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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

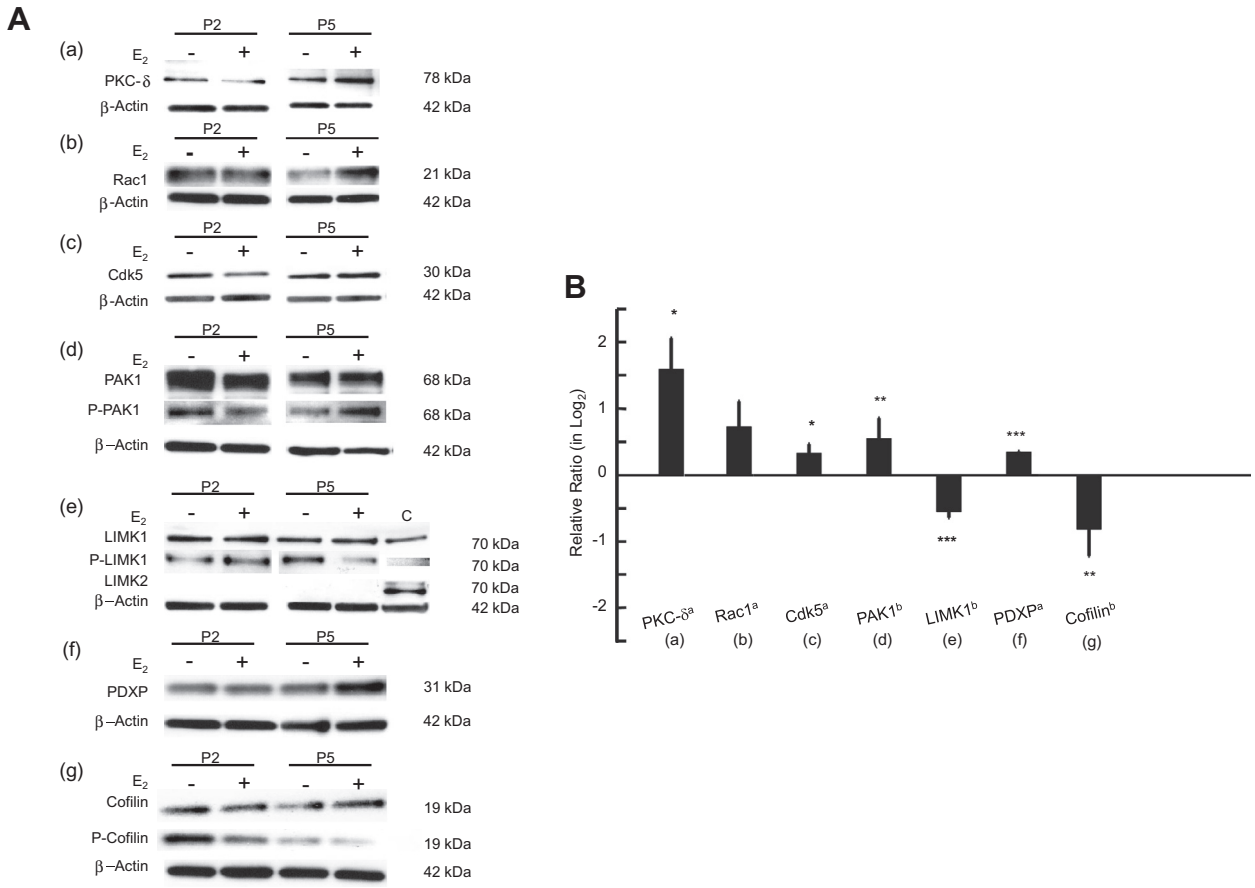

Fig. 1. Estrogen-signaling proteins. (A) Western blot analysis of proteins involved in estrogen signaling at postnatal days 2 (P2) and 5 (P5). Newborn rats were divided into two groups: those treated with 17β-estradiol (E_2^+) at birth and those without treatment (E_2^-) as controls. Rats were decapitated after the treatment, and POA containing SDN was subjected to preparation of protein samples. A total of 10 μg of protein was resolved by SDS–PAGE and then analyzed by Western blotting with the antibodies against the following proteins: (a) PKC-δ, (b) Rac1, (c) Cdk5, (d) PAK1, (e) LIMK1/2, (f) PDXP, and (g) cofilin. Phosphorylated proteins were examined for PAK1 (P-PAK1), LIMK1 (P-LIMK1) and cofilin (P-cofilin). β-Actin was used to normalize the protein amount. The molecular weights of the proteins are shown on the right. C: A lysate of the adult rat whole brain as a control. (B) The expressions of the indicated proteins in response to E_2 at P5 are summarized. Results are presented as the Log_2-transformed fold changes of protein amounts of E_2-treated samples relative to those of control samples. Superscripts a and b indicate the changes in the total amount of protein after E_2 treatment, and the changes in the amount of phospho-protein relative to that of total protein after E_2 treatment, respectively. The amounts of protein were normalized by that of β-actin. Results were obtained from at least three independent experiments and are expressed as mean ± SD. $^{*}p < 0.1$, $^{**}p < 0.05$ and $^{***}p < 0.01$ for the difference between E_2-treated and control samples.

is involved in the differences of the sexual morphology in SDN-POA induced by E_2, we examined representative proteins involved in this signaling. For example, the expression level of either Akt or ERK1/2, which are related to anti-/pro-apoptotic signaling via PKC-δ, was not changed after E_2 treatment (data not shown). Then, we examined Rho-family small G-proteins to evaluate the potential involvement of PKC-δ in cell migration. Members of Rho-family small G-proteins, RhoA, Rac1, and Cdc42, are responsible for the formation of actin stress fibers/focal adhesion complexes, lamellipodia/focal complexes, and filopodia, respectively [15–17]. Interestingly, only Rac1, but not RhoA or Cdc42, was regulated by E_2. The expression of RhoA was not changed after E_2 treatment, and the expression of Cdc42 was not detectable at all (data not shown). In contrast, the expression of Rac1 was up-regulated by E_2 at P5 (Fig. 1A-(b) and B-(b)), although it was not a statiscally significant level ($p = 0.12$) due to its low-sensitive detection.

Cyclin-dependent kinase 5 (Cdk5) is a postmitotic neuron-specific effector for Rac1 [13]. The expression of Cdk5 was significantly up-regulated ($p = 0.06$) by E_2 at P5 (Fig. 1B-(c)). Cdk5, together with its regulator p35, phosphorylates PAK1 (p21-activated kinase 1) at the threonine at position 212 (Thr212) [18].

PAK1 is another well-characterized downstream effector of Rac1. We examined total PAK1 expression in the presence or absence of E_2. There were no effects of E_2 on the total amount of PAK1 at P5 ($E_2^+/E_2^- = 1.025 \pm 0.113$, $n = 5$). When the ratio of the amount of PAK1 phosphorylated at Thr212 to that of total PAK1 was examined in the presence or absence of E_2, a significant up-regulation ($p = 0.05$) was observed (Fig. 1B-(d)). The amount of PAK1 phosphorylated at Thr212 by Cdk5 is high in embryonic and early postnatal days, but not in the central nervous system of adult rats [19]. In SDN-POA, the amount of phospho-PAK1 (Thr212) was increased at P5 after the treatment with E_2 (Fig. 1A-(d)). PAK1 is extensively phosphorylated at Ser as well as Thr residues, and there are six phosphorylation sites [20]. A conserved Ser at position 144 in the kinase inhibitory domain (amino acids 75–149) is directly implicated in the modulation of PAK1 kinase [21]. In addition, phospho-PAK1 (Thr212) mediates signals in the downstream activation pathways such as the phosphorylation of LIM kinase 1/2 (LIMK 1/2) at Thr508/505. In contrast to the phosphorylation of PAK1 at Thr212, the amount of PAK1 phosphorylated at Ser144 was slightly decreased after the treatment with E_2 ($E_2^+/E_2^- = 0.84 \pm 0.14$, $n = 5$). It was not demonstrated that a decreased amount of phospho-PAK1 (Ser144) directly affects the phosphorylation status of LIMK1, but the ratio of phospho-LIMK1 (Thr508) to total LIMK1 was significantly ($p = 0.006$) decreased by E_2 (Fig. 1B-(e)). The treatment with E_2 increased the amount

of total LIMK1 ($E_2^+/E_2^- = 1.39 \pm 0.13$, $n = 4$) and decreased that of phospho-LIMK1 (Thr508) ($E_2^+/E_2^- = 0.07 \pm 0.06$, $n = 4$). Meanwhile, there was no expression of LIMK2 in SDN-POA in spite of its expression in the whole brain of adult rats (Fig. 1A-(e)). These results indicate that SDN-POA does not involve the RhoA/ROCK/LIMK2 pathway but rather the Rac1/PAK1/LIMK1 pathway, supporting the result of the lack of an effect of E_2 on RhoA expression.

A downstream effector of LIMK1 is actin-depolymerizing factor (ADF) or cofilin. LIMK1 phosphorylates cofilin at Ser3. A decrease in the phosphorylation of LIMK1 induced by E_2 was consistent with a significant decrease ($p = 0.046$) in the phosphorylation of cofilin by E_2 (Fig. 1B-(g)). The total amount of cofilin was mostly constant ($E_2^+/E_2^- = 1.03 \pm 0.24$, $n = 4$), while the amount of phosphorylated cofilin was decreased after the treatment with E_2 ($E_2^+/E_2^- = 0.393 \pm 0.097$, $n = 3$). Besides, PDXP/chronophin, a phosphatase highly specific to cofilin, was significantly up-regulated ($p = 0.001$) by E_2 (Fig. 1B-(f)).

3.3. Phospho- and unphospho-cofilin colocalizes at SDN-POA

Cofilin participates in cell motility and morphogenesis as a regulator of actin dynamics. Cofilin is activated by dephosphorylation at Ser3. Active cofilin is translocated to the region in which actin filaments are highly active. Therefore, we examined the distribution of phosphorylated cofilin (phospho-cofilin) or unphosphorylated cofilin (unphospho-cofilin) in SDN-POA (Fig. 2). Western blot analysis was carried out using protein lysates prepared from the brain tissues obtained by punching out a larger region containing SDN from POA, so quantitative results could include the expression of the protein around SDN. Each specimen was prepared from a rat that was decapitated 4 days after E_2 treatment. While the immunoreactive signal for total cofilin was not clear, that of phospho-cofilin was observed exactly at SDN (indicated by a white square). When the images of total and phospho-cofilin were merged, a strong signal was observed at SDN, suggesting that active (unphospho-cofilin) and inactive cofilin (phospho-cofilin) are colocalized at SDN-POA.

3.4. Localization of the upstream regulators of cofilin

Next, we further carried out immunohistochemistry for the upstream regulators of cofilin, Cdk5, Pak1 and LIMK1 (Fig. 3). Calbindin was used as a marker for SDN-POA. A merged image for each protein indicated that the upstream regulators of cofilin were localized at SDN-POA as well.

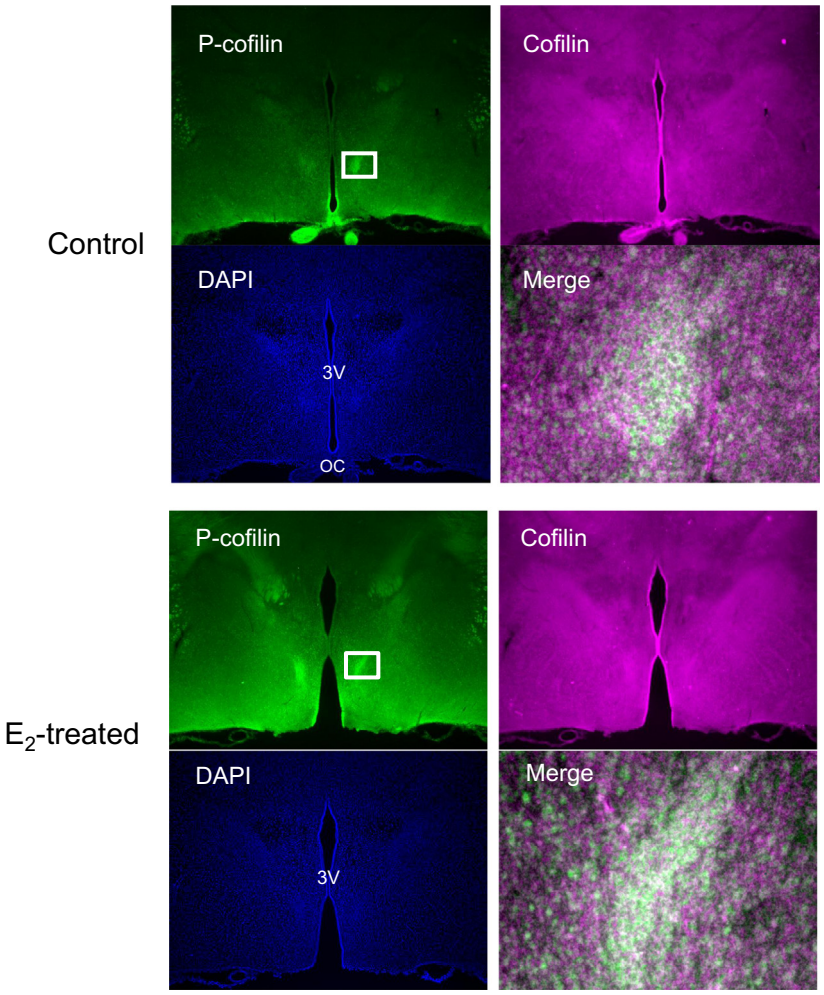

Fig. 2. Localization of total cofilin and phospho-cofilin (Ser3) at SDN-POA. Images were obtained by immunostaining for total cofilin (magenta) and phospho-cofilin (Ser3) (P-cofilin; green) in the coronal sections from E_2-treated or control female pups at P5. SDN-POA was detected by nuclear staining with DAPI (blue). Images were digitized using a BZ-9000 fluorescent microscopy system. A signal of P-cofilin in SDN is shown by a white square. Merged images of cofilin and P-cofilin in the white square are shown at a higher magnification (×40) than those of each separately staining (×4). 3V: third ventricle, OC: optic chiasm.

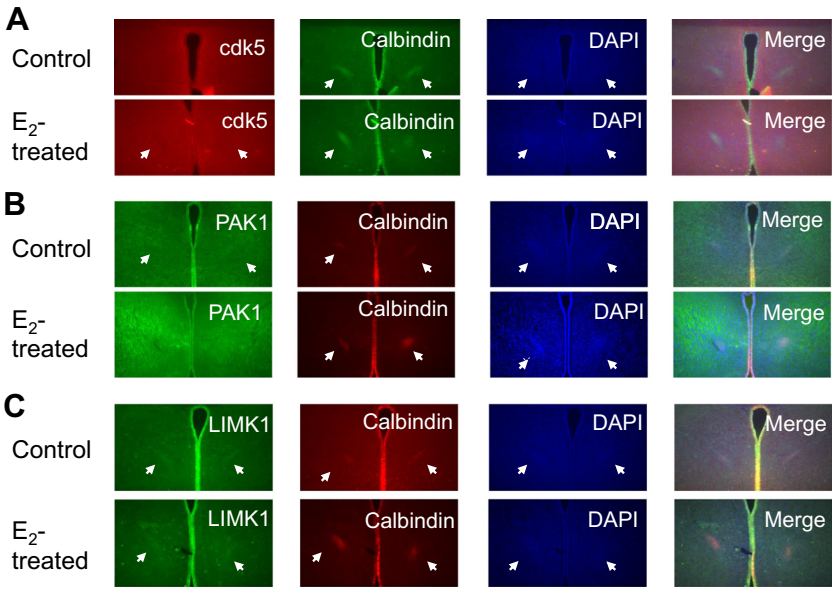

Fig. 3. Immunostaining of upstream regulators of cofilin in SDN-POA. Coronal sections were obtained from E_2-treated or control female pups at P5. SDN-POA was detected by nuclear staining with calbindin (green or red) and DAPI (blue). Images were digitized using a BZ-9000 fluorescent microscopy system. A: Cdk5 (red), B: PAK1 (green), and C: LIMK1 (green). The location of SDN-POA is shown by a white arrow. Magnification (×4).

4. Discussion

Firstly, we focused on PKC-δ according to the results of expression profiling of estrogen-responsive genes by DNA microarray analysis. PKC-δ has been well examined from several functional aspects during the last decade. Most recently, an important cascade for fibroblast migration and the development of pulmonary fibrosis was reported, in which PKC-δ was activated by phosphorylation of a hydrophobic motif by lysophosphatidic acid via $G\alpha_{12}$ and mammalian target of rapamycin complex 2 (mTORC2) [22]. We considered that significant up-regulation of PKC-δ by E_2 occurred through a G protein-coupled receptor, although which type of Gα was involved was not examined. For radial migration of cortical neurons, PKC-δ stabilizes the level of p35, a Cdk5 activator, to promote the migration of newborn neurons [23]. In the present study, the expression of Cdk5 was increased by E_2, suggesting PKC-δ to be an upstream regulator in cell migration.

Cdk5 phosphorylates PAK1 at Thr212. The biological meaning of phosphorylation of Thr212 in PAK1 by Cdk5 is controversial. p35/Cdk5 complex serves to decrease the kinase activity of PAK1 to allow the switching of PAK1 between active and inactive states in order to regulate actin dynamics [24]. Phosphorylation of PAK1 at Thr212 by Cdk5 does not directly affect the kinase activity of PAK1, but rather regulates efficient cytoskeletal remodeling through phosphorylation of multiple substrates [18,25].

The phosphorylation of PAK1 at Ser144 was slightly reduced after the treatment with E_2. An endogenous PAK1 inhibitor, CRIPak, binds to amino acids 132–270. Interesting data were obtained that E_2 induced the expression of CRIPak which modulated the PAK1-mediated estrogen receptor (ER) transactivation [26]. It is possible that the reduced phosphorylation of PAK1 at Ser144 was due to the activation of CRIPak.

As to the relationship of E_2 with cofilin in the rodent brain, it was reported that E_2 stimulated the phosphorylation of cofilin via LIMK1 and this phosphorylation promoted filopodial extension and new spine formation in NG108-15 neuroblastoma cells [27]. This experiment was carried out on the basis of the fact that E_2 modulates the morphology of the dendritic spine at the hippocampal CA1 region in female rodents. Before this work, the same group investigated LIMK as a communication molecule from E_2 to the actin cytoskeletal network involved in spine morphology, and reported that E_2 stimulated its phosphorylation [28,29]. As to the effect of E_2 on the phosphorylation of LIMK and cofilin, there was

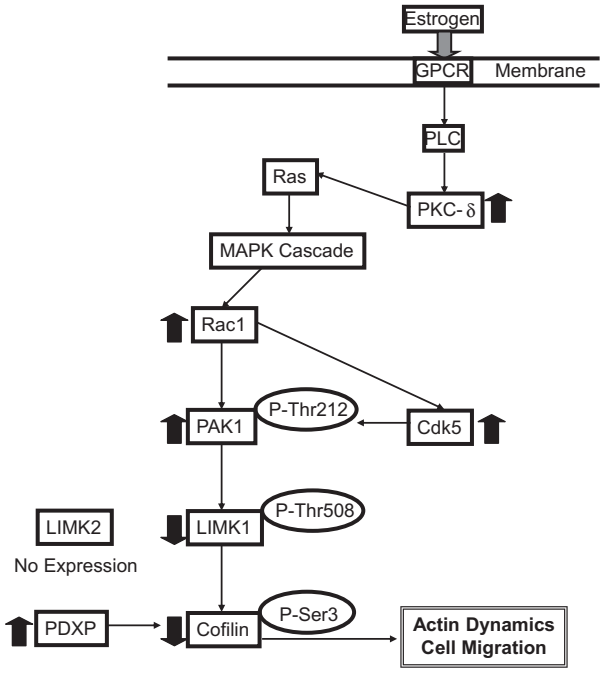

Fig. 4. A schematic diagram of E_2-signal transduction cascades in SDN-POA. E_2 activates a G-protein coupled receptor (GPCR), which further activates phospholipase C (PLC). PLC catalyzes membrane phospholipids such as phophatidylinositol 4,5-biphosphate to inositol 1,4,5-triphosphate and diacylglycerol (DAG). DAG activates PKC-δ Rac1 is activated by PKC-δ through the Ras/MAPK cascade. Rac1 activates PAK1 and Cdk5. Cdk5 phosphorylates PAK1 at Thr212, an unique phosphorylation site. The phosphorylation of Ser144 is suppressed by E_2 (discussed in the text but not shown in the diagram), leading to the negative regulation of LIMK1. Decreased phosphorylation of LIMK1 and increased expression of PDXP lead to dephosphorylation of cofilin, resulting in actin depolymerization to trigger actin dynamics and subsequent cell migration.

an opposite effect between our study and theirs. They observed that the dendritic spine morphology in the hippocampus and E_2-induced phosphorylation of cofilin promoted the extension of actin filaments. In contrast, we examined the mechanisms of the formation of the sexually differentiating nucleus in POA induced by E_2, and obtained the result that E_2 decreased the phosphorylation of both LIMK1 and cofilin. It should be noted that cofilin is not a sequestering protein but a regulatory factor that elicits the rapid turnover-barbed end growth of actin filaments, and does not simply depolymerize actin filaments. We observed that active cofilin (unphosphorylated form) and inactive cofilin (phosphorylated form) are colocalized at SDN-POA, suggesting that actin dynamics occurs in the area. It was reported that the sexual dimorphism was visible in P5-pups when dams were given ^{3}H-thymidine, and female pups had a tighter cluster of neurons in SDN than males, indicating that, while the number of neurons is the same, the activation of migration of neurons differs between the sexes [6]. Proposed E_2-induced signaling pathways for cell migration to explain the sexually dimorphism at SDN-POA are summarized in Fig. 4.

The present results demonstrated for the first time that the signaling cascade induced by E_2 at SDN in POA was directed to cell migration by enhancing actin dynamics during the critical period for establishing sexually differentiated morphology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.117.

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