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Continuous *de novo* synthesis of neurosteroids is required for normal synaptic transmission and plasticity in the dentate gyrus of the rat hippocampus

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ABSTRACT

Both *in vivo* and *in vitro* studies have shown that neurosteroids promote learning and memory by modulating synaptic functions in the hippocampus. However, we do not know to what degree endogenously synthesized neurosteroids contribute to the hippocampal synaptic functions. Cytochrome P450scc is the enzyme that converts cholesterol to pregnenolone (PREG), which is required for the biosynthesis of all other neurosteroids. To investigate the physiological roles of endogenous neurosteroids in synaptic functions, we electrophysiologically examined the effects of aminoglutethimide (AG), a selective inhibitor of P450scc, on the synaptic transmission and plasticity in the dentate gyrus of rat hippocampal slices. The application of AG (100 μM) decreased the slope of the field excitatory postsynaptic potentials (fEPSPs) in granule cells by 20–30% in 20 min through the modulation of postsynaptic AMPA receptors, while it did not affect the presynaptic properties, including the paired-pulse ratio and the probability of glutamate release from presynaptic terminals. The AG-induced depression was nearly completely rescued by exogenously applied 500 nM PREG or by 1 nM dehydroepiandrosterone sulfate (DHEAS), one of the neurosteroids synthesized from PREG, suggesting that the AG-induced depression was caused by the loss of DHEAS. AG also reduced NMDA receptor activity, and suppressed high-frequency stimulation (HFS)-induced long-term potentiation (LTP). These findings provide novel evidence that the endogenous neurosteroids locally synthesized in the brain are required to maintain the normal excitatory synaptic transmission and plasticity in the dentate gyrus of the rat hippocampus.

1. Introduction

Neurosteroids are synthesized *de novo* in the central nervous system independently of the peripheral endocrine glands. The first step in the neurosteroidgenesis is the conversion of cholesterol to pregnenolone (PREG) by P450scc in the mitochondria of neuronal and glial cells (Baulieu and Robel, 1998; Zwain and Yen, 1999; Kimoto et al., 2001). PREG is then converted by various enzymes into other neurosteroids such as PREG sulfate (PREGS), dehydroepiandrosterone (DHEA), estradiol and so on.

These neurosteroids are believed to play important roles in memory and cognitive performance during development and into adulthood. For instance, changes in the brain neurosteroid levels are related to improved memory acquisition and retention, including increased performance in the Morris water maze test in rats (Vallée et al., 2001). There is also a correlation between the circulating steroid levels and cognitive performance in humans (Barrett-Connor and Edelstein, 1994). These observations suggest that neurosteroids are important endogenous modulators of spatial and/or relational learning and memory processes in the hippocampal formation (Corpéchot et al., 1981). The synaptic and molecular mechanisms by which these neurosteroids modulate such brain functions have been extensively studied. PREGS and DHEA sulfate (DHEAS) modulate glutamate release from the presynaptic terminals in hippocampal neurons (Partridge and Valenzuela, 2001; Meyer et al., 2002; Mameli et al., 2005; Dong et al., 2007), and act as potent positive modulators of AMPA and NMDA receptors (Wu et al., 1991; Yaghoubi et al., 1998; Sliwinski et al., 2004; Shirakawa et al., 2005; Chen et al., 2006b; Xu et al., in press) and negative modulators of GABA receptors (Eisenman et al., 2003; Steffensen et al., 2006). Estradiol also acts as a positive modulator of NMDA receptors (Foy et al., 1999; Bi et al., 2000; Nilsen et al., 2002) and a negative modulator of GABA receptors (Murphy et al., 1998; Rudick and Woolley, 2001), thereby increasing the spine density in the hippocampus (Murphy et al., 1998; Pozzo-Miller et al., 1999). On the other hand, allopregnanolone (AP) and

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tetrahydrodeoxycorticosterone (THDOC) act as positive modulators of GABA receptors and hence are known as GABAergic neurosteroids (Mellon and Griffin, 2002; Belelli and Lambert, 2005). These neurosteroids also exert modulatory effects on the induction of long-term potentiation (LTP) and long-term depression (LTD) in hippocampal slices (Foy et al., 1999; Dubrovsky et al., 2004; Sliwinski et al., 2004; Mukai et al., 2006; Smith and McMahon, 2006; Chen et al., 2006a, 2010). As such, mounting evidence indicates that some neurosteroids enhance the learning and memory processes in the hippocampus through modulation of the excitatory and inhibitory synaptic transmission by altering the pre- and/or postsynaptic properties.

However, as most of the reported studies on the neurosteroid effects on brain synapses were performed exclusively with exogenously applied neurosteroids, the degree to which the locally synthesized endogenous neurosteroids in the brain contribute to these activities in the hippocampus is poorly understood. Recently, it was reported that local endogenous estrogen facilitates the development of synaptic plasticity during high-frequency stimulation (HFS) and induces LTP in the rat medial vestibular nuclei (Grassi et al., 2009). Endogenous estrogen is also needed for the maintenance of spine synapses and synaptic proteins in the rat hippocampal CA1 region and dentate gyrus (Kretz et al., 2004; Prange-Kiel et al., 2006). Granule cells in the dentate gyrus and pyramidal neurons in the CA1 region of the rat hippocampus express high levels of several enzymes involved in neurosteroid synthesis, and the basal concentrations of PREG, PREGS, DHEA, and estradiol in the hippocampus were found to be markedly higher than those in the plasma (Kimoto et al., 2001; Hojo et al., 2004). Presumably, endogenous estrogen contributes to synaptic plasticity in the rat dentate gyrus as well as the medial vestibular nuclei. Moreover, there is a possibility that endogenous neurosteroids other than estrogen also play physiological roles in the modulation of synaptic transmission and plasticity in the rat hippocampus.

Aminoglutethimide (AG), an inhibitor of cytochrome P450scc, inhibits the production of PREG, which is the first neurosteroid in the pathway of neurosteroidgenesis and is required for the synthesis of all other neurosteroids (Foster et al., 1983; Kimoto et al., 2001). Therefore, AG can be used to suppress the *de novo* production of all neurosteroids. To study the physiological roles of locally synthesized endogenous neurosteroids and their synaptic and molecular mechanisms in the central nervous system, we electrophysiologically examined the effects of AG on the synaptic transmission and plasticity in the dentate gyrus of rat hippocampal slices.

2. Materials and Methods

2.1. Animals and housing conditions

Young male Wistar rats (3–4 weeks of age) were housed in a light controlled room under a 12-h light–dark cycle starting at AM 9:00 and maintained at a temperature of 25 °C. The animals had free access to food and water. All procedures were carried out in accordance with the guidelines established by the Institute for Laboratory Animal Research of the Medical School of Nagoya University.

2.2. Hippocampal slice preparation

The rats were decapitated under deep anesthesia with ethyl ether. The brains were quickly removed and then transverse 400-μm-thick slices were cut from the hippocampus using a vibratome in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 128 NaCl, 5 KCl, 1.3 $MgSO_4$, 1.25 KH_2PO_4, 2.41 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose. ACSF was gassed with 95% O_2/5% CO_2 and the pH was adjusted to 7.4. Slices were maintained at least 1 h at room temperature (26–28 °C) in an incubation chamber containing gassed ACSF.

2.3. Chemicals

DL-aminoglutethimide (AG), dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), β-estradiol (E2), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), 1,2,3,4-tetrahydro- 6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX), (±)-3-(2-carboxypiperazin- 4-yl)propyl-1-phosphonic acid (CPP), bicuculline, tetrodotoxin (TTX) were purchased from Sigma (St. Louis, MO). Pregnenolone (PREG) was obtained from Cosmo Bio (Tokyo, Japan). Other chemicals of special grade were obtained from Wako chemical (Osaka, Japan). AG was dissolved in 0.1 M HCl for stock solution and then was diluted with the perfusion ACSF at a final concentration of 0.02% HCl just before use. PREG, DHEA, DHEAS, E2 and letrozole were dissolved in dimethyl sulfoxide (DMSO) for stock solution and then were diluted with the perfusion ACSF at a final concentration of 0.01% DMSO just before use. Trilostane dissolved in DMSO was diluted with the perfusion ACSF at a final concentration of 0.05% DMSO. The treatment with 0.01%, 0.05% DMSO alone or 0.02% HCl alone had no effect on the synaptic transmission. Other drugs were dissolved in distilled water and stock solutions were maintained at −20 °C.

2.4. Electrophysiology

A single hippocampal slice was transferred to the recording chamber and superfused continuously with gassed ACSF at a rate of 2–2.5 mL/min at room temperature. A stimulating electrode (monopolar stimulation) was positioned in the molecular layer 50–100 μm from the granule cell layer in the dentate gyrus in order to activate the medial perforant path. Constant-current pulses (100 μs) were supplied by a stimulator (SEN-3301, Nihon Kohden, Tokyo, Japan) every 30 s. The intensity of the test stimuli was adjusted to evoke approximately 50% of the maximum responses. For extracellular recording, field excitatory postsynaptic potentials (fEPSPs) were recorded from the middle molecular layer in the dentate gyrus using a glass pipette filled with 2 M NaCl (2–3 MΩ). The stability of the baseline was established by delivering test stimuli for 20–30 min before recordings. The paired-pulse ratio (PPR) of fEPSPs was elicited by paired-pulse stimulation with four inter-pulse intervals (25, 50, 75, 100 ms). Long-term potentiation (LTP) was induced with 100 pulses at 50, 100, and 200 Hz. For whole-cell patch clamp recording, a patch electrode was filled with a pipette solution containing (in mM) 140 caesium gluconate, 10 NaCl, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 3 Mg-ATP, 0.3 Na-GTP, 5 QX-314, with 6–8 MΩ of resistance. Whole-cell patch-clamp recordings were made from granule cells near the outer border of the granule cell layer in the dentate gyrus. Granule cells were imaged with an IR-DIC optics (BX51WI with 20× water immersion objective lens, OLYMPUS, Japan). Holding potentials were compensated for the junction potential between the pipette solution and the ACSF. AMPA receptor-mediated miniature excitatory postsynaptic currents (EPSCs) were recorded in voltage-clamp mode at a membrane potential (Vm) of −70 mV and in the presence of voltage-dependent Na^+ channel blocker (0.5 μM TTX), NMDA receptor antagonist (20 μM CPP) and $GABA_A$ receptor antagonist (20 μM bicuculline). To measure the AMPA-evoked inward currents in granule cells, AMPA (100 μM) was applied to granule cells using pressure ejection (FemtoJet, Eppendorf AG, Hamburg, Germany) from a micropipette (~10 μm in diameter) in the presence of TTX (0.5 μM). The micropipette was placed near the medial perforant path-patched granule cell synapses in the middle molecular layer. The AMPA-evoked inward currents were completely abolished by 20 μM NBQX. AMPA receptor-mediated EPSCs (AMPAR EPSCs) were recorded in voltage-clamp mode at −70 mV, and were isolated by blocking NMDA and $GABA_A$ receptors. To measure NMDA receptor-mediated EPSCs (NMDAR EPSCs), cells were held at +40 mV to relieve the voltage-dependent Mg^{2+} block. NMDAR EPSCs were isolated by blocking AMPA receptors (20 μM NBQX) and $GABA_A$ receptors. These isolated AMPAR and NMDAR EPSCs were completely abolished by each specific antagonist (20 μM NBQX and CPP). Access resistance was monitored continuously during the experiment, and the obtained data were discarded if the access resistance fluctuated over 20%. Signals were amplified and filtered at 5 kHz using an Axopatch 200B amplifier (Axon Instruments, CA, USA). Data acquisition and analysis were performed using pCLAMP 9.0 software (Axon Instruments, CA, USA).

2.4.1. Dentate granule cell dissociation

Dentate granule cells were prepared by a modified version of the methods that have been reported previously (Eliot and Johnston, 1994; Van Sickle et al., 2002). In brief, the cells were dissociated from hippocampal slices (400 μm) prepared from 18- to 23-day-old Wistar rats. The hippocampal dentate gyrus region was dissected on ice and was incubated for 7 min in 1 mg/ml papain and 0.2 mg/ml L-cysteine at 37 °C in oxygenated (95% O2/5% CO2) PIPES buffer containing (in mM) 120 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 25 D-glucose, and 20 PIPES at pH 7.0. The preparations were washed 4× in PIPES buffer with 1 mg/ml bovine serum albumin (BSA) and were triturated using fire-polished Pasteur pipettes in 1 mL of ice cold PIPES. The resulting cell suspension was plated on poly-lysine-coated (2 mg/ml, poly lysine) cover glasses for at least 15 min before recording. Although the vast majority of neurons in the dentate gyrus of the hippocampus are granule cells (Seress and Pokorny, 1981), we recorded selectively from identified granule cells with relatively small soma (10–15 μm) having just a single prominent process (Eliot and Johnston, 1994). For whole-cell patch clamp recordings from acutely isolated granule cells, a patch electrode was filled with a pipette solution containing (in mM) 140 K gluconate, 10 NaCl, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 3 Mg-ATP, 0.3 Na-GTP, with 6–8 MΩ of resistance. To measure the AMPA-evoked inward currents in the granule cells, AMPA (200 μM) was applied to the granule cells using a pressure ejector with a micropipette