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Sexually dimorphic effects of estrogen on spines in cultures of accessory olfactory bulb

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ABSTRACT

A sex difference has been reported in the responsiveness of the vomeronasal (VN) system to pheromones. In the present study, to clarify a direct and acute influence of 17β-estradiol (E2) on the accessory olfactory bulb (AOB) neurons, we investigated the effect of E2 on dendritic spines in cultured AOB cells derived from male and female neonatal rats. After 17–18 days in vitro (DIV), cultured AOB cells were transfected with GFP expression vectors. At 21–23 DIV, cells were treated with E2, and time-lapse images of transfected AOB neurons identified as granule cells were taken under a confocal laser scanning microscope for 3 h. The dendritic spine head area of granule cells was quantitatively evaluated, and spine heads were classified into larger ($\geq$1 μm^2) and smaller (<1 μm^2) ones before E2-treatment (0 h). In cultured cells derived from both sexes, the larger spines were not significantly changed at 1, 2 and 3 h after E2-treatment. In contrast, E2-treatment significantly enlarged the head area of the smaller spines of granule cells derived from the female, whereas E2 did not cause any significant effects on those from the male. Our results provide evidence for the sexually-dimorphic effect of E2 on spine development in AOB granule cells.

1. Introduction

The accessory olfactory bulb (AOB) is one of the constitutes of the vomeronasal (VN) system and plays a critical role in the perception and processing of pheromonal signals. Sensory neurons in the VN organ (VNO) project to the AOB, which in turn projects to the bed nucleus of stria terminalis (BST) and the vomeronasal amygdala [17,19,30,35]. Neurons in those areas then project to various hypothalamic nuclei and the preoptic area (POA) to regulate hormone secretions and sociosexual behaviors [5,7,23].

It has been indicated that circulating sex steroids strongly affect responses to pheromones through modulating the processing of pheromonal information in the VN system. Especially, E2 is one of the sex steroids known with potent influence on the VN system [2,3,10,11]. Halem et al. [10] have demonstrated that E2 affects the activity of VNO neurons. They showed that E2-treatment significantly increased Fos-immunoreactive neurons in the VNO after exposure to soiled male bedding. Sulfated steroids, present in mouse urine, were recently found to activate VNO neurons [27]. Meeks et al. [21] have showed that 12 sulfated steroids, including sulfated androgens and estrogens, directly cause widespread changes in the activity of VNO neurons via vomeronasal receptors (VRs) and each VNO neuron expresses one or more VRs whose expressions are regulated by sex steroids including E2 [1]. Furthermore, the activity of AOB neurons and its downstream neurons is known to be influenced by circulating steroids. For example, ovariectomized female rats treated with E2 showed increases in neuronal Fos-immunoreactivity in the AOB, vomeronasal amygdale, BST and medial POA after exposure to soiled bedding from sexually active males, compared to those in ovariectomized animals [2,3,10,11]. Taken together, these findings suggest that information of E2 can be transmitted to AOB neurons, change their activities via projections of VNO neurons, and then transmitted to the downstream neurons. On the other hand, E2 is also indicated to affect the AOB through centrifugal feedback projections, since the AOB receives considerable feedback projections from the amygdala and BST that richly express estrogen receptors (ERs) [25]. Fan and Luo [8] recently showed that ERs are expressed in a substantial number of neurons in the amygdala and BST projecting to the AOB. These results suggested that central hormonal cues may modulate the processing of pheromonal signals at the AOB through

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feedback projections. Therefore, the AOB receives information of E2 at least via two projections; one from the VNO and the other from the central area such as the amygdale and BST.

However, it remains still unsolved whether E2 affects AOB neurons directly. In the present study, we aimed to examine direct effects of E2 on AOB neurons. The AOB, which has a distinct three laminar structure of the glomerular layer (GL), the mitral cell layer (MCL) and the granule cell layer (GCL), consists mainly of three morphologically discernible neuronal types: mitral/tufted (MT) cells, granule cells and periglomerular cells. MT cells are the principal neurons of the AOB, which receive fibers from the VN neurons and transmit pheromonal signals to the amygdala [4,12,13]. Granule cells are axonless GABAergic neurons, processing reciprocally synaptic input and output. Dendritic spines of granule cells receive excitatory inputs from dendrites of MT cells via glutamate release, and then provide a feedback inhibition to MT cells via GABA release [33]. Since spinodendritic synapses of granule cells have been suggested to be important for pheromonal processing [13,20], we focused on the effect of E2 on dendritic spines of granule cells in the present study. According to Dominguez-Salazar et al. [6], the increase in Fos-immunoreactive cells in the GL of the AOB were already detected at 90 min after E2-treatment. Therefore, in order to examine the direct effect of E2 on dendritic spines of granule cells, changes in spine structures during the early phase (up to 3 h) after E2-treatment were followed and quantitatively analyzed in cultured AOB neurons transfected with GFP-expressing plasmids.

2. Materials and methods

Primary culture of AOB cells was prepared according to a previously described procedure with some modifications [14,24]. In brief, the AOB region was removed from neonatal (<24-h-old) Wistar rats (P0). All animals were treated according to the Guidelines for the Care and Use of Animals of Tokyo Metropolitan Institute for Neuroscience. We totally used 129 and 165 neonatal males and females, respectively. The AOB dissected from males and females was separately dissociated with papain (Wortington Biochemical Corp., Freehold, NJ) and triturated through a pipette. Dissociated cells were then suspended in a serum culture medium that consisted of Dulbecco's modified Eagles's medium (DMEM) and F-12 (1:1) (DMEM/F12, Gibco, Invitrogen, Carlsbad, CA) supplemented with 5% newborn calf serum (NBCS), 5% horse serum and 50 U/ml penicillin G (Meiji Seika, Tokyo, Japan). The resulting cell suspension was plated at a density of 6.0×10^5 cells/cm^2 on φ12 mm glass bottom dishes (Matsunami Glass Ind, Osaka, Japan) coated with polyethylenimine (Sigma–Aldrich, St. Louis, MO). We obtained totally 43 and 55 dishes for male and female cells, respectively. Cultures were maintained in 5% CO_2 at 37 °C. After the cells were cultured for 2 h, the medium was changed to the normal medium [DMEM/F12 (1:1) supplemented with 0.1 M L-ascorbic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan), 0.025 M 2-mercaptoethanol (Sigma–Aldrich), 50 U/ml penicillin G and 2% B27 serum-free additive (Gibco)]. The cell cultures were maintained for 21–23 days in an incubator with 5% CO_2 humidified atmosphere at 37 °C. Half the culture medium was changed to a fresh one once a week.

The pCAGGS plasmid [26] was kindly provided by Professor Jun-ichi Miyazaki (Osaka University). A GFP fragment was made from pAcGFP (Clontech, Takara Bio USA, Mountain View, CA) by PCR, which was conjugated with MunI sites at the 5′ end (before the Kozak sequence) and at the 3′ end (after the stop codon). To create pCAGGFP, the fragment was digested by *MunI*, and was subsequently recombined into EcoRI-digested pCAGGS. The direction of GFP-insertion was checked by DNA sequence (Applied Biosystems 3130). The AOB neurons were then transiently transfected with GFP expression vectors between 17 and 18 DIV using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfected neurons were observed under a confocal laser microscope (TCS SP5, Leica) at 21–23 DIV.

At 21–23 DIV, 100 nM E2 (Wako, Japan) or solvent ethanol was added to the medium. E2 was first dissolved in ethanol to a concentration of 10 mM, and then diluted with the normal medium to the final concentration. The solvent (ethanol) was added to control cultures at the corresponding concentration. The movement of spines of GFP-transfected neurons was visualized by time-lapse photography. The digital microscopic images were first captured just before the treatment (0 h), and thereafter images were acquired at regular intervals (at 1, 2 and 3 h after the treatment), using a confocal laser scanning microscope (TCS SP5, Leica). Three-dimensional images were converted into two-dimensional images, and then subjected to the measurement of the area of spine heads with a computer-assisted image analysis system (TCS SP5, Leica). The area of spine heads was measured on the two-dimensional images by manually tracing borders of spine heads with a light pen. Culture series consist of four cell groups: control female cell group, E2-treated female cell group, control male cell group and E2-treated male cell group. Statistical comparisons of changes in the spine head area among cell groups were carried out using a one-way analysis of variance (ANOVA) followed by Scheffe's test.

3. Results

Similar to our previous study [14], GFP-transfected neurons were largely classified into two types (Fig. 1). One type was a projection neuron (MT cell), which had a large soma (more than 100 μm^2) and thick multipolar dendrites (A in Fig. 1). The other was a small neuron whose soma was less than 100 μm^2. Among neurons of

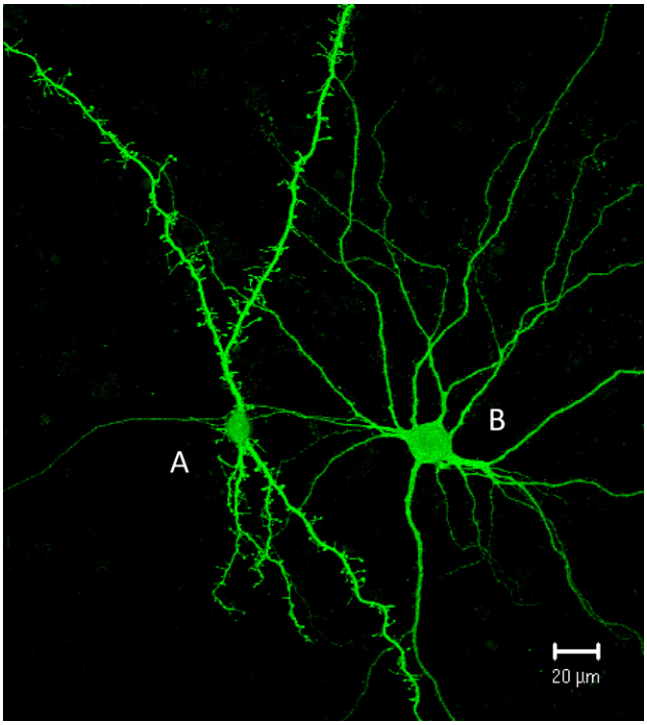

Fig. 1. Two types of neurons identified in cultured accessory olfactory bulb (AOB) on the basis of their morphological characteristics. (A) Granule cell: a bipolar neuron, which has a small soma (less than 100 μm^2) and thin bipolar dendrites. (B) MT cell: a multipolar neuron, which has a large soma (more than 100 μm^2) and thick multipolar dendrites. Scale bar indicates 20 μm.