Neuroscience Letters 594 (2015) 127–132

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Effect of sex steroid hormones on the number of serotonergic neurons in rat dorsal raphe nucleus

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HIGHLIGHTS

- In adult rat brain, sex steroid hormones do not affect serotonin neuron number.
- These hormones do not affect tryptophan hydroxylase-immunoreactive cell number.
- Serotonin-immunoreactive signal intensity in dorsal raphe is sexually dimorphic.
- This subregion-specific sexual dimorphism is independent of sex hormone levels.

ARTICLE INFO

Article history:
Received 27 January 2015
Received in revised form 6 March 2015
Accepted 26 March 2015
Available online 28 March 2015

Keywords:
5-Hydroxytryptamine
Sex steroid hormone
Tryptophan hydroxylase
Sex difference
Serotonergic system

ABSTRACT

Disorders caused by the malfunction of the serotonergic system in the central nervous system show sex-specific prevalence. Many studies have reported a relationship between sex steroid hormones and the brain serotonergic system; however, the interaction between sex steroid hormones and the number of brain neurons expressing serotonin has not yet been elucidated. In the present study, we determined whether sex steroid hormones altered the number of serotonergic neurons in the dorsal raphe nucleus (DR) of adult rat brains. Animals were divided into five groups: ovariectomized (OVX), OVX + low estradiol (E2), OVX + high E2, castrated males, and intact males. Antibodies against 5-hydroxytryptamine (5-HT, serotonin) and tryptophan hydroxylase (Tph), an enzyme for 5-HT synthesis, were used as markers of 5-HT neurons, and the number of 5-HT-immunoreactive (ir) or Tph-ir cells was counted. We detected no significant differences in the number of 5-HT-ir or Tph-ir cells in the DR among the five groups. By contrast, the intensity of 5-HT-ir showed significant sex differences in specific subregions of the DR independent of sex steroid levels, suggesting that the manipulation of sex steroid hormones after maturation does not affect the number and intensive immunostaining of serotonergic neurons in rat brain. Our results suggest that, the sexual dimorphism observed in the serotonergic system is due to factors such as 5-HT synthesis, transportation, and degradation but not to the number of serotonergic neurons.

1. Introduction

Several disorders, such as migraine, depression, and eating disorders, show a sex-specific prevalence. For example, migraines are three times more prevalent in women than in men [1], and women are twice as likely as men to have depression [2]. Ninety percent of eating disorders develop in women [3]. A malfunction of the serotonergic system in the central nervous system has been considered an etiology of these diseases [4–6]. The serotonergic system shows sex differences in mammals. For example, in female rats, serotonin (5-hydroxytryptamine, 5-HT) levels are significantly higher in the brainstem and limbic forebrain than those in male rats, and 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT, also shows significantly higher levels in the several regions of female brain [7]. Conversely, the rate of 5-HT synthesis in women is 52% lower than that in men [8]. In addition, the binding capacity of 5-HT_2 receptors for 5-HT is higher in men than it is in women [9]. These data suggest a relationship between sex steroid hormones and the serotonergic system.

There is considerable evidence for the involvement of sex steroid hormones in the serotonergic system. Estrogen increases the mRNA expression of tryptophan hydroxylase (Tph)-2, which contributes to 5-HT synthesis, in certain subregions of rat median and dorsal raphe nucleus (DR) through the estrogen receptor-β (ERβ) [10,11]. Androgen and estrogen increase 5-HT_{2A} receptor mRNA and the density of ligand-5-HT_{2A} receptor binding sites in both

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http://dx.doi.org/10.1016/j.neulet.2015.03.060
0304-3940/

female and male rat brains [12]. In addition, DR serotonergic neurons express ERβ in rats, mice, and monkeys [13–15] while, ERα expression in serotonergic neurons has been found in mice [14]. Progesterone receptor (PR) expression in serotonergic neurons has been detected in mice and monkeys [16,17]. Androgen receptors have been observed in the DR of male rats and mice; however, most of these receptors are found in non-5-HT-immunoreactive (ir) cells [14]. Although there are some species differences, this evidence strongly supports the hypothesis that sex steroid hormones modify the brain serotonergic system. Additional evidence that 5-HT neurons are regulated by sex steroid hormones is that, the involvement of 5-HT neurons in the midbrain DR has been reported in the regulation of reproductive activities, such as sexual behavior, ovulation, and pregnancy [18–20]. Thus, numerous studies have examined the relationship between the brain serotonergic system and sex steroid hormones; however, there is no clear evidence for an effect of sex steroid hormones on the number of serotonergic neurons. Because it has been reported that ovariectomy decreased serotonin neuronal number and gene expression in female macaques, it is possible that sex steroid hormones alter the number of serotonergic neurons [21]. Therefore, the aim of this study was to determine whether manipulation of sex steroid hormones alter the number of serotonergic neurons in rats.

2. Materials and methods

2.1. Animals and treatments

Adult female and male Wistar rats (200–300 g body weight) were purchased from Kiwa Laboratory Animals Co. Ltd. (Wakayama, Japan) and housed under controlled temperature (24 ± 2 °C) and lighting (lights on from 06:00 to 20:00) conditions with ad libitum access to food and water. The estrous cycle was monitored using daily vaginal smears, and animals showing at least two consecutive 4-day cycles were used. Animals were divided into five groups. [1] OVX group: female rats were bilaterally ovariectomized (OVX) 2 weeks before perfusion. [2] Low estradiol-17β (E2) group: OVX animals immediately received subcutaneously (s.c.) with Silastic tubing (1.57 mm inner diameter; 3.18 mm outer diameter; 37.0 mm in length; Dow Corning, Midland, MI), filled with E2 (Sigma–Aldrich, St. Louis, MO) dissolved in sesame oil at 20 μg/ml. In 37.0 mm of overall Silastic tubing length, the length filled with E2 oil was 25.0 mm, and the length of 6.00 mm at both ends were filled with silicone paste (Shin–Etsu Polymer, Tokyo, Japan). This low E2 level produces a negative-feedback level of plasma E2 for 1 week [22]. [3] High E2 group: OVX animals immediately received s.c. with Silastic tubing (1.02 mm inner diameter; 2.16 mm outer diameter; 32.0 mm in length; Dow Corning) filled with crystalline E2 to produce a positive-feedback level of plasma E2 for 3 days [23]. In 32.0 mm of overall Silastic tubing length, the length filled with crystalline E2 was 20.0 mm, and the length of 6.00 mm at both ends were filled with silicone paste. This high E2 level induces daily luteinizing hormone surges in OVX rats. [4] Castration (Cast) group: male rats were bilaterally gonadectomized 2 weeks before perfusion. [5] Intact male group. All surgical procedures were performed under isoflurane anesthesia. All studies were conducted according to the National Institutes of Health's (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Experimentation Committee, Nippon Medical School.

2.2. Tissue preparation

After each treatment was completed, all animals were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Animals were perfused transcardially with saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed, postfixed in the same fixative solution at 4 °C overnight, and cryoprotected in 30% sucrose in PB at 4 °C for 4 days. Coronal sections (40 μm) through the midbrain were cut on a sliding microtome (Leica CM3050, Heidelberg, Germany) and stored in cryoprotectant solution at −25 °C until immunohistochemistry was conducted.

2.3. Immunohistochemistry

Sections were washed in 0.1 M PB containing 0.9% NaCl and 0.3% Triton X-100 (PBST) and incubated with PBST containing 10% normal rabbit serum at room temperature (RT) for 1.5 h. Sections were incubated with a goat anti-5-HT antibody (Immunostar, Hudson, WI) diluted 1:5000 in PBST overnight at 4 °C, or with a monoclonal mouse anti-Tph antibody (Sigma–Aldrich) diluted 1:1000 in PBST for 5 days at 4 °C. Sections were incubated with a biotinylated secondary antibody followed by streptavidin conjugated with horseradish peroxidase (HISTOFINE SAB-PO kit, Nichirei Biosciences Inc., Tokyo, Japan) diluted 1:1 in PBST for 2 h at RT each. After each step, sections were washed in PBST. Staining was developed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma–Aldrich) with 0.009% hydrogen peroxide. Sections mounted on glass slides were dehydrated with a graded ethanol series, immersed in xylene, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

For double fluorescence immunohistochemistry, sections were washed in PBST and incubated with PBST containing 10% normal donkey serum at RT for 1.5 h. Sections were incubated overnight with a monoclonal mouse anti-Tph antibody (Sigma–Aldrich) diluted 1:500 in PBST at 4 °C, followed by the goat anti-serotonin antibody (1:500) for 4 days at 4 °C. Sections were incubated with an Alexa Fluor 488-conjugated donkey anti-mouse IgG antibody (1:500, Invitrogen, Carlsbad, CA) and an Alexa Fluor 568-conjugated donkey anti-goat IgG antibody (1:500, Invitrogen) in PBST for 2 h at RT. After each step, sections were washed in PBST. Thereafter, sections were mounted with VECTASHIELD (Vector Laboratories, Burlingame, CA) on glass slides.

2.4. Image analysis

The brain sections containing 5-HT or Tph immunoreactivity were examined under a light microscope (BX-50, Olympus, Tokyo, Japan). The number of DR 5-HT-immunoreactive (ir) cells in 10 sections, between bregma −7.32 and −8.28 mm, and the number of Tph-ir neurons expressed in the section corresponding to bregma −8.40 mm [24], were counted for each rat on a computer display using NIH ImageJ software with the cell counter plug-in (version 10.2).

The signal intensity of each DR 5-HT-ir neuron in three sections was verified using photographs. The three sections were collected from the DR, rostral (ROSTRAL), mid (MID), and caudal (CAUDAL), at bregma −7.32, −7.80, and −8.28 mm, respectively [24]. Images were inverted using ImageJ. Since 5-HT immunoreactivity was heterogeneous among subregions in the DR, the DR was divided into three subregions, lateral, dorsal, and ventral regions, and 5-HT immunoreactivity was verified at each subregion of each anatomical level (Fig. 2A–C) [10]. From the ROSTRAL and MID sections, 10 neurons were randomly selected from each subregion. From the CAUDAL sections, 4, 10, and 10 neurons were randomly chosen from the lateral, the dorsal, and the ventral subregions, respectively. The mean gray value of the selected 5-HT-ir neurons was obtained with ImageJ, and was divided by the mean gray value of the background. The number obtained from this calculation was used for statistical analysis.