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Sex differences in estrogen receptor promoter expression in the area postrema[☆]

Chunxiao Zhang¹, Tomohiro Hamada²

¹ Department of Physiology, Norman Bethune College of Medicine, Jilin University, Changchun 130021, Jilin Province, China

² Department of Physiology, Nippon Medical School, Tokyo 113-8602, Japan

Abstract

Estrogen receptor α is widely distributed in the rat brain, but the tissue- or target-specificity of the estrogen receptor α gene promoters remains unknown. In the present study, we used transgenic rats expressing enhanced green fluorescent protein under the control of the estrogen receptor α O/B promoter to examine expression driven by this promoter in two significant nuclei that regulate cardiovascular activity, the area postrema and the nucleus tractus solitarius. Immunohistochemistry showed that enhanced green fluorescent protein-labeled cells were distributed in the area postrema and the nucleus tractus solitarius of both female and male transgenic rats, and a neural network of enhanced green fluorescent protein-positive fibers was seen between the area postrema and the nucleus tractus solitarius. The number of enhanced green fluorescent protein-labeled cells in the area postrema of female rats was significantly higher than in the males, but no significant difference was found in the number of enhanced green fluorescent protein-labeled cells in the nucleus tractus solitarius. The sex differences in the number of enhanced green fluorescent protein-labeled cells in the area postrema was not affected after ovariectomy or 17β -estradiol benzoate treatment in adult rats. Our results suggest that the effects of estrogen in the area postrema are related to the expression of estrogen receptor α under the control of the O/B promoter, and changes in the sex hormone environment in the adult period do not affect estrogen receptor α expression in the area postrema or the nucleus tractus solitarius.

Key Words

neural regeneration; basic research; estrogen receptor α promoter; green fluorescent protein; sex differences; area postrema; nucleus tractus solitarius; medulla; estrogen; rats; grant-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

- (1) Enhanced green fluorescent protein under the control of the estrogen receptor α O/B promoter was abundantly expressed in the area postrema and the nucleus tractus solitarius of transgenic rats.
- (2) The number of enhanced green fluorescent protein-labeled cells in the area postrema was significantly larger in the females than in the males, but the number of enhanced green fluorescent protein-positive cells in the nucleus tractus solitarius showed no sex difference.
- (3) The number of enhanced green fluorescent protein-labeled cells in the area postrema and the nucleus tractus solitarius was not affected by manipulation of the gonadal steroid milieu (ovariectomy or 17β -estradiol benzoate treatment) in the adult rat.

Chunxiao Zhang[☆], Ph.D.,
Associate professor.

Corresponding author:
Tomohiro Hamada,
Department of Physiology,
Nippon Medical School,
Tokyo 113-8602, Japan,
hamada@nms.ac.jp.

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INTRODUCTION

Estrogen is a pleiotropic hormone that plays an important role in the modulation of reproduction and is also involved in several nonreproductive functions, including neurotrophic and neuroprotective effects, and some age-related diseases in women^[1]. 17 β -estradiol has been used as a replacement hormone in postmenopausal women to achieve a wide range of health benefits, including cardiovascular protection against hypertension and stroke^[2-3], and cardiac repair after ischemic infarction^[4]. In a number of experimental models of hypertension, it has been shown that ovariectomy exacerbated, and estrogen replacement attenuated the course of hypertension^[5-6], and that the incidence of hypertension and vascular dysfunction was lower in females than in males in an adverse fetal environment^[7]. Recent studies have also shown that blood pressure is increased by chronic angiotensin II or aldosterone/NaCl more severely in female than in male mice and that ovariectomy exacerbates hypertension in females^[8-10]. Intracerebroventricular infusions of 17 β -estradiol inhibited the ovariectomy-induced increase in blood pressure, and the estrogen receptor antagonist ICI182780 blocked these effects^[8, 10]. Moreover, knockdown of the estrogen receptor gene increased the hypertensive effects of angiotensin II^[11], indicating that estrogen and the expression of estrogen receptor, especially estrogen receptor α , in the central nervous system play a significant role in the development of hypertension^[11].

Estrogen acts through its receptor on many aspects of brain function, including acetylcholinesterase activity, lipid peroxidation^[12] and expression of nitric oxide synthase^[13]. These cellular and molecular events occur in the whole central nervous system. Estrogen receptors in the brain include estrogen receptor α and estrogen receptor β , and estrogen receptor α is widely distributed in the brain. It is known that expression of estrogen receptor is controlled by several promoters^[14]; however, the tissue- or target-specificity of different estrogen receptor α gene promoters remains unknown.

The area postrema and the nucleus tractus solitarius are two important nuclei in the modulation of cardiovascular activity^[15-16] and are located in the medulla. The area postrema is one of the circumventricular organs, unique central structures that communicate information between circulating peptide hormones and the brain, and both estrogen receptor α and estrogen receptor β were shown to be highly expressed in the area postrema^[17-18]. The area

postrema is also known to send dense projections to the nearby dorsomedial and dorsolateral nucleus tractus solitarius^[19], which have been shown to express estrogen receptor^[17, 20].

To investigate the localization of estrogen receptor α in the brain, we examined the distribution of enhanced green fluorescent protein and the sex differences in enhanced green fluorescent protein distribution in the area postrema and the nucleus tractus solitarius using a transgenic rat expressing enhanced green fluorescent protein under the control of the estrogen receptor α O/B promoter^[21].

RESULTS

Quantitative analysis of experimental animals

A total of 20 transgenic rats expressing enhanced green fluorescent protein under the control of the estrogen receptor α O/B promoter were divided into male ($n = 5$) and female ($n = 15$) groups. The female rats were randomly assigned to normal ($n = 5$), ovariectomized (OVX, $n = 5$) and 17 β -estradiol benzoate-treated (OVX + EB, $n = 5$) subgroups. The OVX and OVX + EB groups were subjected to ovariectomy, and the 17 β -estradiol benzoate-treated rats were additionally subcutaneously implanted with a silastic capsule containing 17 β -estradiol benzoate. All rats were included in the final analysis.

Distribution of enhanced green fluorescent protein-expressing cells in the area postrema and the nucleus tractus solitarius

Many enhanced green fluorescent protein-expressing cells were found in the area postrema and the nucleus tractus solitarius of both male and female adult transgenic rats. Enhanced green fluorescent protein-labeled and -immunoreactive cell bodies and fibers were observed throughout the area postrema (Figure 1). Enhanced green fluorescent protein fluorescence and immunoreactivity in the area postrema tended to be higher than in the other areas in the medulla. In the nucleus tractus solitarius, enhanced green fluorescent protein-positive cell bodies showed a typical distribution along the anteroposterior axis. In the anterior part of the nucleus tractus solitarius, enhanced green fluorescent protein-positive cells were localized in the lateral nucleus tractus solitarius and enhanced green fluorescent protein-positive fibers were found running toward the area postrema (Figure 1). The distribution of the enhanced green fluorescent protein-positive cells

was shifted to the dorsomedial part and enhanced green fluorescent protein-positive fibers ran horizontally in the posterior part of the nucleus tractus solitarius (Figures 1, 2C, D). Furthermore, a number of enhanced green fluorescent protein-positive neurons projected fibers that crossed the border between the area postrema and the

nucleus tractus solitarius (Figures 2A, B), indicating that there was a neural network between the area postrema and the nucleus tractus solitarius and that estrogen receptor α -expressing neurons in the area postrema could regulate the functions of neurons in the nucleus tractus solitarius.

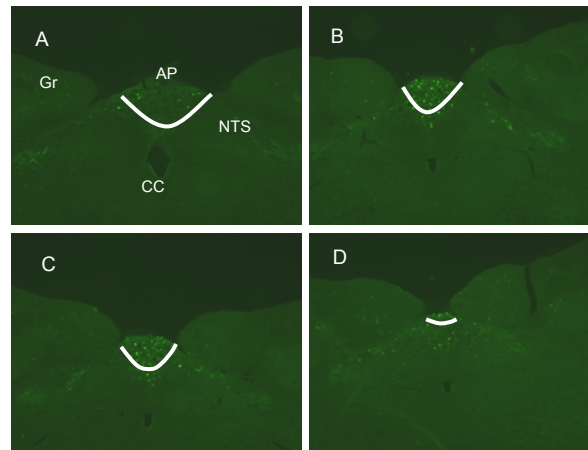


Figure 1 Anteroposterior pattern of EGFP expression in the AP and the NTS of a female transgenic rat (immunohistochemical staining, fluorescence microscope).

(A–D) EGFP-positive cells were distributed throughout the AP. At these levels, EGFP-positive cells were localized in the lateral to dorsomedial part of the NTS. Scale bar: 400 μ m. EGFP is shown in green. The white lines in A–D are borders between the AP and the NTS.

EGFP: Enhanced green fluorescent protein; AP: area postrema, CC: central canal, Gr: gracile nucleus, NTS: nucleus tractus solitarius.

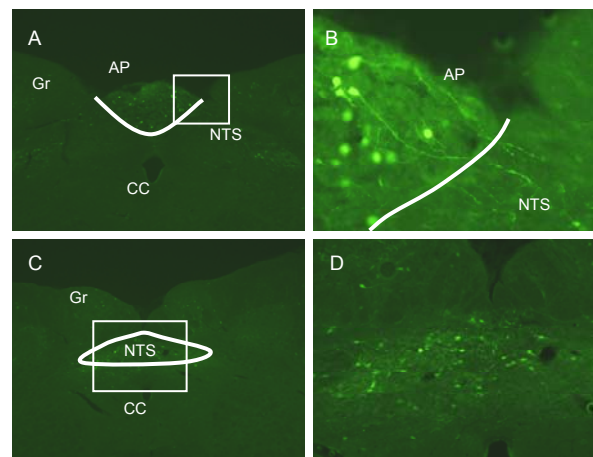


Figure 2 Neural network of the EGFP-labeled fibers between the AP and the NTS in a female transgenic rat (immunohistochemical staining, fluorescence microscope).

Scale bars: 400 μ m (A, C), 200 μ m (D) and 100 μ m (B), respectively. EGFP is shown in green.

(A) EGFP-positive fibers crossed the border (white line) between the AP and the NTS.

(B) Higher magnification of the box in A.

(C) EGFP-positive cells were localized in the dorsomedial part of the posterior NTS (white line circle).

(D) Higher magnification of the box in C showed that EGFP-positive fibers were found running horizontally in the posterior NTS.

EGFP: Enhanced green fluorescent protein; AP: area postrema; CC: central canal; Gr: gracile nucleus; NTS: nucleus tractus solitarius.

Sex differences in enhanced green fluorescent protein expression in the area postrema of the transgenic rats

The expression of enhanced green fluorescent protein in the area postrema exhibited sex differences in female and male transgenic rats. The expression of enhanced green fluorescent protein in the area postrema in female transgenic rats was significantly greater than in male transgenic rats ($P < 0.01$; Figure 3A), and that there was no sex difference in the expression of enhanced green fluorescent protein in the nucleus tractus solitarius ($P > 0.05$; Figure 3B). Manipulation of gonadal steroids, such as ovariectomy or 17 β -estradiol benzoate treatment, had no marked effect on the number of enhanced green fluorescent protein-positive cells in the area postrema and the nucleus tractus solitarius ($P > 0.05$; Figure 3), suggesting that the expression of enhanced green fluorescent protein, and thus of estrogen receptor α , in the area postrema and the nucleus tractus solitarius was not changed by manipulation of gonadal steroids in adulthood.

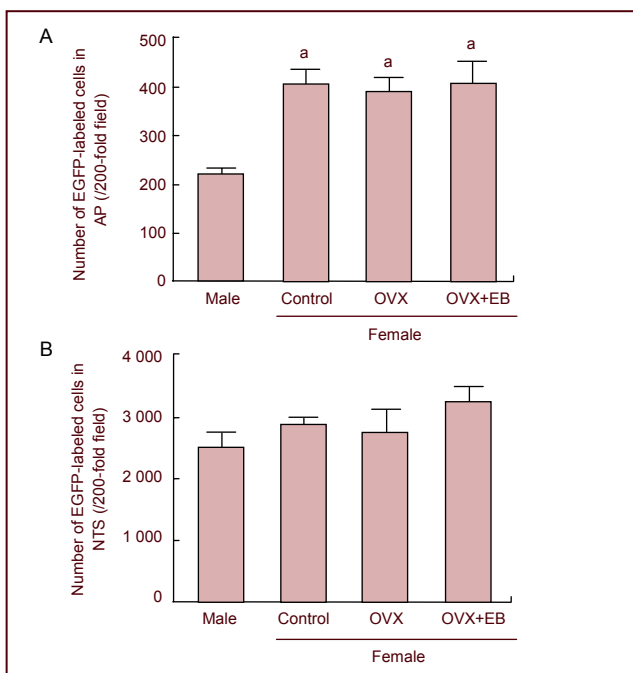


Figure 3 The number of EGFP-labeled cells in the AP and the NTS in male and female rats.

Data are presented as mean \pm SD. One-way analysis of variance was used to analyze sex differences and the effects of hormonal manipulation in the AP and the NTS.

(A) EGFP expression in the AP was greater in female rats than in male rats and was not affected by OVX or OVX + EB. $^aP < 0.01$, vs. male rats.

(B) There were no sex differences in the number of EGFP cells in the NTS in both sexes, and OVX or OVX + EB treatment had no effect on EGFP expression in the NTS.

EGFP: Enhanced green fluorescent protein; OVX: ovariectomy; EB: 17 β -estradiol benzoate; AP: area postrema; NTS: nucleus tractus solitarius.

DISCUSSION

Many studies have suggested that the response is different in male and female organisms when presented with physical or chemical factors. Brain function and behavior show sex differences in acute psychosocial stress and posttraumatic stress disorder^[22-23]. In addition, striatal dopamine D2/D3 receptor availability is reduced more severely in male smokers than in female smokers^[24], and there are also sex differences in angiotensin II^[8-9] and mineralocorticoid/NaCl-induced hypertension^[10]. These sex differences are related to sex hormones and their receptors.

A previous study reported that there are at least four promoters for the estrogen receptor α gene in the rat, but their tissue-specificity is unknown^[14]. In this study, we used transgenic rats expressing enhanced green fluorescent protein under the control of the estrogen receptor α O/B promoter to analyze the activity of the O/B promoter in the medulla. Results showed that intense enhanced green fluorescent protein fluorescence was specifically expressed within the area postrema and the nucleus tractus solitarius, which are strongly implicated in the regulation of cardiovascular activity^[15-16]. Therefore, we believe that the enhanced green fluorescent protein-positive cells in the area postrema and the nucleus tractus solitarius will be useful in investigations of the molecular mechanisms of the cardiovascular-related functions of estrogen and estrogen receptor α . Because sex differences in the development of hypertension are related to estrogen and its receptor in the brain^[5-10], we examined whether there were sex differences in the number of enhanced green fluorescent protein-positive cells in the area postrema and the nucleus tractus solitarius between the female and the male rats. Results showed that the number of enhanced green fluorescent protein-positive cells was larger in the area postrema in the female than in the male transgenic rats, but that there was no sex difference in the number of enhanced green fluorescent protein-positive cells in the nucleus tractus solitarius.

Despite the fact that estrogen receptor is known to be highly expressed in the area postrema^[17-18] and that its expression is densest in cardiovascular-related brain nuclei^[18], sex differences in estrogen receptor expression in these areas have not yet been reported. This is the first finding of sex differences in estrogen receptor α expression in the area postrema, demonstrated by enhanced green fluorescent protein expression. In

addition, sex differences have been found in angiotensin-II^[8-9] and mineralocorticoid/NaCl-induced hypertension^[10], and central estrogen receptor α plays a key role in the process of hypertension^[11, 25]. However, little is known about molecular mechanisms mediated through any estrogen receptor α gene promoter. Angiotensin II is known to centrally modulate baroreflex function *via* actions at the level of the area postrema^[15, 25-26]. We thus hypothesized that the sex differences in hypertension and the typical antihypertensive effects of estrogen were mediated, at least in part, through estrogen receptor α expressed under the control of the O/B promoter.

In vertebrates, there are sex differences in several regions in the central nervous system^[27], and the sex differences in each region can be established in either the perinatal period or in adulthood. For example, sex differences in the expression of the somatostatin gene in the medial preoptic nucleus depend on the gonadal hormonal environment in the perinatal period, and the expression of the gene in females is masculinized by estrogen but not altered by castration in adulthood^[28]. The volume of the posterodorsal medial amygdala, however, depends on adult testosterone^[29]. In the present study, manipulation of gonadal steroids, such as ovariectomy or 17 β -estradiol benzoate treatment, had no effect on the sex differences in enhanced green fluorescent protein expression in the area postrema in adulthood. These results suggest that the sex differences in the enhanced green fluorescent protein expression in the area postrema were probably established during the perinatal period. While 17 β -estradiol benzoate treatment of OVX rats resulted in down-regulation of O/B promoter-driven enhanced green fluorescent protein expression in the preoptic area-bed nucleus of the stria terminalis continuum, expression did not change in the subgranular zone of the dentate gyrus^[30]. This involves the site-specific and developmentally regulated use of alternative promoters of the estrogen receptor α gene^[31].

In conclusion, estrogen receptor α expression was regulated by the O/B promoter occurred in the area postrema and the nucleus tractus solitarius in the medulla, and sex differences were found in the area postrema. These results should be useful for studying estrogen receptor α promoter-mediated signaling in the area postrema and the nucleus tractus solitarius, two areas involved in the regulation of cardiovascular activity. Further studies should be conducted using the enhanced green fluorescent protein-expressing area postrema and nucleus tractus solitarius neurons to understand the

mechanisms of the effects of estrogen on cardiovascular functions.

MATERIALS AND METHODS

Design

A randomized, controlled, animal study.

Time and setting

The experiments were performed at the Department of Physiology, Nippon Medical School, Japan from May to November 2007.

Materials

Transgenic rats expressing enhanced green fluorescent protein under the control of the estrogen receptor α O/B promoter were provided by the Laboratory Animal Center of Nippon Medical School, Japan. The transgenic rats were generated as follows. Briefly, a plasmid containing the estrogen receptor α O/B promoter sequence was amplified and *SacI/AflI* fragments of the plasmid were injected into the pronucleus of fertilized oocytes of Wistar rats and the rats were identified by southern blot analysis of tail DNA using ³²P-labeled probes^[21]. We used five adult male and 15 female transgenic rats, aged 8 weeks or 4 weeks (for manipulation of the gonadal steroid milieu, below). The rats were allowed free access to water and food and were housed under a 14 hour light/10 hour dark cycle at 23°C.

Methods

Manipulation of the gonadal steroid milieu

Female transgenic rats (4 weeks old, $n = 5$) were anesthetized, ovariectomized and subcutaneously implanted with a 5-mm silastic capsule (inner diameter, 1.47 mm; outer diameter, 1.96 mm; Dow Corning, Midland, Michigan, USA) containing 17 β -estradiol benzoate (Sigma, St Louis, MO, USA)^[32]. These 17 β -estradiol benzoate capsules have been reported to maintain the serum estradiol titer within the proestrus range^[32]. Female rats without 17 β -estradiol benzoate treatment ($n = 5$) were also anesthetized and ovariectomized at 4 weeks of age.

Sampling method

The distribution patterns of the fluorescent cells were determined in the area postrema and the nucleus tractus solitarius of male and female transgenic rats at 8 weeks of age ($n = 5$). All animals were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused through the heart with ice-cold 1% heparinized

saline, followed by ice-cold 4% paraformaldehyde in 0.1 M PBS (pH 7.3). Brains were removed and postfixed in the same fixative at 4°C overnight and then transferred into 30% sucrose (Sigma) in 0.1 M PBS until they settled. According to the rat atlas by Paxinos and Watson^[33], coronal sections (30- μ m thick) containing the area postrema and the nucleus tractus solitarius were cut on a freezing microtome. All sections were collected and stored at 4°C in 0.1 M PBS.

Detection of enhanced green fluorescent protein in the area postrema and the nucleus tractus solitarius by immunohistochemistry

All sections containing the area postrema and the nucleus tractus solitarius were subjected to immunohistochemical staining with an antibody against enhanced green fluorescent protein to avoid the attenuation of enhanced green fluorescent protein fluorescence during observation. Briefly, free-floating sections were rinsed with 0.05 M PBS and then incubated in 3% H₂O₂/methanol to remove endogenous peroxidases. After further washes in PBS, sections were incubated with 1.5% normal goat serum (Molecular Probes, Eugene, OR, USA) in PBST (0.05 M PBS/0.05% TritonX-100) for 30 minutes at room temperature. Sections were then reacted with rabbit anti-green fluorescent protein polyclonal antibody (1:1 000; Molecular Probes) for 60 hours at 4°C, followed by visualization of the immunolabeling using an Alexa 488-conjugated anti-rabbit IgG (1:800; Molecular Probes). The sections were then mounted, dehydrated and coverslipped with Vecta Mount (Vector Laboratories, Burlingame, CA, USA). The number of enhanced green fluorescent protein-labeled and -immunoreactive cells in the area postrema and the nucleus tractus solitarius was determined under an Olympus BX50 fluorescence microscope (Tokyo, Japan) with a digital camera system (Nikon, Tokyo, Japan).

Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and presented as mean \pm SD. One-way analysis of variance was used to analyze the sex differences and the effects of hormonal manipulation on the nucleus tractus solitarius and the area postrema. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Chunxiao Zhang designed the study, collected data, and wrote the manuscript. Tomohiro Hamada participated in study guidance, statistical processing and manuscript authorization. All authors approved the final version of the manuscript.

Ethical approval: The housing and use of the animals were supervised and approved by the Nippon Medical School Institutional Animal Care and Use Committee.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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