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Sex differences in cells expressing green fluorescent protein under the control of the estrogen receptor-α promoter in the hypothalamus of mice

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ABSTRACT

Estradiol that originates from testicular testosterone and binds to estrogen receptor-α (ERα) during developing period acts to organize the male-type brain in mice. Here, we examined transgenic mice expressing green fluorescent protein (GFP) under the control of the ERα promoter, in which ERα-expressing cells in the brain can be visualized by GFP. Fluorescence microscopy revealed the existence of many GFP-expressing cells in the medial preoptic area, medial preoptic nucleus (MPN), bed nucleus of the stria terminalis (BNST), and striohypothalamic nucleus (StHy) of adult transgenic mice. Neuronal nuclear antigen, a neuron marker, but not glial fibrillary acidic protein, an astrocyte marker, was mostly expressed by GFP-expressing cells. Analysis of GFP expression area showed that adult females had higher GFP expression in a region including the ventral part of the BNST, StHy, and dorsal part of the MPN than in adult males. Such female-biased sex difference was also found in transgenic pups on postnatal day 5 and 8. The GFP expression area of adult females was decreased by postnatal treatment with testosterone or estradiol. These results indicate that GFP visualizes a sex difference of ERα-expressing neurons. The transgenic mice may be useful for the analysis of the sexual differentiation of the brain.

1. Introduction

Estradiol is a sex steroid that modulates brain function by binding with its receptor. Two types of nuclear receptors for estrogens were identified and termed estrogen receptor-α and -β (ERα and ERβ). Both ERα and ERβ are widely distributed in the brain, but have differential expression patterns (Mitra et al., 2003; Merchenthaler et al., 2004; Perez et al., 2003), suggesting differing roles in the brain. In fact, defects in the ERα gene induce infertility and abnormalities in sexual, parental, and aggressive behaviors in male and female mice (Ogawa et al., 1996, 1997, 1998a,b; Korach et al., 1996; Lubahn et al., 1993), while ERβ knockout mice are fertile and display sexual behavior in both sexes (Ogawa et al., 1999; Krege et al., 1998). However, aggressive behavior in male mice is increased by deletion of the ERβ gene (Ogawa et al., 1999; Nomura et al., 2006).

In rodents, estradiol, which is locally synthesized in the brain from testosterone by aromatase during the perinatal period, has masculinizing and defeminizing effects on the brain (MacLusky and Naftolin, 1981; MacLusky et al., 1979; McEwen et al., 1977). The sexually differentiated brain contains nuclei exhibiting morphological sex differences that are termed sexually dimorphic nuclei (SDNs). The principal nucleus of the bed nucleus of the stria terminalis (BNSTp) is a male-biased SDN having a larger volume and containing many more neurons in males of mice and rats (Gilmore et al., 2012; del Abril et al., 1987; Hines et al., 1992). In contrast, the anteroventral periventricular nucleus (AVPV) is a female-biased SDN that has a larger volume and number of cells in female rodents than in males (Bleier et al., 1982; Simerly et al., 1985). Almost all brain regions including SDNs express ERα in mice and rats (Merchenthaler et al., 2004; Perez et al., 2003; Chakraborty et al., 2003). In addition, there may be a sex difference in the expression of ERα in SDNs, because ERα expression of the BNSTp and AVPV is higher in female mice and rats than in males (Brock et al., 2015; Cao and Patisaul, 2011; Kelly et al., 2013). Thus, neurons that express ERα are key components of SDNs in the rodent brain. The

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morphological sex differences in the BNSTp and AVPV can be eliminated and reversed in rats and mice by castration in newborn males and treatment with testosterone or estradiol in postnatal females (Hisasue et al., 2010; Murakami and Arai, 1989). We previously revealed that masculinization of the BNSTp and defeminization of the AVPV in male mice are disrupted by deletion of aromatase and ERα genes, but not the ERβ gene (Tsukahara et al., 2011; Kanaya et al., 2014). These findings indicate that aromatized testosterone binding to ERα during the postnatal period plays an essential role in the formation of male-type SDNs. However, there are reports showing the involvement of ERβ in SDN formation (Hisasue et al., 2010; Bodo et al., 2006).

Recently, transgenic (tg) mice expressing green fluorescent protein (GFP) under the control of the ERα promoter (ERα-GFP tg mice) were generated (Matsuda et al., 2013). In ERα-GFP tg mice, ERα-expressing cells can be visualized by GFP. This characteristic of the tg mice may be useful for the analysis of the mechanisms responsible for SDN formation by monitoring ERα-expressing cells with GFP. In the current study, we examined the preoptic-anterior hypothalamus, where ERα is abundantly expressed, of adult and postnatal ERα-GFP tg mice to determine whether sex differences in ERα-expressing cells can be visualized by GFP.

2. Materials and methods

2.1. Animals

Male and female heterozygous ERα-GFP tg mice used in this study were obtained from litters resulting from mating between male heterozygous ERα-GFP tg mice and female wild-type mice. Genotypes of offspring were determined by polymerase chain reaction amplification of tail DNA using a specific primer set for the enhanced GFP gene (forward primer: 5′-CGACGTAAACGGCCACAAGT-3′; reverse primer: 5′-GATGTTGCCGTCCTCCTTGA-3′). All animals were housed in a room maintained at 22 °C with a 12 h light/12 h dark cycle with free access to a standard diet and tap water. Animal procedures were conducted according to the Guidelines for the Care and Use of Experimental Animals of Saitama University.

2.2. Experimental design

2.2.1. Analysis of GFP expression in the preoptic-anterior hypothalamus of adult ERα-GFP tg mice

Gonadally intact adult male and female ERα-GFP tg mice (8–10 weeks old) were histologically processed to obtain coronal brain sections. Estrous cycles of female mice were checked by vaginal smear, and were sacrificed on proestrous day. The brain sections of adult male ($n = 7$) and female ($n = 6$) ERα-GFP tg mice were used for fluorescence microscopy of GFP to observe GFP-expressing cells in the preoptic-anterior hypothalamus. In addition, using the brain sections of 5 of 7 males and 4 of 6 females, we performed a screening analysis of GFP expression area in the preoptic-anterior hypothalamus to determine whether it differs between sexes. In this study, we divided the preoptic-anterior hypothalamus into six divisions (Fig. 1A; see Section 2.5 for details). The area of GFP expression in each division was then measured.

The brain sections obtained from adult male and female ERα-GFP tg mice were subjected to fluorescence immunohistochemistry for neuronal nuclear antigen (NeuN), a neuron marker, and glial fibrillary acidic protein (GFAP), an astrocyte marker ($n = 3$ of each sex for each target). The immunostained brain sections were observed under a fluorescence microscope to determine the cell type of GFP-expressing cells in the brain. Regarding NeuN, we counted the numbers of GFP-expressing cells with or without its immunoreactivity and then calculated the percentage of NeuN-immunoreactive cells in GFP-expressing cells.

2.2.2. Effects of postnatal sex steroids on GFP expression in the preoptic-anterior hypothalamus of adult ERα-GFP tg mice

ERα-GFP tg female pups were treated with a single subcutaneous injection of testosterone propionate (TP; 100 μg in 0.02 ml sesame oil, $n = 4$) or dihydrotestosterone (DHT; 100 μg in 0.02 ml sesame oil, $n = 3$) on PD1, or estradiol benzoate (EB; 2 μg in 0.02 ml sesame oil per day, $n = 5$) on 5 consecutive days from PD1 to PD5. Female pups ($n = 5$) were injected with sesame oil during PD1–5 (0.02 ml per day) to serve as controls. Eight to ten weeks after birth, all animals were sacrificed for histological processing to obtain control brain sections. The brain sections were observed under a fluorescence microscope, and the area of GFP expression in the preoptic-anterior hypothalamus was measured after it was divided into six divisions (Fig. 1A; see Section 2.5 for details) to determine the effects of postnatal treatment with sex steroids.

2.2.3. Analysis of GFP expression in the preoptic-anterior hypothalamus of postnatal ERα-GFP tg mice

Postnatal ERα-GFP tg mice were histologically processed on postnatal day (PD) 2 (male, $n = 3$; female, $n = 4$), PD5 (male, $n = 4$; female, $n = 5$), and PD8 (male, $n = 4$; female, $n = 6$) to obtain coronal brain sections. The day of birth was defined as PD1. The brain sections were observed under a fluorescence microscope. In addition, as well as in adult ERα-GFP tg mice, the area of GFP expression in the preoptic-anterior hypothalamus of PD5 and PD8 mice was measured to examine sex differences. The preoptic-anterior hypothalamus was divided into six divisions (Fig. 1B; see Section 2.5 for details), and the area of GFP expression in each division was measured.

2.3. Tissue preparation

Adult mice were anesthetized by intraperitoneal injection with sodium pentobarbital (60 mg/kg body weight). They were perfused with 0.05 M phosphate-buffered saline (PBS; pH 7.5), followed by perfusion fixation with 4% (w/v) paraformaldehyde in 0.05 M phosphate buffer (PB; pH 7.5). Brains were postfixed with the same fixative at 4 °C overnight and placed in 30% (w/v) sucrose-0.05 M PB at 4 °C for 2 days. Thirty-micrometer-thick coronal brain sections were made using a cryostat. Serially cut brain sections were collected at 60 μm intervals and used for fluorescence microscopy with or without immunohistochemistry of NeuN or GFAP. To determine the location of GFP-expressing cells, the brain sections obtained from some adult tg mice were subjected to 4′,6-diamidino-2-phenylindole (DAPI) staining by using DAPI Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL, USA).

Postnatal pups were decapitated to obtain brain samples. The brains were immersed in 4% (w/v) paraformaldehyde in 0.05 M PB at 4 °C overnight. The fixed brains were placed in 0.05 M PBS at 4 °C overnight, followed by immersion in 30% (w/v) sucrose-0.05 M PB at 4 °C for 1–2 days. Brains were coronally sectioned at a thickness of 50 μm using a cryostat. Serially cut brain sections were mounted on gelatin-coated glass slides and used for florescence microscopy.

2.4. Fluorescence immunohistochemistry of NeuN and GFAP

Brain sections were rinsed in 0.05 M PBS containing 1% (v/v) Triton X-100 (PBST) and treated with 5% (v/v) normal goat serum (NGS) in PBST for 1 h at room temperature. For fluorescence immunohistochemistry of NeuN, the sections were then reacted with mouse anti-NeuN antibody (1:1000, MAB377; Chemicon)

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