not been well elucidated, due to the extremely low levels of steroidogenic proteins in the brain [4]. Sex steroids (e.g., 17β-estradiol and testosterone) have not been considered to be brain neurosteroids, because of the reported absence of cytochrome P45017α in adult mammalian brain [5,6]. In particular, because sex steroids cannot be synthesized without P45017α, which converts PREG to DHEA (a precursor steroid), they are thought to reach the brain via blood circulation [7]. To date, because a good correlation between

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CORT, corticosterone; cytochrome P450scc (CYP11A1), cytochrome P450 having cholesterol side-chain cleavage activity; cytochrome P45017α (CYP17A), cytochrome P450 catalyzing the conversion of pregnenolone to dehydroepiandrosterone; cytochrome P450arom (CYP19), cytochrome P450 catalyzing aromatization of androstenedione and testosterone; DHEA, dehydroepiandrosterone; GABA, γ-aminobutyric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; NMDA, *N*-methyl-D-aspartate; PREG, pregnenolone; PREGS, pregnenolone sulfate; RIA, radioimmunoassay; StAR, steroidogenic acute regulatory protein

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progesterone synthesis and its nerve regeneration had mainly been shown for peripheral Schwann cells [8,9], the term 'neurosteroids' has been used to refer to neuroactive steroids produced not only in the brain, but also in the peripheral nerves and glial cells.

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. In addition to hormones derived from the endocrine glands, hippocampal neurons are exposed to locally synthesized brain neurosteroids. In contrast to the classical genomic effects of peripheral steroids, many neurosteroids induce non-genomic effects by means of putative cell surface receptors [10–12]. There is increasing evidence that neurosteroids modulate neurotransmissions rapidly (< 30 min) and with either excitatory or inhibitory effects, in the hippocampus, the center for learning and memory [2,13]. PREGS potentiates the Ca^{2+} conductivity of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors [14,15] but suppresses the Cl^- conductivity of the γ-aminobutyric acid (GABA) receptors in cultured rat hippocampal neurons [16,17]. Taken in combination, these actions could facilitate the excitation of neurons at the postsynaptic level [16]. DHEA potentiates the GABA-induced Cl^- current but DHEA sulfate suppresses it [16–18]. Several studies have reported the observation of specific, non-genomic effects induced by estradiol on neuronal excitability in the hippocampus, which indicates the non-reproductive actions of sex steroids [13,19–21].

Neurosteroids are indicated to be effective in enhancing animal learning and memory. The administration of PREGS and DHEA enhanced the retention of foot-shock avoidance in mice when injected directly into the hippocampus [22,23]. An injection of PREGS into the hippocampus has also been reported to temporally improve the spatial memory performance of aged rats [24–26].

Until recently, the cellular location and activity of the neurosteroidogenic machinery in the brain had not been sufficiently elucidated. This is due primarily to the very low level of expression of the mRNAs of steroidogenic enzymes in the cerebrum and cerebellum [4]. For example, the concentration of P450scc mRNA expressed in the brain is reported to be only 10^{-4}–10^{-5} of that in the adrenal gland [6,27]. As a result, the presence of P450scc mRNA could be demonstrated only by the reverse transcription–

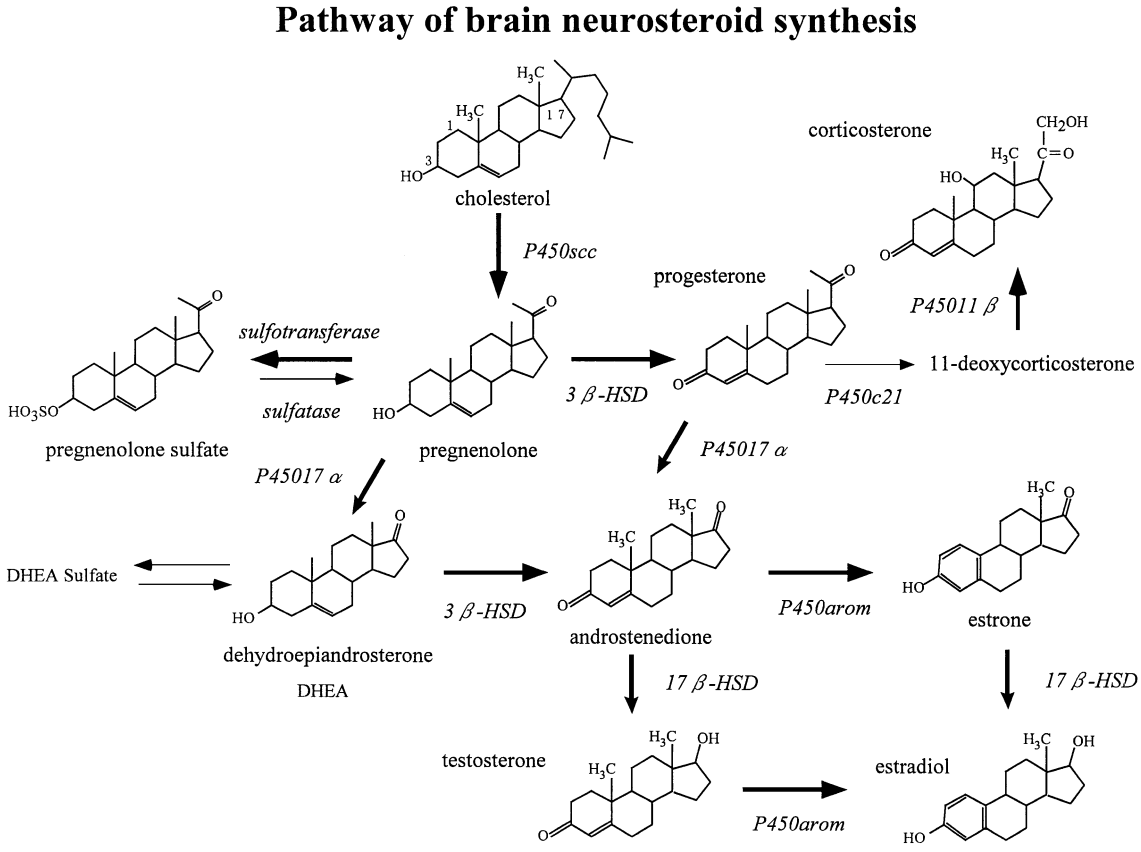

Fig. 1. Flow chart of brain neurosteroid synthesis in hippocampal neurons. The structures of brain neurosteroids and enzymes responsible for biotransformation are indicated. Thick arrows indicate pathways demonstrated in the hippocampus. Thin arrows indicate possible pathways expected to be present in the hippocampus.

polymerase chain reaction (RT-PCR) method. The role of neurons in steroid synthesis had not yet been clearly determined, although glial cells had been subjected to extensive investigation [28,29]. The localization, in neurons of several steroidogenic proteins, has been demonstrated by means of in situ hybridization. For example, mRNAs for both steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNA (10^{-2}–10^{-3} of the levels in the adrenal gland) were observed to be localized along the pyramidal cell layer in the CA1–CA3 regions and the granule cell layer in the dentate gyrus [30]. Expression of mRNAs for steroidogenic factor-1 in colocalization with StAR and P450arom has been demonstrated in rat and marmoset hippocampus [31]. There had been still poor demonstration of the neuron-specific localization of steroidogenic P450s in the hippocampus, although the neuronal localization of P450scc and 3β-HSD had been demonstrated in Purkinje neurons in the rat cerebellum [32].

In this work, the localization of the neurosteroidogenic machinery in hippocampal neurons is described, along with the associated synthesis of a variety of brain neurosteroids (including sex steroids). The rapid, non-genomic effects of neurosteroids on synaptic transmission are also described. Let the term, 'brain neurosteroid' refer to a steroid that is synthesized de novo in the brain by P450 systems. This includes all of the steroids illustrated in Fig. 1.

2. Localization of neurosteroidogenic systems in the adult rat hippocampus

2.1. Immunohistochemical and Western immunoblot analysis

Adult male Wistar rats aged 3 months were used, and the hippocampi were frozen-sliced coronally at 20 μm thickness with a cryostat. A significant localization of cytochromes P450scc, P45017α and P450arom was observed in pyramidal neurons in the CA1–CA3 regions, as well as in granule cells in the dentate gyrus, by means of the immunohistochemical staining of hippocampal slices [28,29,33,34]. The colocalization of P450s with hydroxysteroid sulfotransferase and StAR has also been demonstrated in pyramidal neurons and granule cells [29].

To determine the localization of P450scc, an immunohistochemical staining was performed with anti-rat P450scc antibodies against amino acid sequence 421–441 (Fig. 2) [29]. The resulting intense immunoreaction with P450scc IgG was restricted to pyramidal neurons in the CA1–CA3 regions, and to granule cells in the dentate gyrus. The colocalization of immunoreactivity against P450scc and NeuN confirmed the presence of P450scc in these neurons. Immunohistochemical staining was also performed to determine the presence of other cytochrome P450s, such as P45017α and P450arom, using anti-guinea pig P45017α IgG (gift from Dr. Shiro Kominami) and anti-human P450arom IgG (gift from Dr. N. Harada). The observation of an intense immunoreaction with all of these antibodies was limited to pyramidal neurons in the CA1–CA3 regions and granule cells in the dentate gyrus (Fig. 3) [28]. The 3β-HSD activity was also observed to be localized in these neurons, the activity of which was stained by means of formazan accumulation. Both hydroxysteroid sulfotransferase and StAR were also stained with antibodies against rat hydroxysteroid sulfotransferase (gift from Dr. Hiro-omi Tamura) and mouse StAR (gift from Dr. Douglas Stocco) [29]. These results, taken together, imply that pyramidal neurons and granule cells are equipped with complete steroidogenic systems which catalyze the conversion of cholesterol to PREG, DHEA, testosterone and estradiol, and their subsequent sulfation to PREGS and DHEAS.

The expression of these steroidogenic proteins was confirmed by Western immunoblot analysis. As illustrated in Fig. 4, a single protein band was observed for each of these P450s [34]. The resulting molecular weights obtained for P450scc, P45017α and P450arom were almost identical to those obtained from peripheral steroidogenic organs. The relative levels of these P450s in the hippocampus were approximately 1/500 (P450scc) and 1/200 (P45017α and P450arom) of that in the testis (P450scc and P45017α) and the ovary (P450arom), respectively.

In marked contrast to the peripheral steroidogenic organs, only the full-length 37-kDa species of StAR was observed in the mitochondria from the control hippocampus (Fig. 5) [29]. The level of StAR in the hippocampus was approximately 1/100 of that in rat testis. When the hippocampus was stimulated with 100 μM NMDA for 30 min, conversion of StAR from the 37-kDa species to the truncated 30-kDa species was observed. The processing of StAR proteins, which is probably performed by mitochondrial proteases, was dependent on an NMDA-mediated-Ca^{2+} influx. This processing of StAR may be correlated with the Ca^{2+}-dependent movement of StAR-bearing cholesterol from the outer to the inner membranes of mitochondria, which supplies cholesterol to P450scc. This possibility was further investigated using genetically engineered Chinese hamster ovary (CHO) cells. Upon heat-shock treatment at 43 °C for 2 h, this stable transfectant CHO line expressed NMDA receptors which were mouse GluRε1(NR2A) with GluRξ1(NR1) subunits [35]. When these CHO cells were transfected with StAR plasmid for 48 h, a large proportion of full-length, 37 kDa StAR (approximately 45%) was observed by a Western blot analysis of the mitochondrial fractions. Upon application of 100 μM NMDA for 10 min, a sustained Ca^{2+} elevation induced the conversion of approximately 95% of the full-length StAR to the truncated 30-kDa species (K. Shibuya, H. Ishii, H. Mukai and S. Kawato, unpublished results).

For decades, neurosteroidogenesis had been extensively studied in glial cells. This line of investigations was

motivated by the absorption of anti-bovine P450scc antibodies by white matter throughout the rat brain [36,37], and by the many reports which have indicated the presence of steroidogenic proteins and mRNAs in astrocytes, oligodendrocytes and white matter [36,38,39]. We therefore examined the possible existence of P450s in glial cells. The distributions of astroglial cells and oligodendroglial cells, however, displayed very different patterns from those characteristic of the P450-containing cells (see Fig. 2) [29]. The distributions of astroglial and oligodendroglial

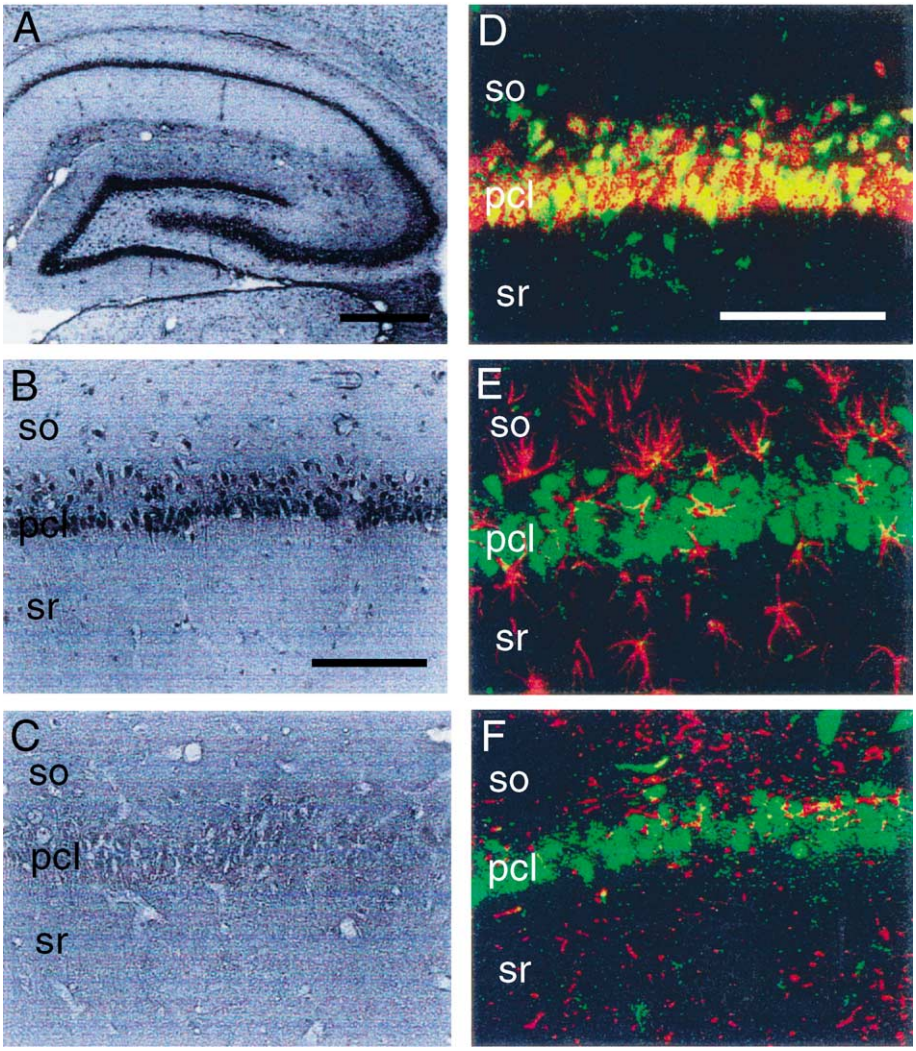

Fig. 2. Immunohistochemical staining of cytochrome P450scc, astroglial cells and oligodendroglial cells in hippocampal slices of an adult male rat. Adult male Wistar rats aged 3 months (from SLC Japan Co.) were deeply anesthetized with pentobarbital and perfused transcardially with phosphate-buffered saline, followed by a fixative solution (4% paraformaldehyde) at 4 °C. The hippocampi were postfixed for 24–48 h in the fixative solution at 4 °C and were frozen-sliced coronally at 20 μm thickness with a cryostat (Leica CM1510, Germany) at −17 °C. All experiments using animals were conducted according to the institutional guidelines. (A) Low-magnification image of the whole hippocampus, stained with antibodies against rat cytochrome P450scc. The somata layer of pyramidal neurons is characterized as a mirror image of C-shaped curve throughout the CA1–CA3 regions of the hippocampus. Granule cells in the dentate gyrus (DG) showed a characteristic arrowhead distribution. (B) The hippocampal CA1 region stained with antibodies against rat P450scc. (C) Preadsorption of the antibody with excess purified bovine P450scc antigen is observed to result in the disappearance of P450scc immunoreactivity in all of the positively stained cells in CA1 region, due to cross-reaction of the anti-rat P450scc antibodies. (D) Fluorescence dual staining of P450scc (green) and NeuN (red). (E) Fluorescence dual staining of P450scc (green) and glial fibrillary acidic protein (GFAP) (red). (F) Fluorescence dual staining of P450scc (green) and myelin basic protein (MBP) (red). A superimposed region of green and red fluorescence is represented in yellow color. (G) Astroglial cells stained with antibody GFAP. (H) Oligodendroglial cells stained with antibody against MBP. B and C, D–F, G and H are at the same magnification. Scale bar, 800 μm (A), 120 μm (B and C, G and H), and 100 μm (D–F). so, stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. A–C, G and H are obtained using the avidin–biotin–peroxidase complex (ABC) technique according to the free-floating method, and immunoreactive cells are visualized by diaminobenzidine–nickel staining. Fluorescence immunohistochemistry of P450scc in E and F is carried out in the same manner as ABC staining except that the avidin–horseradish peroxidase complex is replaced by streptavidin–Oregon Green 488 complex. Detection of neuronal/glial marker proteins was achieved with Cy3-labeled anti-mouse IgG without avidin–biotin amplification. Fluorescence signals are observed using a confocal microscope. (A–F are taken from Kimoto et al. [29].)

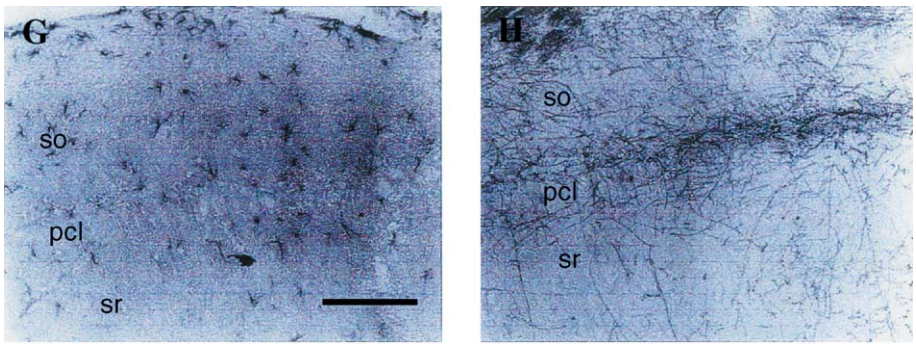

Fig. 2 (*continued*).

cells (each stained with their specific antibodies) are clearly different from those of P450-reactive cells. Glial cells were observed mainly in the stratum radiatum and the stratum oriens (see Fig. 2). This indicates that the majority of P450-containing cells are neither astroglial cells nor oligodendroglial cells.

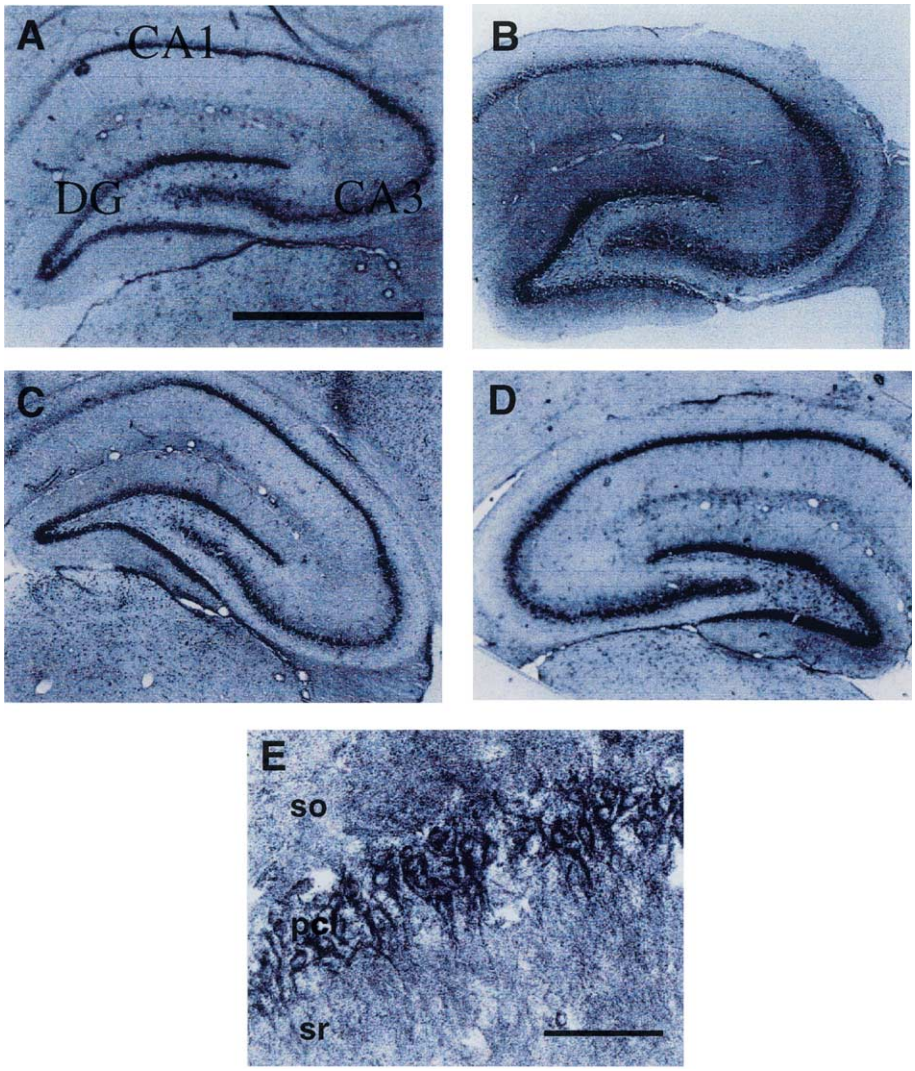

Fig. 3. Immunohistochemical staining of P45017α, P450arom, the sulfotransferase and StAR in the hippocampus of adult male rats. (A) P45017α in the whole transverse section of the hippocampus; (B) P450arom; (C) the sulfotransferase; (D) StAR protein; (E) activity of 3β-HSD in the CA1 region as revealed by nitro-BT staining. Observation of P45017α, P450arom, the sulfotransferase, StAR and 3β-HSD is restricted to pyramidal neurons in the CA1–CA3 regions and granule cells in the dentate gyrus (DG). so, stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. (A–D) A low magnification; (E) the high magnification. Scale bar, 800 (A–D) and 120 μm (E). Immunoreactive cells in A–D are visualized with diaminobenzidine–nickel staining. (Taken from Kimoto et al. [29].)

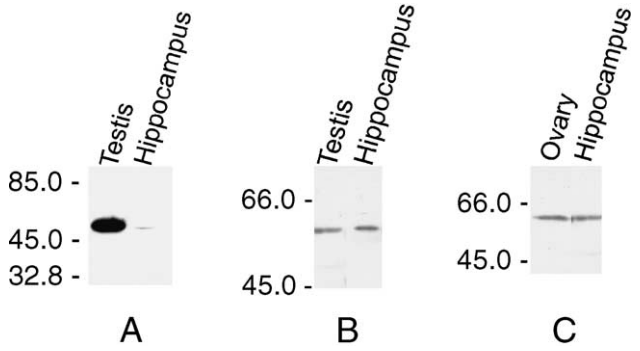

Fig. 4. Western immunoblot analysis of steroidogenic proteins in hippocampal tissues of adult male rats. (A) P450scc in mitochondria; (B) P45017α in microsomes; (C) P450arom in microsomes. For each panel, the left lane indicates a positive control protein band in rat testis (1 μg protein for A and 0.5 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 direction indicate molecular weights. None of these protein bands are observed in the lung used as a negative control. (Taken from Kawato et al. [28,34].)

2.2. Transcripts for P450s in the hippocampus

The PCR amplification of mRNA transcripts for cytochromes P450scc, P45017α and P450arom was performed using a total RNA Purification Kit (Nippongene, Japan). The hippocampal tissues from adult male rats aged 3 months were used. The relative number of P450 transcripts expressed in the hippocampus was demonstrated to be $1/10^4$–$1/10^5$ for P450scc, approximately 1/300 for both P45017α and P450arom (A. Furukawa and S. Kawato, unpublished results), when compared with those expressed in the adrenal gland (for P450scc), the testis (for P45017α) and the ovary (for P450arom). Not only the PCR amplification but also the ribonuclease (RNase) protection assay demonstrated the presence of StAR transcripts with an expression level of approximately 1/200 of the levels in the adrenal gland. The RNase protection assay for P450scc in the hippocampus, which was performed using a ^{32}P-labelled rat P450scc antisense riboprobe, however, yielded no detectable specific hybridization signals [30]. Collectively, the relative level of mRNA expressed in the hippocampus was lowest for P450scc and highest for StAR, with that of P45017α and P450arom expressed at intermediate levels.

The mRNA levels obtained for P45017α and P450arom in the hypothalamus were almost twice of those obtained in the hippocampus (A. Furukawa, unpublished results). In the

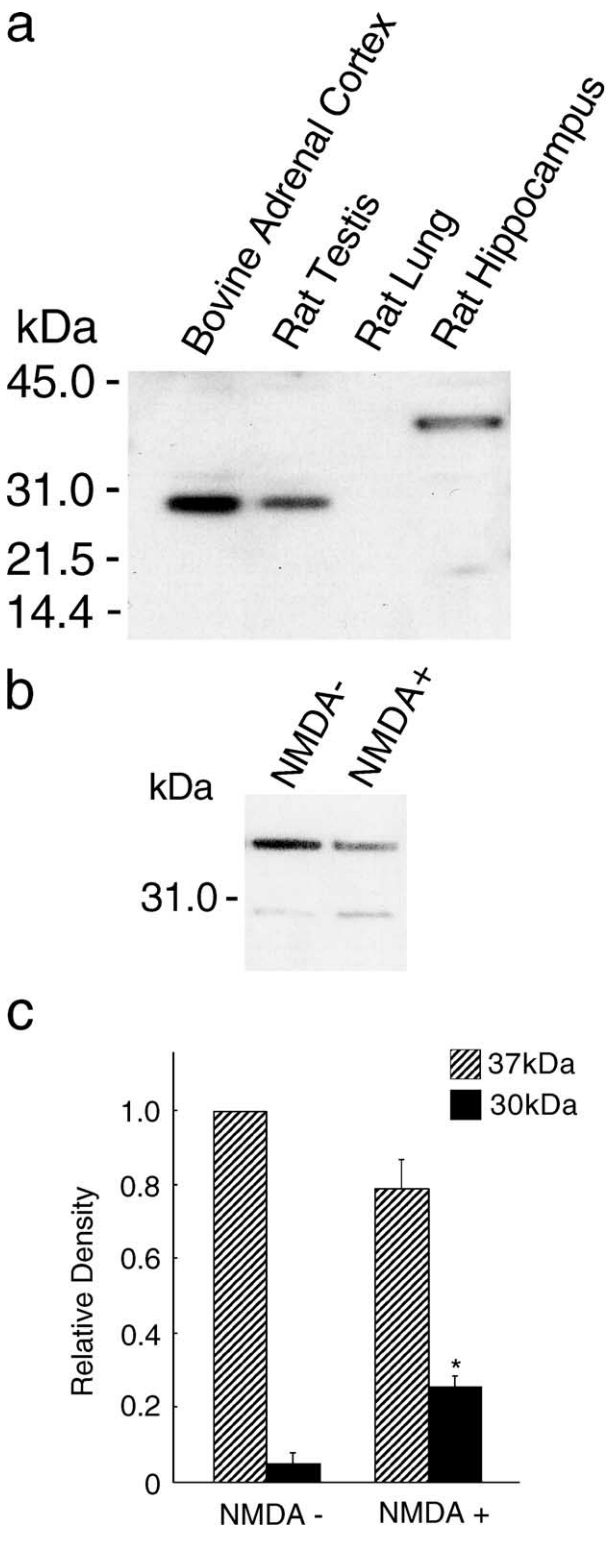

Fig. 5. Western immunoblot analysis of StAR in mitochondria from the hippocampal tissues of adult male rats. (a) From left to right, mitochondria from bovine adrenal cortex (1 μg protein), rat testis (1 μg protein), rat lung (50 μg protein), and rat hippocampus (50 μg protein). Rat lung mitochondria 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 and lower bands correspond to 37 000 and 30 000 in molecular weight, respectively. Each lane contained 50 μg protein. (c) Quantitative comparison for immunoblots of StAR in mitochondria from the hippocampus. Data represent the mean ± S.E. from three independent experiments and are expressed as a relative density compared with that of the 37-kDa StAR bands in the mitochondria obtained without stimulation by NMDA. *$P<0.05$ compared with the density of 30 kDa StAR from the hippocampus incubated without NMDA. (Taken from Kimoto et al. [29].)

cerebral cortex, however, the mRNA expression for P45017α was not detectable within the experimental error, which indicates that the cerebral cortex levels were lower than those in the peripheral steroidogenic organs by at least a factor of 10^{-4}.

3. Neurosteroid synthesis

3.1. Analysis with specific radioimmunoassay (RIA)

The activity of the steroidogenic system in the hippocampus from adult male rats was measured by means of RIA using a RIA kit from ICN (USA) for PREG and 17β-estradiol [29,33,34]. Note that PREGS was analyzed using antibodies against PREG after solvolysis to PREG [29]. The purification of neurosteroids from very fatty brain tissues required the application of a set of sophisticated methods, which included purification with organic solvent, column chromatography and high-performance liquid chromatography (HPLC), etc. [29,40,41]. The basal concentrations of PREG and PREGS which were measured in the hippocampus were 0.17 and 0.29 pmol/mg protein (i.e., 0.016 and 0.028 pmol/wet weight, 16 and 28 nM), respectively, which were roughly eight times greater than those typical of plasma [29]. To demonstrate the acute net production of neurosteroids during neuron–neuron communication, the NMDA-stimulated production of PREG and PREGS was investigated in hippocampal cubic slices [29] (see Fig. 6). Upon stimulation with 100 μM NMDA for 30 min at 37 °C, the hippocampal level of PREG and PREGS increased to 0.35 and 0.60 pmol/mg protein (33 and 57 nM), respectively, which represents an approximate doubling of the basal levels. Stimulation of PREG and PREGS production with NMDA was completely suppressed by either the application of MK-801 (a specific blocker of NMDA receptors), or by the depletion of extracellular Ca^{2+}. This suggests that the NMDA-induced production of PREG was mediated by the influx of Ca^{2+} through NMDA receptors. The application of aminoglutethimide (an inhibitor of P450scc) completely blocked the PREG production induced by NMDA stimulation. This indicates that the PREG production in the hippocampus was due solely to the P450scc enzyme activity. The

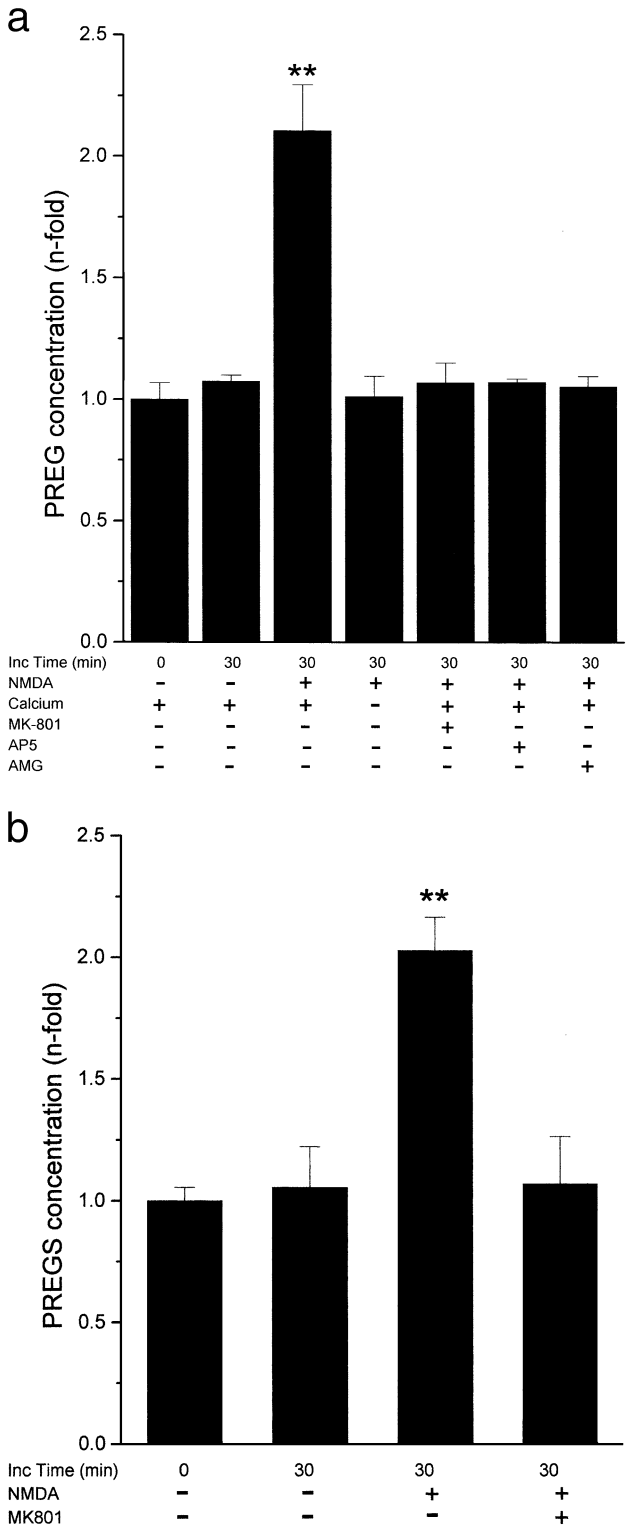

Fig. 6. RIA analysis of the synthesis of PREG and PREGS in hippocampal cubes. (a) PREG concentration. From left to right, basal PREG (without incubation); PREG after incubation in the absence of NMDA and inhibitors; PREG after incubation with 100 μM NMDA; PREG after incubation with 100 μM NMDA in Ca^{2+}-depleted medium; PREG after incubation with 100 μM NMDA in the presence of 50 μM MK-801; PREG after incubation with NMDA in the presence of AP5; and PREG after incubation with NMDA and 1 mM aminoglutethimide. (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; PREGS after incubation with NMDA in the presence of MK-801. All incubations were performed for 30 min at 37 °C. Vertical scale in each panel indicates 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 represents the mean ± S.E. of four to seven independent determinations, each analyzed in duplicate. $^{**}P < 0.01$ compared with the PREG or PREGS concentration in the case of the 30-min incubation without NMDA stimulation. (Taken from Kimoto et al. [29].)

concentration of 17β-estradiol was also investigated. The basal concentration of estradiol was approximately 0.006 pmol/mg protein (600 pM) which is roughly six times greater than that typical of plasma. When the hippocampal slices were stimulated with 100 μM NMDA for 30 min at 37 °C, the estradiol concentration increased to approximately 0.013 pmol/mg protein (1.3 nM).

3.2. Analysis with HPLC

The synthesis of DHEA and estradiol in the hippocampal cubic slices was also investigated by means of HPLC analysis [28,34]. The elution solvent employed consisted of hexane/isopropanol/acetic acid = 97:3:1 or 98:2:1. The significant conversion of [^{3}H]-PREG (10^6 cpm) to [^{3}H]-DHEA (approximately 7000 cpm) was observed after incubation with the slices for 30 min to 5 h at 20 °C [28]. When [^{3}H]-DHEA (10^6 cpm) was incubated with hippocampal slices for 5 h at 20 °C, the production of significant amounts of [^{3}H]-androstenedione, [^{3}H]-testosterone and [^{3}H]-estradiol (approximately 4000 cpm) was observed. The approximate relative ratio of production was androstenedione/testosterone/estradiol = 1:2:17, which demonstrates the effective synthesis of estradiol. It should be noted that the production of [^{3}H]-estradiol obtained from [^{3}H]-PREG as the initial substrate was much smaller than that obtained from [^{3}H]-DHEA. This is probably due to the additional multiple steroidogenic pathways from PREG, as compared to pathways from DHEA.

4. Rapid action of neurosteroids

4.1. NMDA receptor-mediated Ca^{2+} signals

The NMDA receptor-mediated elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) was investigated by means of digital fluorescence microscopy (ARGUS-50 system, Hamamatsu Photonics, Japan), using the Ca^{2+}-sensitive indicator, fura-2 or Calcium Green-1 [42,43].

4.1.1. Effect of PREGS

For isolated hippocampal neurons taken from 3-day-old rats and cultured for 8–10 days, the application of 100 μM NMDA induced a transient elevation in $[Ca^{2+}]_i$ which lasted for approximately 20–60 s in 86% of the neurons, in the absence of steroids, and in Mg^{2+}-free medium. Preincubation with 100 μM PREGS for 20 min at 37 °C increased both the peak amplitude of the Ca^{2+} transients by 1.4-fold, and the population of NMDA-responsive neurons from 86% to 92%. Application of PREGS caused no considerable change in the time course of NMDA-induced Ca^{2+} transients [42,43].

The PREGS-induced enhancement of the Ca^{2+} transients was also examined in genetically engineered CHO cells using imaging analysis. Upon heat-shock treatment at 43 °C for 2 h,

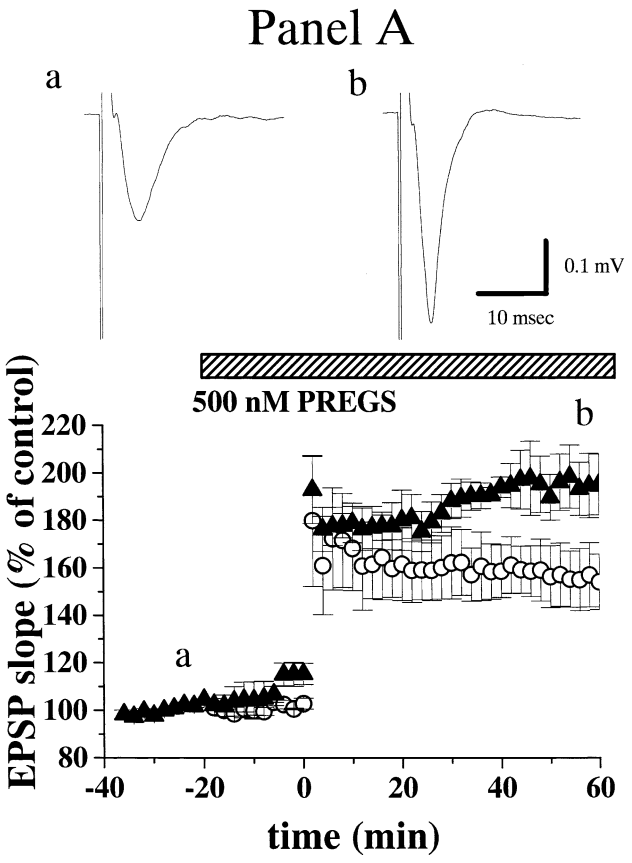

Fig. 7. Electrophysiological measurements of the LTP induction of the field EPSP in the CA1 pyramidal neurons in hippocampal slices from 4-week-old rats. (Panel A) Enhancement of LTP induction by perfusion of PREGS. Upper panel: (a and b) EPSP slopes in the presence of 500 nM PREGS, showing sample recordings taken before (a) and after (b) tetanus stimulation, at times corresponding to similarly lettered points on the graphs. Lower panel: Changes in slopes of the EPSP, plotted against the ordinate scale. Here, 100% refers to the response value before tetanic stimulation, irrespective of the test condition. Tetanic stimulation was delivered at time, $t = 0$. The applied concentrations of PREGS are 0 (drug free, open circle) and 500 nM (closed triangle). Points for each of the two conditions illustrated represent the means of 12 observations. Hatched bar above the graph indicates the period of time during which PREGS was administered. (Panel B) Suppression of LTP induction by perfusion of estradiol. Upper panel: (a and b) EPSP slopes in the presence (a and b) and absence (c and d) of 10 nM estradiol, taken before (a and c) and after (b and d) the tetanus stimulation. Lower panel: Changes in the slope of the EPSP upon the tetanus stimulation at time, $t = 0$. Estradiol concentration is 0 (open circle), 0.1 (closed circle), 1.0 (open triangle), 10 (closed triangle) and 50 nM (open square), respectively. Hatched bar above the graph indicates period of time during which estradiol was administered. (Panel C) Suppression of LTP induction by perfusion with CORT. Upper panel: (a and b) EPSP slopes in the presence (a and b) and absence (c and d) of 10 μM estradiol, taken before (a and c) and after (b and d) the tetanic stimulation. Lower panel: Changes in the slopes of the EPSP upon the tetanic stimulation. CORT concentration is 0 (open circle) and 10 μM (closed triangle). Hatched bar above the graph indicates the period of time during which CORT was administered. [Panel A, unpublished results of N. Takata and S. Kawato. Panels B and C, unpublished results of N. Yasumatsu and S. Kawato.]

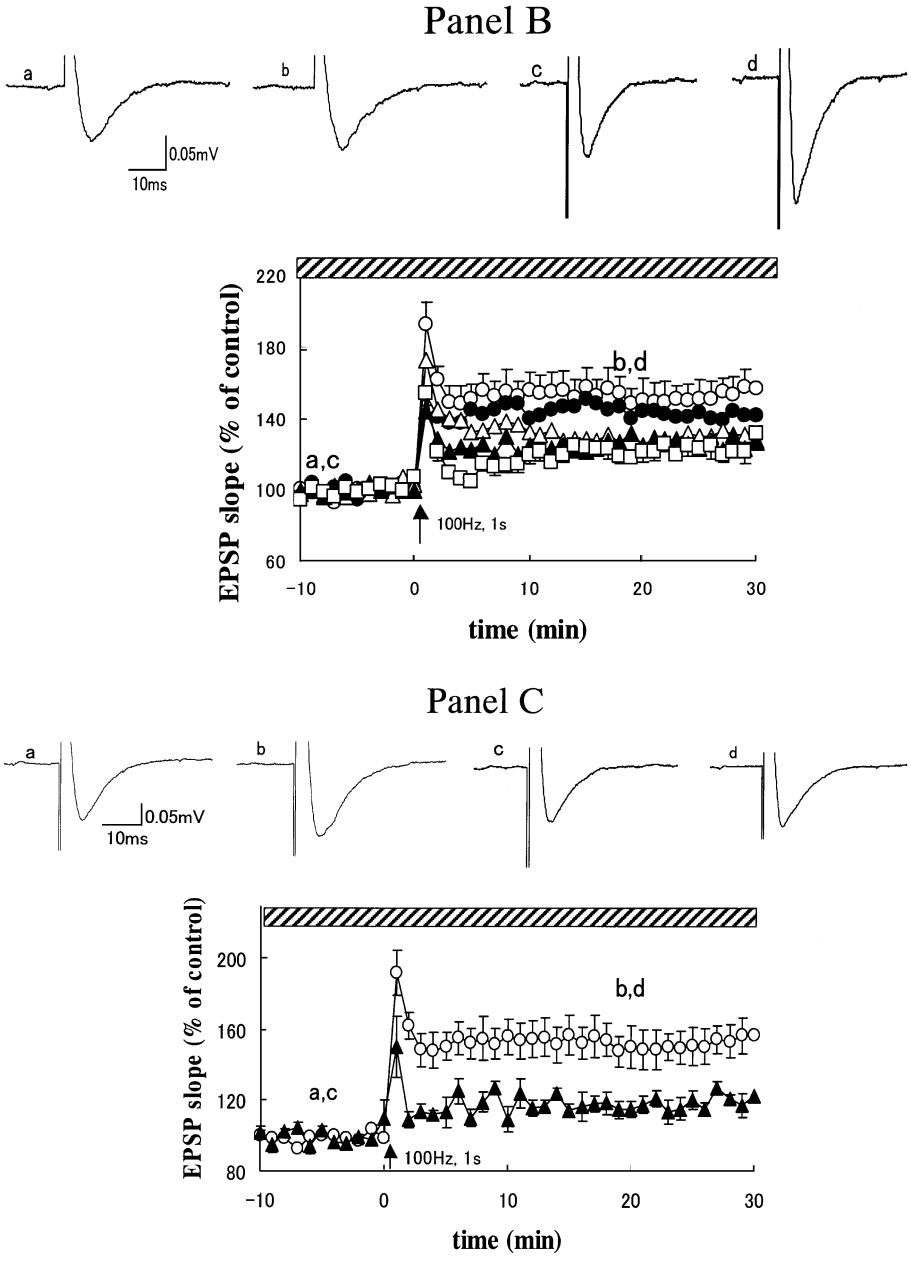

Fig. 7 (*continued*).

this stable transfectant CHO line expressed NMDA receptors which were either mouse GluRε1(NR2A) with GluRζ1(NR1) subunits or GluRε2(NR2B) with GluRζ1(NR1) subunits [35]. In contrast to hippocampal neurons, these CHO cells demonstrated a sustained Ca^{2+} elevation upon NMDA stimulation. The application of 50 μM PREGS for 20 min enhanced the NMDA-induced Ca^{2+} elevation by approximately 2-fold [44]. This PREGS-induced enhancement was canceled by the coapplication of PREGS with other sulfated steroids (e.g., DHEAS and estradiol sulfate), indicating that the sulfate residue is essential for their action on NMDA receptors. Taken in combination with intracellular electrophysiological measurements combined with NMDA stimulation [17,45,46], these results imply that PREGS increases the opening probability of NMDA receptors.

4.1.2. Effect of corticosterone (CORT)

CORT is a principal glucocorticoid that is synthesized in the rodent (e.g., rat and mouse) adrenal cortex and secreted in response to stress [47]. To date, little has been reported concerning the rapid effects (i.e., those which appear within 30 min of application) of CORT on neurotransmitter-mediated signal transduction in hippocampal neurons. We examined the acute effects of CORT using digital fluorescence microscopy, with the Ca^{2+}-sensitive indicator, fura-2 [42,43]. CORT induced a rapid effect on NMDA receptor-

mediated Ca^{2+} signaling at 37 °C in cultured hippocampal neurons isolated from 3-day-old rats. Following a 20-min preincubation of neurons with 0.5–50 μM CORT in the absence of extracellular Mg^{2+}, the application of 100 μM NMDA induced an extremely prolonged Ca^{2+} elevation which was maintained over the experimental time range of 5–20 min. This prolonged $[Ca^{2+}]_i$ elevation was terminated either by the blocking of NMDA receptors with MK801 or by washout of CORT. The rapid effect of CORT was also investigated in hippocampal slices from adult male rats aged 3 months. NMDA stimulation at 300 μM induced a transient elevation in $[Ca^{2+}]_i$ which consisted of a rapid rise (which occurred within 10 s) followed by a slow decay to a plateau (approximately 60–70% of the maximal $[Ca^{2+}]_i$ rise), which was maintained within the experimental time range of 5 min. A 10-μM CORT perfusion for 20 min suppressed this NMDA-induced transient $[Ca^{2+}]_i$ elevation by enhancing the slow decay phase to 40–50% of the maximal $[Ca^{2+}]_i$ rise, although the initial rapid rise phase was unaffected (M. Harada and S. Kawato, unpublished results). These results suggested that the effects induced by CORT on the $[Ca^{2+}]_i$ signaling in cultured neurons from pups differ from those in neurons of slices from adult rats.

4.2. Electrophysiological investigations of the long-term potentiation

In the electrophysiological field potential measurements of the hippocampal slices, the long-term potentiation (LTP) of CA1 pyramidal neurons is observed as an approximate 1.5- to 1.6-fold increase in the initial slope of the excitatory postsynaptic potential (EPSP), which is attendant upon the high-frequency tetanic stimulation of Schaffer collaterals with 100 Hz for 1 s. The following experiments were performed using the hippocampal slices from young male Wistar rats, aged 4 weeks in the presence of a high concentration of Mg^{2+} (1 mM) at 30 °C.

4.2.1. Effect of PREGS

A 20-min preperfusion of hippocampal slices with 500 nM PREGS potentiated the induction of LTP, as judged from a significant increase in the EPSP slope (approximately 1.95-fold) (Fig. 7). Interestingly, even without tetanic stimulation in the presence of 1 mM Mg^{2+}, a 100-μM PREGS perfusion induced an immediate increase in both the slope and the peak magnitude of EPSP [the EPSP slope attained a peak of approximately 1.4 times the basal level after 20 min (N. Takata and S. Kawato, unpublished results)]. The LTP induction by PREGS is probably due to PREGS's reported ability to potentiate NMDA receptor-mediated Ca^{2+} currents [17,45]. Because PREGS has been shown to suppress the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) type of glutamate receptors [16], the enhancement of LTP induction implies that the potentiation of NMDA receptors overcomes the suppression of AMPA receptors in synaptic signal transmission.

4.2.2. Effect of estradiol

The preperfusion of hippocampal slices with 0.0, 0.1, 1.0, 10 and 50 nM estradiol for 20 min reduced, in a dose-dependent fashion, the induction of LTP as indicated by an approximate 1.51-, 1.42-, 1.29-, 1.26- and 1.22-fold enhancement of the EPSP slope, respectively (see Fig. 7) (N. Yasumatsu and S. Kawato, unpublished results). This rapid suppressive effect by physiological concentrations of estradiol in hippocampal slices from 4-week-old rats is essentially the same as those described by Ito et al. [21].

4.2.3. Effect of CORT

A 20-min preperfusion of hippocampal slices with 10 μM CORT resulted in a significant suppression of the LTP induction, as observed by a reduction in the increase in the EPSP slope attendant upon a 100-Hz tetanic stimulation [i.e., from 1.51 ± 0.10 (control without CORT) to only 1.18 ± 0.04-fold (see Fig. 7)] (N. Yasumatsu and S. Kawato, unpublished results). These results imply that a high concentration of CORT (possibly secreted during stress) may acutely inhibit the synaptic signal transduction.

5. Possible pathway of steroidogenesis in the hippocampus

Taken in combination with previous reports, our results indicate that brain neurosteroid synthesis in the hippocampal neurons is likely to be catalyzed by the biotransformation of cholesterol to various steroids by the cytochrome P450-containing monooxygenase systems. This process is illustrated hypothetically in Fig. 1. The proposed process of neurosteroid synthesis is as follows. First, cholesterol is transported to the inner membrane of mitochondria along with StAR. In the mitochondria, cytochrome P450scc catalyzes the side-chain cleavage of cholesterol, resulting in the formation of PREG. PREG then reaches the microsomes (endoplasmic reticulum), where cytochrome P45017α catalyzes the conversion of PREG to DHEA. Following the transformation of DHEA to androstenedione by 3β-HSD, cytochrome P450arom catalyzes the conversion of androstenedione to testosterone. This is followed by a further transformation to 17β-estradiol by 17β-hydroxysteroid dehydrogenase (17β-HSD) (type 3). It appears likely that 17β-estradiol may also be formed by 17β-HSD (type 3) from estrone, which is converted from androstenedione by P450arom. Hydroxysteroid sulfotransferase converts PREG and DHEA to their sulfate forms, PREGS and DHEAS. Our study demonstrated the neuron-specific localization of P450scc, P45017α, P450arom and sulfotransferase in the hippocampus. The presence of mRNAs for 17β-HSD type 1 and type 3 has also been demonstrated in the human and rat hippocampus without a specification of cell type [48]. 17β-HSD type 1 and 17β-HSD type 3 catalyze the conversion of estrone to estradiol, and the conversion of androstenedione to testosterone [49,50]. It is possible that CORT is also a

member of brain neurosteroid synthesized in the hippocampus as illustrated in Fig. 1. In the microsomes, PREG is metabolized to progesterone by 3β-HSD [51]. Cytochrome P450c21 (CYP21) converts progesterone to deoxycorticosterone and deoxycortisol, which then reach the mitochondria, where P45011β (CYP11B) converts them to CORT and cortisol. Evidence supporting the synthesis of CORT from PREG is still fragmentary. Thus far, only the conversion of [^{3}H]-CORT from [^{3}H]-deoxycorticosterone has been reported [52]. Although the cell-specific localization of P450c21 and P45011β has not been demonstrated, the presence of the mRNAs for these P450s has been reported in the rat and human hippocampus [52–55].

It is important to consider whether the local concentration of brain neurosteroids is sufficiently high to allow action as local mediators. Dimensional conversion from picomoles per milligram protein to molars (moles per liter) was performed by considering that 10 mg wet weight of the hippocampal tissue contained 0.96 mg of protein. The concentration of PREGS detected in the hippocampus is then about 28 and 57 nM before and after the NMDA stimulation, respectively. The local concentration of PREGS in the pyramidal neurons is likely to be 10- to 20-fold higher than the bulk concentration of 57 nM, due to the relatively small volume of the P450-immunoreactive cells in the total hippocampus. These considerations suggest that the local concentration of PREGS could be as high as 0.6–1.2 μM. NMDA stimulation also increased the concentration of 17β-estradiol from 0.6 (basal) to 1.3 nM, which is estimated to correspond to a 13- to 26-nM local concentration within neurons. These levels are sufficient to allow PREGS and estradiol to act as local mediators that modulate LTP and NMDA receptors (this work, Refs. [20,21,56]). The concentration of PREGS and estradiol may change in a time-dependent fashion, due to further conversion to other steroids.

The physiological mode of action of brain neurosteroids could be as local mediators for brain neurons. However, the solid demonstration of local synthesis and action for 'neurosteroid' has been performed primarily in the peripheral glial cells and nerves. Sex steroids and DHEA had not been recognized as brain neurosteroids, because their endogenous synthesis had been poorly demonstrated in the brain especially in adult mammals. Only PREG(S), pregnenolone, allopregnenolone and allotetrahydrocorticosterone have been considered to be 'true' endogenously synthesized brain neurosteroids. Recently, progesterone has demonstrated to be another brain neurosteroid synthesized in the cerebellum [57]. Progesterone-induced dendrite growth of Purkinje neurons reported in the cerebellum is also indicative of the neurotrophic action of progesterone in the brain [58].

As reported in a number of studies over past decades, the absence of P45017α and its activity in the brain of adult rats has discouraged the investigation of the endogenous synthesis of sex steroids and DHEA [5–7]. Incubations of [^{3}H]-PREG(S) with brain slices, homogenates and microsomes, primary cultures of mixed glial cells, or astrocytes and neurons from rat and mouse embryos, had never produced a radioactive metabolite with the chromatographic behavior characteristic of [^{3}H]-DHEA [59]. In neonatal stage, however, the expression of mRNA for P45017α as well as an associated DHEA synthesis activity has recently been demonstrated in cultured cortical astrocytes and neurons [60,61]. Many attempts to demonstrate the immunohistochemical reactivity for P45017α in the adult rat brain had been unsuccessful [5]. The inability to detect mRNA for P45017α had been reported for both RNase protection assays and RT-PCR [6]. It was therefore generally concluded that the expression of mRNA for P45017α occurs only transiently, during rat embryonic and neonatal development [60–62], with the exception of one report that indicated its presence in the adult rat brain [53].

It has therefore been believed that DHEA and testosterone are supplied to the hypothalamus and the amygdala (where P450arom is expressed) via blood circulation, where they are converted to estradiol. The action of estradiol has been investigated mainly in female rats in relation to the estrous cycle as well as experimentally induced estrogen depletion and replacement (to modulate the estrogen level in blood circulation). Our elucidation of the NMDA-dependent machinery of estradiol synthesis (see description in Sections 3.1 and 3.2) [29,34], which begins with endogenous cholesterol and proceeds to estradiol and testosterone through DHEA, introduces essentially a new class of brain neurosteroids, with a new role in the process of signal transduction in the brain. This role is clearly different from their reproductive actions, as evinced by the observation of endogenous estradiol synthesis within the male brain.

Our ability to observe cytochrome P45017α in the adult rat hippocampus could be explained by the application of several experimental improvements. For example, to expose the antigens of P45017α, (1) we used fresh frozen slices instead of paraffin sections for immunostaining experiments, and (2) we used a slightly higher Triton X-100 concentration (0.5%). In the Western immunoblot experiments, we used very fresh microsome preparations, and included a careful treatment with protease inhibitors to suppress protease digestion of trace amounts of P45017α proteins, before gel electrophoresis. For the RT-PCR, probes were carefully designed using computer simulations, to ensure that the selected probe sequences were free from hairpin-loops.

So far, few studies have been reported which demonstrate the neuronal distributions of steroidogenic P450 'proteins' in the hippocampus. In the rat cerebellum, on the other hand, the neuronal localization of both P450scc and 3β-HSD has been demonstrated in Purkinje neurons and granule cells by immunohistochemistry or in situ hybridization [32,57]. PREGS has been observed to enhance the electrical activity of Purkinje neurons, an effect of which may be due to the suppression of GABA neurons. [32]. A significant amount of PREG (although a much lower amount of PREGS) has been observed in the rat cerebellum. A small level of neuronal expression of P450scc mRNA has

also been reported in other rat nervous systems, including neurons in the retinal ganglion, and sensory neurons in the dorsal root ganglia [6,27,53]. The NMDA-dependent synthesis of PREG(S) by P450scc has been reported in the rat retinal neurons [63,64]. The neural expression of P450s has been indicated in the frog brain [32,65].

6. Possible mechanisms of rapid modulation of signal transduction by brain neurosteroids

The combined results of recent experimental studies indicate that PREGS facilitates postsynaptic signal amplification, as illustrated in Fig. 8 [16,17,46]. In particular, an NMDA-gated Ca^{2+} influx triggers a cascade of steroidogenesis by StAR and P450scc [29]. This increases the production of PREG and PREGS, which in turn potentiates an NMDA receptor-mediated Ca^{2+} influx. By this means, PREGS facilitates the excitation of neurons at the postsynaptic level. The observation that the production of PREG and PREGS in the hippocampus was found to be enhanced by an approximate factor of 2 upon stimulation with NMDA strongly suggests the existence of positive feedback between NMDA receptor activation and the production of PREGS [29]. This possible rapid (<30 min) postsynaptic signal amplification through a "PREGS → NMDA receptor → Ca^{2+}" cycle could directly contribute to the LTP of hippocampal pyramidal neurons, during which PREGS acts as a mediator of the postsynaptic LTP induction. Our observation of the enhancement of LTP induction by PREGS in the CA1 pyramidal neurons strongly supports this hypothesis. The ability of a 500-nM PREGS perfusion to achieve potentiation of LTP induction is significant, because this concentration of PREGS is estimated to lie within the physiological, local concentration range typical of the hippocampus (0.6–1.2 μM). Before our study, there had been difficulties in explaining the physiological significance of the action of PREGS in the hippocampus, because PREGS requires micromolar concentrations (e.g., 20–100 μM) to display its potentiation effect [16,17,44,46].

The rapid action of estradiol on glutamate-mediated neuronal excitability was observed to be suppressive for 4-week-old rats (our results; Ref. [21]). Although the production of estradiol was enhanced by an NMDA-gated Ca^{2+} influx, this increase of estradiol appears to acutely suppress LTP induction. This suppressive action by estradiol may serve to prevent neurons from an overshoot excitation induced by the "PREGS → NMDA receptor → Ca^{2+}" cycle, because estradiol synthesis occurs much more slowly than PREGS (as judged from Fig. 1) (see Fig. 7). The effect of estradiol on hippocampal neurons is dependent on experimental conditions, and is also somewhat controversial. When a 1-s, 100-Hz tetanic stimulation was applied, a pretreatment of 0.1–50 nM estradiol suppressed the induction of LTP in hippocampal neurons taken from 4-week-old rats. In slices from adult rat (3 months old), on the other hand, an identical preperfusion with estradiol had no effect on the induction of LTP [21]. When theta-burst stimulation (e.g., five applications of 100 Hz for 200 ms each, in 10 s intervals) was applied to slices from an adult rat, a 20-min preperfusion with 0.1–10 nM estradiol

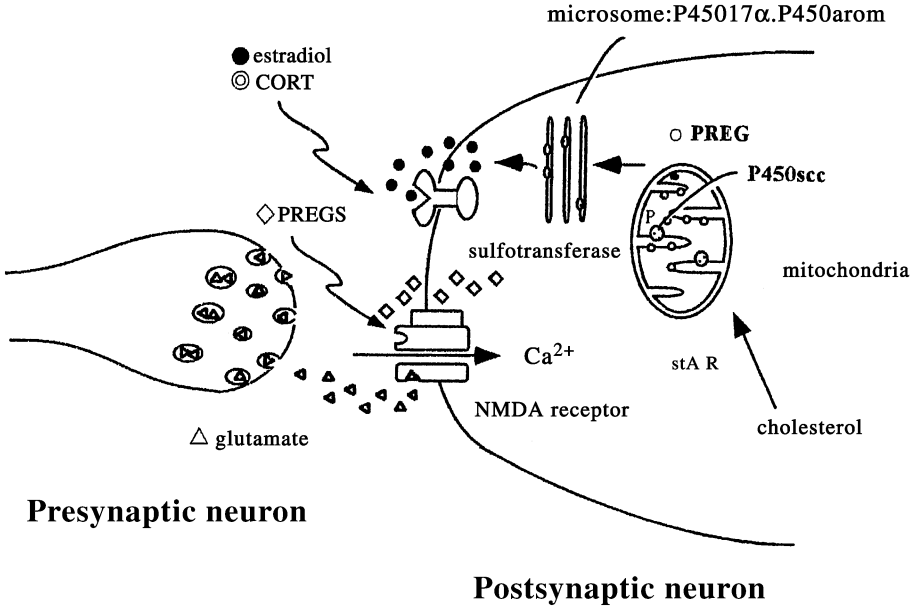

Fig. 8. Proposed postsynaptic signal amplification cascade mediated by PREGS in the hippocampus: NMDA-gating Ca^{2+} influx → StAR transports cytosolic cholesterol into mitochondria → P450scc converts cholesterol to PREG → conversion to PREGS → potentiation of NMDA receptor-mediated Ca^{2+} influx → StAR → P450scc → …. Possible modulation by PREGS, estradiol and CORT may be performed either by (1) direct binding to NMDA receptors or (2) binding to specific membrane receptors, followed by interactions with NMDA receptors. For illustrative purposes, the AMPA type of glutamate receptors is omitted.

was alternately effective at inducing LTP, as indicated by an approximate 2-fold enhancement of the slope and the peak magnitude of EPSP [20,56], or ineffective in inducing LTP as indicated by the near absence of either an enhancement or depression in the slope and the peak magnitude of EPSP (K. Ito, personal communication). In other reports, a 20-min preperfusion with 0.1–10 nM estradiol increased both the primed burst potentiation and the population spike amplitude [19]. It should be noted that we measure different types of responses when different types of stimulations are employed, for example, tetanic stimulation-induced LTP is essentially dependent on NMDA receptors, and theta-burst stimulation-induced LTP may be dependent on interneurons. Further experiments should therefore be performed to resolve the complicated effects of estradiol on neuron–neuron communication.

Although the intracellular signaling pathway, from estrogen receptors to NMDA receptors for rapid estradiol action, has not well been elucidated, the involvement of src tyrosine kinase has been indicated in the enhancement of LTP [56]. Estradiol has also been demonstrated to protect against the degradation of hippocampal NMDA receptors, upon only a 10-min exposure to estradiol, during which time MAP kinase-dependent protection of NMDA receptors occurred [56].

In this article, we focus on a consideration of the neurosteroid-induced modulation of NMDA receptors and LTP, because NMDA receptor-dependent LTP is likely to be the synaptic mechanism that implements memory. This view is supported by the observation that selective NMDA antagonists impair hippocampus LTP when delivered to the brain. Furthermore, the NMDA-dependent strengthening of CA1 synapses has been demonstrated to be essential for the acquisition and storage of spatial memory by transgenic mice in which the NMDA receptors in the CA1 pyramidal cells had been selectively deleted [66,67].

7. Putative membrane receptors for brain neurosteroids

Because classical nuclear steroid receptors mediate delayed genomic processes (which normally require hours to days), the rapid action (within 30 min) of brain neurosteroids may be mediated via novel membrane steroid receptors. The rapid enhancing effects of PREGS on NMDA receptors have been extensively studied in neurons from the hippocampus, cortex and hypothalamus [14,16,46]. The rapid modulation of PREGS has also been demonstrated for NMDA receptors expressed in *Xenopus* oocytes [46] and CHO cells [44]. It may therefore be deduced that PREGS could have specific binding sites on NMDA receptors. This hypothesis is further supported by the fact that no cytoplasmic/nuclear receptor has been observed for PREGS. PREGS is likely to have specific binding sites on GABA receptors as judged from investigations using electrophysiology and ligand binding assay [68,69]. There is also the possibility that novel membrane receptors (different from NMDA receptors) exist for estradiol [11,56,70–73]. This idea is supported by the observation that the rapid Ca^{2+} transients are induced by the application of 1–100 nM estradiol (alone) in both cultured rat hippocampal neurons (this work) and cultured dopaminergic neurons from the mouse embryonic midbrain [74].

The existence of putative surface CORT receptors also appears likely, and would serve to explain CORT's acute non-genomic effects [43]. This hypothesis is supported by studies which demonstrate that the immunoreactivity of antibodies against GR is associated with plasma membranes from hippocampal and hypothalamic neurons [75], and by reports that specific CORT binding to neuronal membranes may occur in several brain areas [76,77].

Further investigation is required to determine the primary and 3-D structure of these membrane steroid receptors.

8. Classical genomic effect of peripheral steroids

Brain neurosteroids act not only via rapid signaling pathways but also via classical cytoplasmic/nuclear steroid receptors. The concomitant classical genomic effect has been studied extensively in the past few decades. In the classical view of steroid hormone actions, steroids are considered to require binding to intracellular nuclear steroid receptors after reaching neurons via the circulation. Because activation of both the transcriptional and translational machinery of the cell is necessary to invoke classical steroid actions, a time-lag of hours to days must be present between the beginning of the steroid actions and their physiological consequences.

The chronic genomic effects of estradiol on synaptic plasticity have been extensively investigated. For example, the dendritic spine density in CA1 pyramidal neurons is sensitive to both naturally occurring estrogen fluctuations in rats [78], and experimentally induced estrogen depletion and replacement [79]. Recent evidence suggests that estrogens mediate these morphological changes by means of NMDA receptors. Estradiol increases the binding of NMDA agonist, as well as the NR1 levels in CA1 dendrites [80,81]. Moreover, estrogen-induced increases in dendritic spine density are blocked by NMDA receptor antagonists [82,83], and the electrophysiological properties of NMDA receptor-mediated transmission are altered by estrogens [20,84,85].

The administration of a 1-year therapy with 17β-estradiol for female patients of Alzheimer's disease following menopause has been shown to be very effective in improving their capacity for learning and memory [11]. Although this form of therapy requires the application of 17β-estradiol through blood circulation, the investigation of the signaling pathway induced by an endogenous paracrine supply of estradiol, which results in the modulation of neuron–neuron communication, may contribute to an understanding of this therapeutic effect.

With regard to its chronic genomic effects, CORT displays a so-called inverted U-shape type of modulation on neuronal excitability [86]. At endogenous low levels of CORT (0.5–1 nM in plasma), LTP was enhanced [87] in comparison to that observed in the absence of CORT following adrenalectomy. In stressful situations, a high level of CORT (1–10 μM), either produced in the hippocampus or supplied from the adrenal glands, may suppress LTP induction. Stress-induced increase in CORT secretion has been shown to produce neuronal cell damage [88,89]. The exogenous application of a high dose of CORT has also been shown to endanger the neurons in the hippocampus [90,91]. These chronic effects are also considered to be dependent on NMDA receptor-mediated Ca^{2+} conductance. Stress-elevated high levels of glucocorticoids have enhanced Ca^{2+} conductance. The blockage of NMDA receptors and the suppression of glutamate release are effective at inhibiting CORT-induced neuronal atrophy [92].

9. Conclusion and perspective

Brain neurosteroids could function as fourth generation neuromessengers in the brain, at least in the hippocampus. These substances are synthesized within the neurons and are responsible for the rapid modulation of neuron–neuron communication through neurotransmitter receptors. First-generation neuromessengers are neurotransmitters such as glutamate, GABA and acetylcholine. Second-generation neuromessengers are catecholamines such as dopamine and serotonin. Third-generation neuromessengers are neuropeptides such as enkephalin, vasoactive intestinal peptide, and substance P. In contrast with first- to third-generation neuromessengers, which are stored in synaptic vesicles and rapidly exocytosed from presynapses, brain neurosteroids are produced in mitochondria and microsomes, and are released relatively slowly by passive diffusion in neuronal cells. They then may diffuse to fill the interior of the neurons and, due to their amphipathic characters, may also reach other cells near to steroidogenic neurons, resulting in rapid modulation of neurotransmissions. In this sense, brain neurosteroids may serve as intracrine or paracrine modulators.

Several essential challenges must be addressed before we can claim to have a comprehensive understanding of this field. The first is a clear demonstration of the endogenous synthesis of brain neurosteroids in pure hippocampal neurons from the adult mammal. The second is a determination of the molecular structure of the accompanying membrane receptors, which may differ from those of nuclear receptors.

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