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Estrogen-induced cell signaling in the sexually dimorphic nucleus of the rat preoptic area: Potential involvement of cofilin in actin dynamics for cell migration

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ABSTRACT

Estrogen is a key factor to induce the sexually dimorphic nucleus (SDN) in the preoptic area (POA) of the rat brain. Identification of estrogen-dependent signaling pathways at SDN in POA during the critical period is a prerequisite for elucidating the mechanism. In the present study, we treated female rats with/without 17β-estradiol (E_2) at birth, designated as postnatal day 1 (P1), and prepared total RNA from brain slices containing SDN for DNA microarray analysis. Among the estrogen-responsive genes identified, protein kinase C-delta (*PKC-δ*) was significantly up-regulated by E_2 at P5. We examined the downstream effectors of PKC-δ protein by Western blotting and found an E_2-induced PKC-δ/Rac1/PAK1/LIMK1/cofilin pathway. In the pathway, E_2 suppressed the phosphorylation (inactive form) of cofilin. This result was supported by immunohistochemistry, where the phosphorylation/dephosphorylation of cofilin occurred at SDN, which suggests that cell migration is a cue to create sexual dimorphism in POA.

1. Introduction

Sexually dimorphic structure in the mammal brain is important for modulating neuroendocrine functions and regulating sexual behavior. In rats, the preoptic area (POA) is one of the best defined regions showing a sexually differentiated morphology. Striking differences between the sexes are observed in two groups of cells in POA, the sexually dimorphic nucleus of POA (SDN-POA) and the anteroventral periventricular nucleus (AVPV). The sexual dimorphism of the rat brain is formed by the exposure to estrogen, such as 17β-estradiol (E_2), during specific periods for embryonic and early neonatal development. The critical period for the onset of sexually dimorphic structure begins around embryonic day 18 (5 days before birth), and is shut down around postnatal day 10 (P10).

The existence of SDN-POA was first demonstrated by Nissl staining of POA sections [1]; nowadays, the detection of SDN-POA by immunohistochemistry using calbindin is widely used [2]. However, the meaning of volumetric differences between sexes observed by staining is controversial. There are several phenomena induced by E_2 at particular regions of the developing brain: (1) structural differences such as the type or the number of synapses and the size of a particular projection, (2) the period for neuroblast division, (3) cell survival/protection, and (4) cell migration. The first effects of E_2 on the morphology of the dendritic spine may result in volumetric differences even though the number of cells is the same [3]. The third effect of E_2 was examined by focusing on cell death: E_2 regulates the number of neurons by inhibiting cell death in SDN-POA while promoting it in AVPV [4,5]. As to the second and the fourth effects of E_2, there is little evidence. The fact that neurogenesis in SDN-POA occurs simultaneously for both sexes could rule out the second effect of E_2 influencing on the structural differences in the brain [6]. Most of these studies were carried out by treating rats with/without E_2 followed by observation of the phenotype. Therefore, a key factor that induces sexually dimorphic structure in POA in response to E_2 has not been identified yet. In order to search for such a factor, it is essential to examine signaling cascades associated with the phenotype. However, there are no reports that examine multiple molecular signaling pathways triggered by E_2 in SDN-POA, as far as we know.

We previously reported site-specific regulation of neuronal system-related genes (*Chrna4*, *Gabrd*, *Htr6* and *Scl6a13*) induced by E_2 in POA and the ventromedial nucleus of the hypothalamus (VMH) of adult female rats [7]. We examined expression profiles of E_2-induced genes by comprehensive microarray analysis, followed by Western blotting. A series of experiments revealed roles of the networks of these genes in the neuroendocrine system of adult female rats. Since then, a microarray system for examining the effect of E_2

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on gene regulation has been developed [8], and a custom-made microarray consisting of a total of 173 rat estrogen-responsive genes is now available.

2. Materials and methods

2.1. Animal treatment

Pregnant Wistar–Imamichi rats (15–17 gestation days) were purchased from the Institute of Animal Reproduction (Ibaraki, Japan) and housed individually under a controlled temperature (23 °C) in a 14 h light/10 h dark cycle. Food and water were available *ad libitum*. All experiments were carried out upon approval according to the guidelines for the care and use of laboratory animals of Nippon Medical School. A subset of female pups received subcutaneous injection of either 100 μg of E_2 (Sigma–Aldrich, St. Louis, MO) or sesame oil as a control on the day of birth (P1) (Supplementary Fig. 1A). For comparison of the animals during the period sensitive to E_2 with those in the insensitive period, female rats were treated at P10 the same as for newborn rats (a control). Twenty-four hours (P2 or P11 as a control) or 96 h (P5 or P14 as a control) after the treatment, rats were anesthetized by hypothermia and decapitated. In order to dissect the brain tissue containing SDN-POA, we followed Takagi and Kawashima [9] (see Supplementary Fig. 1B and C). Slices of 300 μm thickness encompassing the preoptic area were taken and snap-frozen in liquid nitrogen. The brain slices were stored at −85 °C before the preparation of total RNA or protein.

2.2. cDNA microarray analysis

Total RNA and mRNA were prepared from the brain tissue containing SDN-POA according to the methods as described previously [7,10]. The expression profiles of estrogen-responsive genes were analyzed using a custom-made microarray, which contained a total of 173 estrogen-responsive genes based on the same DNA microarray system for humans [8].

2.3. Western blot analysis

Protein was extracted from the brain tissue containing SDN-POA by homogenization with CelLytic (Sigma–Aldrich) on ice. Western blotting was carried out as described previously [7]. Protein (10 μg) was resolved by SDS–PAGE with a 5–20% gel, and electro-transferred onto nitrocellulose membranes (Millipore, Billerica, MA) using a semi-dry transfer cell (BIO-RAD, Hercules, CA) for 1 h. The membrane was incubated overnight at 4 °C with rabbit antibodies against proteins, PKC-δ, Cdk5, PAK1, phospho-PAK1 (Ser144), LIMK1, phospho-LIMK1 (Thr508), PDXY, cofilin, phospho-cofilin (Ser3) (Cell Signaling Technology, Danvers, MA), Rac1 (BD Transduction Laboratories) or phospho-PAK1 (Thr212) (Signalway Antibody, Baltimore, MD), at a concentration of 1 μg/ml in TBS buffer, or with a mouse monoclonal antibody against the control β-actin (0.37 μg/ml; Sigma–Aldrich). Proteins were visualized using either horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (0.033 μg/ml, Cell Signaling Technology) for the proteins examined or HRP-conjugated horse anti-mouse IgG for β-actin (0.033 μg/ml, Cell Signaling Technology). The signals were detected with ECL plus reagents (Amersham Biosciences, Piscataway, NJ) using the Light-Capture System (ATTO, Tokyo, Japan). The intensity of bands was quantified using a Light-Capture software CS Analyzer (Version 2) (ATTO). The final data were obtained after normalization with the intensity for β-actin.

2.4. Immunohistochemistry

In order to prepare a brain sample for immunohistochemistry, female pups at P5 were sacrificed with an overdose of sodium pentobarbital (>70 mg/kg, ip) and perfused by transcardial infusion with 0.4% paraformaldehyde in 0.1 M phosphate buffer. The brains were collected and postfixed in the same fixative for 2–3 days, and then transferred to 30% sucrose in 0.1 M phosphate buffer until they were completely immersed. Serial coronal sections of 30 μm thickness encompassing the preoptic area were taken with a freezing microtome and stored in 0.1 M PBS containing 0.05% sodium azide at 4 °C. The free-floating coronal sections were washed with 0.01 M PBS and 0.1% Triton in PBS and then incubated in blocking buffer (1% BSA, 3% NGS, 0.1% Triton, in PBS). The sections were incubated overnight at 4 °C with the primary antibody in blocking buffer against the proteins examined, Cdk5 (Santa Cruz, CA), PAK1, LIMK1, cofilin or phospho-cofilin (Ser3) (Abcam, Tokyo), which was followed by visualization with Alexa Fluor 488-conjugated or Alexa Fluor 568-conjugated secondary antibodies (Invitrogen, Eugene, OR). Images were digitized using a BZ-9000 fluorescent microscopy system (Keyence). SDN-POA was detected by nuclear staining with DAPI (Vector Laboratory, Burlingame, CA) and calbindin (Sigma–Aldrich or Millipore).

3. Results

3.1. PKC-δ is a regulator of E_2 signaling during the critical period in SDN-POA

In order to identify factors regulated by E_2 during the critical period for establishing SDN in POA, we first carried out expression profiling of the E_2-responsive genes, using a custom-made microarray. For the analysis, specimens were prepared as described in Section 2. A total of 172 E_2-responsible genes (Supplementary Table 1) were analyzed by microarray assay with the RNA samples to examine gene expression profiles according to functional categories as follows: 1, apoptosis; 2, migration and motility; 3, development of the brain/nervous system; 4, nervous system except the category 3; 5, cell growth, cell cycle and cell proliferation; 6, cell functions except the category 5; and 7, metabolism. Among the profiles of these seven categories, most were not changed during the postnatal days in the presence or absence of E_2, except for categories 1 (apoptosis) and 2 (migration and motility). As shown in Supplementary Fig. 2, the genes related to apoptosis (12 genes) and cell migration (7 genes) showed correlations between the postnatal days examined. The profiling by microarray assays was re-examined quantitatively by *real-time* RT-PCR. Fourteen genes changed their expression after the E_2 treatment (data not shown). In order to examine the expression at the protein level, we carried out Western blot analysis for the genes whose antibodies were commercially available. The most significant effect of E_2 was observed for protein kinase C-δ (PKC-δ). As shown in Fig. 1A-(a) and B-(a), the expression of PKC-δ was significantly ($p = 0.06$) up-regulated after rats were treated with E_2 for 4 days (P5). On the basis of this result, we focused on PKC-δ to investigate upstream regulators in E_2 signaling contributing to the formation of SDN. The effect of E_2 on protein expression was examined using the specimens of P5 in the following experiments.

3.2. E_2 signaling via PKC-δ stimulates cell migration in SDN-POA

PKC-δ is engaged in multiple cellular functions, including neuritogenesis, cell growth and differentiation, anti-/pro-apoptotic signalings, and membrane functions, such as phagocytosis and cell migration [11–14]. In order to determine which function of PKC-δ