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17α-Estradiol is generated locally in the male rat brain and can regulate GAD65 expression and anxiety

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ABSTRACT

Increasing evidence suggests that 17β-estradiol, a sex hormone, is synthesized by neurons. In addition, 17α-estradiol, the stereoisomer of 17β-estradiol, is reported to be the dominant form in the male mouse brain. However, probably because the method to detect these isomers requires unusual and precise experimental design, the presence of this endogenous 17α-estradiol has not been reported subsequently and the actual role is therefore not well elucidated. We first quantified the estradiol level in hippocampal extracts using gas chromatography/mass spectrometry. As a result, 17α-estradiol was found in all of the male rats tested, while that of 17β-estradiol was detected only in a certain subset. The estrogen-biosynthesis inhibitor letrozole decreased the expression of the major presynaptic GABA synthesizing enzyme GAD65 in cultured neurons and the effect was abrogated by exogenously supplied 17α-estradiol. Next, injection of the inhibitor into the brain reduced the 17α-estradiol level, indicating its biogenesis in the brain. Under the same conditions, immuno-staining of GAD65 was also decreased. Furthermore, the inhibitor treatment increased anxiety index of rats in the open field and this was ameliorated by the addition of 17α-estradiol. We showed that 17α-estradiol was generated in the brain and acted as a regulator of inhibitory neurotransmission as well as behavior. These results may have implications for a variety of diseases, such as the menopausal depression and Alzheimer's disease that have been reported to be related to estrogen levels.

1. Introduction

While the small chemical compound 17β-estradiol is known as a female hormone, increasing evidence has demonstrated that it is synthesized by neurons in the brain and functions locally as a neuromodulator (Fester et al., 2011; Srivastava et al., 2013).

In 2005, 17α-estradiol, the stereoisomer of 17β-estradiol, was reported to be the dominant form found in the male mouse brain (Toran-Allerand et al., 2005). This stereoisomer differs in the orientation of the 17th−OH of the steroid structure, and is mainly made from 17α-testosterone, while 17β-estradiol is generated from 17β-testosterone, both by an enzyme called aromatase. However, the presence of the endogenous 17α-estradiol has not been reported subsequently. This is at least partly because the usual quantification methods for 17"β"-estradiol have been immunoassays (RIA, ELISA) that are not specific enough. Accordingly, the role of the endogenous 17α-estradiol has not been elucidated yet.

It is reported in humans that 17α-testosterone, the precursor for 17α-estradiol, is measurable in the serum of 24% of boys and 33% of girls (Courant et al., 2010). Thus, individual variation in the 17α-estradiol concentration is likely. If so, this should be taken into account when considering the therapeutic use of estradiol-related drugs.

We at first detected 17α-estradiol in the male rat brain using gas chromatography/mass spectrometry (GC/MS). Then, since an effect of estradiol on GAD65, a dominant GABA synthetic enzyme in the presynaptic terminals of inhibitory neurons (Ikeda et al., 2006; Nakamura et al., 2004; Rudick and Woolley, 2001) as well as

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direct transcriptional activation (Hudgens et al., 2009) have been reported, we used GAD65 to evaluate the effect of 17α-estradiol in comparison with 17β-estradiol. Next, we sought to inhibit estrogen synthesis in the brain by intracerebroventricular injection of an inhibitor to confirm the biogenesis and role in the male rat brain. We thereby found the first evidence of locally-generated 17α-estradiol and its effects on the GAD level *in vitro* and *in vivo*, as well as effect on anxiety-related behavior.

Since there have been many reports on the different functions of the exogenously applied 17α-estradiol and 17β-estradiol (Barha and Galea, 2010), it seems plausible that this difference could be utilized to confer benefit in certain cases.

The present results provide a basis for further analyses on the variety exhibited in different individuals and the specific functions of 17α-estradiol.

2. Materials and methods

2.1. Animals

All experiments were performed with Wistar/ST rats from SLC (Shizuoka, Japan), according to the Japan Neuroscience Society guide for the care and use of laboratory animals under the approval of the Committee for Animal Experiments of the University of Tokyo.

2.2. Quantification of the estradiol level using GC/MS

Cold phosphate-buffered saline was flushed via the cardiac ventricles to purge blood of ether-anesthetized male rats, then the hippocampi were rapidly dissected on ice and homogenized by a teflon homogenizer at 1000 rpm 10 times in 3 volumes of chilled water. The known amounts of isotopes, 17α-estradiol-2,4-d_2 and 17β-estradiol-2,4,16,16,17-d_5 (CDN isotope Quebec, Canada) were added to the homogenate, so that they served as the internal standards for the extraction, derivatization and all other procedures. They can be detected at the final quantification using GC/MS as molecules having the same retention time but different masses. Ethylacetate (16 vol.) was added to the homogenate, mixed by a vortex mixer for 10 min and centrifuged 2000 × *g* at 10 °C for 10 min. The upper organic layer was collected and the solvent was evaporated under a stream of nitrogen gas at room temperature. 100 μl of 10%TMCS in BSTFA (Pierce) and 60 μl of pyridine (Sigma–Aldrich) were added to the residue, and heated at 70 °C for 60 min to prepare the derivative. The solvent was evaporated and residue was resolved in chloroform.

The Ultra-2 capillary column (25 m long, 0.25 mm in diameter, 0.33 μm film coated; Agilent Technologies, Santa Rosa, CA) using Herium as the carrier gas was used for high-resolution selected ion monitoring mode of GC/MS. (6890N GC, Agilent technologies Santa Rosa, CA, USA, coupled with JMS-SX102A MS, JEOL, Tokyo, Japan). The injection port and transfer line temperature were maintained at 250 °C and 300 °C, respectively. The program of the GC oven temperature was as follows: 70 °C for 5 min, followed by a ramp up to 160 °C at 30 °C/min, then to 300 °C at 7 °C/min, and a final hold for 7 min. The presence of estradiol was confirmed by the mass of *m/z* 416.2567 and that of the structurally expected fragments (*m/z* 326.2066 and *m/z* 285.1675), in addition to having the same retention time with both pure standards and mixed isotopes. We usually load the pure estradiols as external standards just before every set of experiments, and could detect 0.3 pg of the standards in charts, so the detection limit of GC/MS is thought to be comparable to or better than the radioimmuno-assay system.

2.3. Primary neuronal culture

Hippocampal neurons were cultured by a previously described method (Ohba et al., 2005), except using Neurobasal medium free of phenol red (Invitrogen, San Diego, CA) as a possible estrogen-like molecule. In brief, dispersed neurons from the hippocampi (both male and female rats on embryonic day 18) were suspended in medium containing 10% fetal bovine serum (MP Biomedicals Inc., Irvine, CA) and were plated at a density of 50,000 cells/cm^2 on polyethyleneimine (Sigma, St. Louis, MO)-coated glass coverslips (Matsunami Glass Osaka, Japan) attached to the flexiPERM having eight wells of 0.7 × 1.0 cm^2 each (Sartorius, Göttingen, Germany). Medium was replaced with a serum-free one supplemented with 2% B27 (Invitrogen) 24 h after plating. Half of the medium was gently changed every other day. The aromatase inhibitor letrozole (kindly provided by Novartis Pharma AG, Basel, Switzerland), with or without 17α- or 17β-estradiol (Sigma–Aldrich, St. Louis, MO), was added to the new medium at the change on 7 days of *in vitro* and applied for 48 h.

2.4. Intracerebroventricular injection of letrozole

Each Wistar/ST male rat (SLC, 4 wk-old) was anesthetized with pentobarbital, and a guide cannula with an inserted plug was intracerebrally implanted into the lateral cerebral ventricle, one week before the injection. The implantation site was adjusted by the bregma-lambda length to a place corresponding to 0.8 mm anteroposterior, 1.4 mm lateral right and 3 mm dorsoventral in the rat atlas (Paxinos and Watson, Academic Press). A solder-mounted wire ring on the cannula was fixed to the skull with a screw and dental cement. For injection, the plug was transiently removed and vehicle (20% DMSO/80% saline) or letrozole (1.4 μg/kg) was injected every 24 h for three days, then samples were taken 24 h after the last injection. For the behavior test, to avoid stress from the daily handling, a tube from the subcutaneously-implanted osmotic pump (Alzet, Cupertine, CA, USA) was connected to the injection needle in the guide cannula for three days. The dose of letrozole (1.4 μg/kg/day) with or without 17α-estradiol (30 μg/kg/day) was determined according to previous reports (Liang et al., 2002).

2.5. Immunostaining and fluorescence image analysis

The cultured cells were treated sequentially with 4% paraformaldehyde at 4 °C for 30 min, wash, 0.1% Triton X-100 for 15 min, wash and 1% goat serum/PBS for 1 h, and then with primary antibody for GAD65 (GAD-6, mouse monoclonal, 6 μg/ml, DSHB) overnight. The specificity of the GAD-6 was previously characterized as a single band at the size of GAD65 on Western analysis, in a literature (Chang and Gottlieb, 1988) and by ourselves. After washing, the cells were incubated with a secondary antibody (Alexa594-conjugated anti-mouse IgG; dilution, 1:500; Invitrogen) for 2 h at 4 °C.

For immunohistochemistry, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with chilled phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were post-fixed (4 °C, 1.5 h), immersed in 10%, 20%, and 30% sucrose in PB in series (4 °C, 24hr in total), frozen and coronally sectioned at a thickness of 30 μm. Sections mounted on glass slides were incubated in 0.1% Triton X-100 for 10 min, 5% goat serum for 1 h at 4 °C, GAD-6 antibody in 0.25% goat serum at 4 °C overnight, and then with the Alexa594 labeled antibody in 1% goat serum with the addition of NeuroTrace 435/455 (fluorescent Nissl, Invitrogen) for 1 h at room temperature.

All 8-bit images were captured with an Orca-II cooled-CCD camera (Hamamatsu photonics, Shizuoka, Japan) on a Nikon TE300 microscope (10 × objective lens). Care was taken not to include intensity-saturated pixels. Compared images were taken in the same conditions.

For neuronal culture, the image analysis method had been developed in the previous study (Ikeda et al., 2006). Briefly, 10 image frames (340 × 340 μm^2) were randomly selected from a well according to marks on the glass bottom assigned in a blind manner. To omit background staining, the average of mode + 2 × standard deviation of the intensity value in the images from control wells (cells with no reagent) of each batch of culture was calculated and defined as a threshold value. This nearly matches the intensity value from the glass surface without any evident neurons, possibly from antibodies remaining on the coated glass. Values greater than the threshold were integrated for each image and averaged, normalized to control. The intensity was measured by Scion Image software (Scion Corp., Frederick, MD).

For the slice imaging, the focus points and image frames were determined using filter-set for Nissl staining then changed to that for Alexa594 to preserve fluorescence and to keep unbiased selection. The sub-region was also objectively defined by Nissl images taken together and the raw intensity was used for quantification. The thresholding procedure was not done because of the apparently low background. The analysis was done by ImageJ (NIH) and MetaMorph (Universal Imaging Corp., West Chester, PA).

2.6. Behavior test

The male rats were received daily handling before the surgery, until they seemed to be habituated. For the open-field test, each rat was put at the center of an unfamiliar, round open-top arena with a wall (37.5 cm diameter, 80–100 lux) and a movie was taken from above for 5 min. Each image from the movie was binarized for body shape and the center of the mass position was examined to determine whether it came into the center of the arena (22.5 cm diameter) or not and used to draw the track in Fig. 5A.

2.7. Statistical analysis

Group data are presented as mean ± SEM. Welch's modified *t*-test for comparison of two groups or Tukey's test for multiple comparisons after ANOVA was applied by Microsoft Excel or R, respectively.

3. Results

3.1. Quantification of estradiol in the male rat hippocampus

For the initial step, we quantified the 17α- and 17β-estradiol present in the hippocampus utilizing their different retention times in GC due to the large conformational difference between these stereoisomers. When preparing the samples, we flushed the blood out and added an internal control for quantification. As seen in the