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

Takamitsu Ikeda a, Yukiko Makino b, Maki K. Yamada a,c,*

a Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
b Laboratory of Chemistry and Biology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
c PRESTO, The Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

A R T I C L E   I N F O

Article history:
Received 25 September 2014
Accepted 18 October 2014
Available online 6 November 2014

Keywords:
Estrogen
Female hormone
Inhibition
Anti-anxiety
Aromatase
AKR1C

A B S T R A C T

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.

© 2014 Elsevier Ltd. All rights reserved.

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 17β-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
direct transcriptional activation (Hudgens et al., 2009) have been reported, we used GAD65 to evaluate the effect of 17α-estriodiol 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 (Barba 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 ventricle 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 5 volumes of chilled water. The known amounts of isotopes, 17α-estradiol-2,4-d, and 17β-estradiol-2,4,6,16,17-d6 (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 at 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 100TMS 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) using Herium as the carrier gas was used for high-resolution selected ion monitoring mode of GC/MS. The injection port and transfer line temperature were maintained at 250 °C/C2 overnight, then with the Alexa594 labeled antibody in 1% goat serum with the addition of CAT (Boehringer Mannheim) for 2 h at 4 °C. The injection port and transfer line temperature were maintained at 250 °C, and 24 hr 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) were randomly selected from a well according to marks on the glass bottom assigned in a random 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 agent) 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
3.2. The effect of 17α- and 17β-estradiol on cultured hippocampal neurons

17α-Estradiol has been described as “inactive” in certain previous studies (Lingham et al., 1988; Womble et al., 2002). Therefore, to clearly show the effect of 17α-estradiol, we added 17α- and 17β-estradiol to the medium of a relatively pure neuronal population together with an inhibitor of aromatase, an enzyme required for estrogen biogenesis. After confirming a decrease in the GAD65 signal induced by letrozole, as in the previous findings (Ikeda et al., 2006), the dose for recovery was studied. As the result, 17α-estradiol was found to be comparable to or more effective than 17β-estradiol in all of them, while 17β-estradiol was detected in only a portion.

3.3. Decrease of estradiol by a brain injection of an aromatase inhibitor

To further inhibit the biogenesis of estradiol in the brain separately from other tissues, such as the adrenal glands and adipose tissue, we injected letrozole directly into the brain ventricles. Since quantitative data (Fig. 1A–C), we detected 17α-estradiol in all of the samples, but only three out of five rats exhibited a detectable amount of 17β-estradiol. Of the 17 rats tested, including those in Fig. 3, we found a detectable level of 17α-estradiol in all of them, while 17β-estradiol was detected in only a portion.
the brain is separated from other tissues by the blood—brain barrier, this route of administration limits the effectiveness of the drug in the brain. As a result, a significant decrease in the 17α-estradiol level was found in the letrozole-treated rats (Fig. 3, \( ^*P = 0.0097 \), \( N = 5 \) and 7 rats).

3.4. An aromatase inhibitor down-regulated the brain GAD65 level

After 3-days of letrozole injection i.c.v., immunostaining by anti-GAD65 antibody (specificity: a single band on Western analysis) detected a decrease in the immunoreactive signals (Fig. 4A, B). In the vehicle control sections, a potent GAD65 signal was seen around the pyramidal cell layer (stratum pyramidale, SP) and a weak signal in the stratum oriens (SO) and stratum radiatum (SR). By semiquantification of the GAD65-signal intensity, a significantly lowered signal was found in the letrozole-treated rats in the pyramidal cell layer (Fig. 4C, 85 \( \pm \) 5\%, \( ^*P = 0.038 \)). The difference was not evident in CA1-SO (97 \( \pm \) 3\%, \( P = 0.51 \)) containing basal dendrites, or CA1-SR (92 \( \pm \) 2\%, \( P = 0.05 \)) containing apical dendrites. It was, however, observed in CA3a (which is closest to CA1 among three CA3 subregions, 68 \( \pm \) 6\% in the SO and 75 \( \pm \) 4\% in the SP, \( Ps < 0.05 \)) and in the molecular layer of the dentate gyrus (75 \( \pm \) 7\%, \( P < 0.05 \)) in the hippocampal formation.

3.5. Rat behavior was altered by letrozole treatment and this effect was canceled by the addition of 17α-estradiol

We analyzed anxiety-related behavior in the open field test of the letrozole-treated rats based on the fact that GABA-enhancing drugs are commonly used as anti-anxiety drugs. In this test, a shortened length of time spent in the center is generally considered as an index of the level of anxiety. After 3 days of letrozole injection into cerebroventricles, the time in the center was shorter in the letrozole-treated group. A simultaneous injection of 17α-estradiol abrogated the effects of letrozole. (Fig. 5A, B left, \( ^*P = 0.032 \) vehicle vs letrozole, \( ^*P = 0.047 \) letrozole vs letrozole + 17αE, \( N = 12, 10, 11 \) rats.) The traveled distance was not significantly different (Fig. 5A, B right, \( P > 0.05 \)).

4. Discussion

We detected 17α-estradiol in the male rat brain. We also demonstrated that aromatase inhibitor injection in male rat cerebral ventricles decreased 17α-estradiol levels, indicating that this molecule is generated in the brain, even in males. In addition, aromatase inhibition altered GAD65 expression as well as anxiety-related behavior, which effects were abrogated by the addition of 17α-estradiol. These results suggest that the 17α-estradiol generated in the brain changes mood, possibly via modulation of transmission.

4.1. 17α-Estradiol is produced in the brain

It has been proposed that 17α-estradiol is produced in the brain since it was detected in the mouse brain and its presence was not affected by ovariectomy or adrenalectomy (Toran-Allerand et al., 2005). Our result of an intracerebroventricular injection of letrozole supports this previous report.

However, a few papers have reported detection by chromatography of brain 17β-estradiol without 17α-estradiol. We consider this discrepancy to be explained by the difference in methods and/or the strain of animal used.

In terms of the method, there are possible advantages as follows: 1, a high-resolution selected ion monitoring mode by GC/MS; 2, high temperature in GC favorable to dissociating disturbing molecular interactions; 3, the detection system of MS that minimizes steps to the detection; 4, quick perfusion and dissection under chilled conditions; 5, homogenization with water so as to osmotically punctuate cellular organelles; 6, internal standards maintained throughout the sample processing.

Otherwise, differences in the sample source, such as animals being from a different strain/colony, might also lead to a difference.
in the detected stereoisomer. Even within the single closed-colony strain of rats we used (Wistar/ST), the ratio of 17α- and 17β-estradiol in each individual varied considerably (Fig. 1). This strongly suggests that the generation system can vary even within animals of the same strain. The most probable cause is a genetic variation, such as single nucleotide polymorphisms in the AKR1C family, 17α-hydroxysteroid dehydrogenase that generates the precursor 17α-testosterone for the aromatase (Faucher et al., 2006). Although it is not yet possible to resolve the cause underlying the observed heterogeneity in stereoisomer expression, future analyses of the variance of this and other related enzymes, such as those in the AKR1C family, should shed light on such inter-individual variations in laboratory animals as well as in humans.

Despite the differences among various reports in the literature, the presence of endogenous 17α-estradiol in the brain at least in this strain of rats was clearly confirmed in the present study.

4.2. 17α-Estradiol is a positive regulator of the inhibitory synaptic molecule, GAD65

We used a neuronal culture system for the detailed recovery experiments (Fig. 2), because this system has the advantage that any effect other than that from neurons is negligible. As a result, clear data were obtained. The similar dose dependency of 17α-to 17β-estradiol confirmed that the residual contamination of 17β-estradiol in the purchased 17α-estradiol should not be the sole effector. The effective estradiol dose in this experiment (~10 nM) can induce ERα to bind the GAD65 promoter (Hudgens et al., 2009). Thus, of the possible affected factors including the major estradiol metabolite estriol, the estradiols were most probably the direct cause of GAD recovery (Kuiper et al., 1997). The recovery showed that, in the presence of letrozol, neurons were able to respond to exogenous 17α estradiol in their GAD regulation and return to baseline. Thus, it is reasonable to think that the halted biogenesis of endogenous estradiol was the main cause of the observed GAD65 decrease. This experiment clearly demonstrated a 17α-estradiol effect on GABAergic function and supports the notion of an endogenous estradiol regulation of GAD65.

4.3. The pattern of GAD65 decrease and its significance

We first found the effect of letrozole that decreases the GAD65 level in the live brain (Fig. 3). In addition, the pattern of decrease around the neuronal soma is suggestive, because this perisomatic inhibition is thought to be important in memory processing (Paulsen and Moser, 1998; Royer et al., 2012). That is, the estradiol produced by neurons accelerates perisomatic inhibition along with GAD65 upregulation and thus helps maintain normal memory function. Together with the reported synergistic effect of BDNF on GAD (Ikeda et al., 2006; Ohba et al., 2005), locally-generated estradiol may exert an effect on memory processing via control of the GAD level.

4.4. The selectivity of 17α-estradiol function in the brain

Although the present paper showed a similarity between the activity of the two isomers, there have been many reported phenomena that are only induced by 17β-estradiol, not 17α-estradiol, and vice versa (Barha and Galea, 2010). Nevertheless, the binding constants (Kd) of both of these isomers to the two known receptors, ERα and ERβ, were shown to be the same (Toran-Allerand et al., 2005) or similar (Kuiper et al., 1997). Considering that a ligand with the same affinity for a given receptor theoretically ought to elicit the same response, this apparent discrepancy may be due to either modulators of the known receptors, ligand-specific carrier proteins or unknown additional receptors that might be more selective for one of isomers (Snyder et al., 2010; Toran-Allerand et al., 2005). Considering purpose-driven nature of organisms, an unknown mechanism for the different responses is suggested by the existence of endogenous 17α-estradiol.

4.5. Possible implications for treatment

We found an increase in the anxiety-related behavior in the letrozole injected rats, consistent with the many reports on estrogen’s effect on relieving anxiety. The present findings add information about the potential role of endogenous 17α-estradiol and the temporal association of behavioral changes with decrease in GAD-intensity. Even though a causal relationship is yet to be determined, our results suggest that some estradiol functions may be induced via GAD regulation. Given this possibility, treatment of menopausal women or letrozole-induced depression with GABA-enhancers should perhaps be considered. Furthermore, if 17α-estradiol safely works more preferentially in the brain than 17β-estradiol, many other therapeutic options may also become available.

The differences in these isomers would be clarified by further investigation involving the wide range of information available on the activity of estradiol (Balthazart and Ball, 2006; Daniel, 2013). Applications for estradiol-related drugs for psychiatric and/or neurological disorders should be carefully examined and analyzed based on these differences as well as individual variations.

Acknowledgments

This work is supported by Grant-in-Aid from Scientific Research (C, 19590060), Priority Areas Integrative Brain Research (20019016) from the MEXT Japan and PRESTO/Sakigake from JST for MKY. We thank Novartis for providing letrozole. Developmental Studies Hybridoma Bank (DSHB, University of Iowa) for the monoclonal antibody for GAD65 named as GAD6 and Emeritus Prof. Dr. Norio Matsuki for supporting our research.

References


