The effect of steroid hormone on the expression of the calcium-processing proteins in the immature rat brain

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Research article

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Abstract

Background The cytosolic calcium concentration is regulated by calcium-processing proteins such as transient receptor potential cation channel subfamily V member 5 (TRPV5), TRPV6, sodium-calcium exchanger 1 (NCX1), and plasma membrane Ca 2+ ATPase 1 (PMCA1). Those calcium-processing proteins are important for physiological functions in the brain. The effects of steroid hormones on calcium-processing protein expressions in the brains are unknown. Thus, the effects of steroid hormones on the distribution, localization, and expressions of calcium-processing proteins in the brain were analyzed. Immature female rats were injected with estrogen (E2), progesterone (P4), dexamethasone (DEX), and their antagonists (ICI 182,780 and RU486).Results We found that TRPV5 and TRPV6 proteins were highly expressed in the cerebral cortex (CT), hypothalamus (HY), and brain stem (BS) compared to that in the olfactory bulb (OB) and cerebellum (CB). Also, the NCX1 protein was highly expressed in CT and BS compared to that in OB, HY, and CB, and PMCA1 protein was highly expressed in CT compared to that in other brain regions. Furthermore, expression levels of TRPV5, TRPV6, NCX1, and PMCA1 proteins were regulated by E2, P4, and/or DEX in the CT and HY. In summary, calcium-processing proteins are widely expressed in the immature rat brain, and expressions of calcium-processing proteins in CT and HY are regulated by E2, P4, and/or DEX and can be recovered by antagonist treatment.Conclusion These results indicate that steroid hormone regulation of TRPV5, TRPV6, NCX1, and PMCA1 proteins may serve as a critical regulator of cytosolic calcium absorption and release in the brain.

Background

Calcium concentration maintenance in nerve cells is needed for their proper development and function [1], and is important in the temporal and spatial control of neuronal function [2]. Transient changes in the free calcium intracellular concentration have a role in the control of numerous neuronal processes ranging from electrical excitability and neurotransmitter release to gene expression and dendritic integration [3, 4]. Calcium homeostasis is the maintenance of (re)absorbed calcium amounts in the body and is important for essential body's functions, cell function, and cell survival [5]. To cope with large variations in calcium input and output, organisms are equipped with regulatory systems to control plasma calcium levels, and calcium fluxes between the extracellular compartment and several organs [2]. Calcium is processed by calcium transport proteins (channels, carriers, and pumps) and through interaction with cytosolic calcium buffers, which are regulated differentially at the plasma membrane and in calcium stores [6].

Transient receptor potential cation channel subfamily V member 5 (TRPV5), TRPV6, sodium-calcium exchanger 1 (NCX1), and plasma membrane Ca^{2+} ATPase 1 (PMCA1) have critical roles in calcium homeostasis and are regulated by steroid hormones in kidney [7]. TRPV5 and TRPV6 are calcium channels involved in intracellular calcium influx. In addition, during the estrus cycle and pregnancy, TRPV6 is affected by steroid hormones involved in duodenal calcium absorption [8]. NCX1 is a counter-transporter membrane protein found in the plasma membrane, mitochondria, and endoplasmic reticulum of excitable cells [9]. PMCA1 is a transport protein in the plasma membrane of cells and is upregulated by

E2 in the uterus [10]. When intracellular calcium concentrations increase, NCX1 and PMCA1 discharge calcium and vice versa [11, 12].

E2 exerts a variety of effects, including electrophysiological, metabolic, neurotrophic, and neuroprotective effects, on neurons in the brain. Also, E2 can induce or maintain the dendritic spine in the hippocampus, protecting neurons. Neuroprotection of E2 is a calcium-dependent process and is related to the regulation of intracellular calcium levels [13]. P4 has an important neuroprotective effect and affects neuronal function by regulating cellular activity and gene transcription through intracellular receptors that are abundant in the central nervous system (CNS) [14, 15]. Glucocorticoids are a class of steroid hormones involved in calcium metabolism and can increase urinary calcium excretion and inhibit calcium absorption in the intestine [16]. They also have an essential role in the hypothalamus-pituitary-adrenal cortex axis [17]. Within the cell, DEX may protect the cell from calcium overload [18].

This study investigated the distribution of calcium-processing proteins in rat brain and the regulation of calcium-processing proteins expression by steroid hormones. We observed high expressions of calcium-processing proteins in the CT and HY and the regulation of expression of calcium-processing proteins by E2, P4, and DEX in immature rat brain. Our findings suggest that calcium-processing proteins are regulated by steroid hormones, which may serve as important regulators of cytosolic calcium absorption and release in the brain.

Methods

Reagents and chemicals

The 17b-estradiol (E2), dexamethasone (DEX), progesterone (P4), mifepristone (RU486), and corn oil were obtained from Sigma-Aldrich Corp (St. Louis, MO, USA), and the ICI 182,780 was obtained from Tocris (Avonmouth, UK). Stock solutions were diluted with dimethyl sulfoxide (DMSO; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Animal treatment

Specific pathogen-free immature female Sprague-Dawley rats weighing 15–20 g at postnatal day 10 were purchased from Samtako (Osan, Republic of Korea). The rats were housed in polycarbonate cages and were allowed to acclimate to their housing in an environmentally controlled room (temperature 23°C ± 2°C, relative humidity 50% ± 10%, frequent ventilation, and a 12 h light:dark cycle).

After approximately 1 week of acclimatization the rats were separated into seven groups (n = 7) and each group was subcutaneously (SC) injection with E2 (50 μg/kg body weight [BW]), DEX (10 mg/kg BW), P4 (20 mg/kg), or vehicle (corn oil) once per day for 5 consecutive days. To evaluate the affected signal pathways, antagonist groups were injected with ICI 182,780 (10 mg/kg BW) and RU 486 (50 mg/kg BW) 30 min prior to hormone administration. At 12 h after final injection, all immature rats were euthanized

with ether and tissue samples were collected. All experimental animal management and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Chungbuk National University.

RNA extraction and real-time PCR

Rats were euthanized by CO2 asphyxiation as described in previously [19]. The brains were removed and sagittally divided and the olfactory bulbs, cerebral cortex, hypothalamus, cerebellum, and brain stem were dissected from the right hemisphere. Total RNA was extracted using the TRIzol (Ambion, Austine, TX, USA), according to the manufacturer's instructions. RNA purity was determined using an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA). Next, first strand cDNA was prepared by subjecting 1 μg total RNA to reverse transcription using Moloney murine leukemia virus reverse transcriptase (ThermoFisher Scientific, Logen, UT, USA) with random primers (9-mers; TaKaRa Bio Inc., Kusatsu Japen). The cDNA template (2 μL) was added to 10 μL of 2× SYBR premix Ex Taq (TaKaRa Bio) and 10 pmol of each specific primer for *ERa, PR*, and *GR* genes. The primer sequences are showed in additional table 1. By using an ABI 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA), quantitative real-time PCR was conducted under the following conditions: 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Fluorescence intensity was measured at the end of the extension phase of each reaction cycle, and the reaction cycle at which the PCR products exceeded the fluorescence intensity threshold in the exponential phase of PCR amplification was considered the threshold cycle (CT). Each data point was analyzed to the internal control (GAPDH) gene for determination of a normalized arbitrary value. Relative expression (R) was calculated by using the equation $R = 2^{-\Delta\Delta CT}$ and was normalized to the vehicle.

Immunofluorescence assay

Immunofluorescence assay was performed as described previously [20, 21]. For examination of TRPV5, TRPV6, NCX1, and PMCA1 immunofluorescence, rat brain was fixed with 4% paraformaldehyde and serial 80 μm think coronal and sagittal sections were cut on a vibratome (Leica, VT1000S, Nussloch, Germany). To improve antibody penetration, brain sections were soaked in 0.5% Triton X-100 for 15min and incubated in blocking solution (5% normal goat serum, normal donkey serum and 0.3% Triton X-100) for 1 h. Primary antibodies used were rabbit anti-TRPV5 antibody (Allomone labs, Israel, ACC-035, 1:500), rabbit anti-TRPV6 (Allomone labs, A2052, 1:500), mouse anti-NCX1 (Swant, Bellinzona, Switzerland, R3F1, 1:500), rabbit anti-PMCA1 (Swant, PMCA1, 1:500), rabbit anti-ERα (Santa Cruz Biotechnology, CA, USA, SC-542, 1:500), mouse anti-ERα (ThermoFisher Scientific, Waltham, MA, USA, MA5-13304, 1:500), rabbit anti-PR (Santa Cruz, SC-538, 1:500), mouse anti-PR (Abcam, Cambridge, UK, ab2765, 1:500), rabbit anti-GR (Santa Cruz, SC-1004, 1:500) and mouse anti-GR (Abcam, ab2768, 1:500) for 24 h at 4°C. Following washing in PBS, appropriate secondary antibodies conjugated with Alexa Fluor dyes

(Invitrogen, Carlsbad, CA, USA, 1:1000) were used to detect primary antibodies, and DAPI (ThermoFisher, D1306, 1:1000) was used to stain nuclei.

Western blot assay

Brain proteins were extracted with Pro-prep (iNtRON Bio, Sungnam, Kyeonggido, Republic of Korea), according to the manufacturer's protocol. Protein concentration was determined by using the BCA assay (Sigma-Aldrich). Next, a 30-40 μg sample was resolved by performing tris-glycine SDS-polyacrylamide gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Taunton, MA, USA). Next, the membrane was blocked for 1 h with 5% skim milk dissolved in TBS-T. The membrane was incubated in primary antibodies diluted in 1% BSA for overnight at 4°C. Primary antibodies to rabbit anti-TRPV5 (Allomone labs, 1:1000), rabbit anti-TRPV6 (Allomone labs, 1:1000), mouse anti-NCX1 (Swant, 1:1000), rabbit anti-PMCA1 (Swant, 1:1000) and GAPDH (Santa Cruz, Biotechnology, 1:1000) were used. The membrane was washed four times for 10 min each with TBS-T. Horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling, Beverly, MA, USA, 1:3000) and anti-mouse IgG (Cell Signaling, 1:3000) were used as a secondary antibody with 2.5% skim milk dissolved in TBS-T for 60 min. The membrane was washed with TBS-T four times as previously mentioned, and immunoreactive proteins were visualized by using the West-One Western Blot Detection System (iNtRON Bio), according to the manufacturer's instructions. Signals were detected with the Chemi Doc EQ (Bio-Rad, Hercules, CA, USA) imaging system and were analyzed by the Quantity One program (Image J, version 1.48; Wayne Rasband, NIH, Bethesda, MD, USA).

Data analysis

Data were analyzed by applying a nonparametric one-way ANOVA followed by Tukey's correction test for multiple comparisons. All experiments consisted of three separate trials and each used a minimum of four samples. Data were analyzed using GraphPad Prism (v.5.0; GraphPad Software, La Jolla, CA) and are presented as mean ± S.E.M values.

Results

Expression of calcium-processing proteins in immature rat brain regions

The expressions of calcium-processing proteins were examined in several regions of the immature rat brain by performing western blot assays (Fig. 1a and Additional file 1). The results showed that calcium-processing proteins were expressed at comparable levels in the olfactory bulb (OB), cerebral cortex (CT), hypothalamus (HY), cerebellum (CB) and brain stem (BS). Significantly higher expressions of TRPV5 and TRPV6 proteins were observed in the CT, HY, and BS, compared to those in the CB and OB (Fig. 1b, c). In addition, the NCX1 protein was significantly more highly expressed in CT and BS than in OB, HY, and CB

(Fig. 1d), and the PMCA1 protein level was significantly higher in CT than in all other brain regions (Fig. 1e).

Localization of calcium-processing proteins in the immature rat brain regions by immunofluorescence staining

Distribution of calcium-processing proteins in the brain was examined by immunofluorescence staining. TRPV5-, TRPV6-, NCX1-, and PMCA1-positive cells were observed in OB, CT, dentate gyrus, thalamus, HY, ventral tegmental area (VTA), CB, and BS. We observed that calcium-processing proteins-positive cells were widely distributed in immature rat brain (Fig. 2).

Regulation of calcium-processing proteins in the CT and HY by steroid hormones

We confirmed relationships between the expressions of TRPV5, TRPV6, NCX1, and PMCA1 proteins and the E2, P4, and DEX signal pathways in the immature rat brain. First, we observed double immunofluorescence staining to examine whether TRPV5-, TRPV6-, NCX1-, and PMCA1-positive neurons are co-expressed with ERα, PR, and GR (Fig. 3a-d). There are no significant differences in expression of *ERa*, *PR*, and *GR* mRNA levels (Fig. 3e, f). We observed that ERα, PR, and GR receptors were expressed with TRPV5, TRPV6, NCX1, and PMCA1 in the neuronal cell.

Next, we examined whether E2, P4, and DEX influence the expressions of TRPV5, TRPV6, NCX1, and PMCA1 proteins in the CT and HY (Fig. 4 and Additional file 1). In the E2-treated group, the expressions of TRPV5, TRPV6, NCX1, and PMCA1 proteins in CT and HY were analyzed by examining western blot results (Fig. 4a-d). In the CT region, the levels of TRPV5, NCX1, and PMCA1 proteins were significantly decreased by E2, but TRPV6 protein expression did not change. TRPV5, NCX1, and PMCA1 protein levels in the CT region were recovered by ICI 182,780 (Fig. 4a, b). In the HY region, the levels of TRPV6, NCX1, and PMCA1 proteins were increased by E2, but TRPV5 protein expression showed no significant change. The levels of TRPV6, NCX1, and PMCA1 proteins were recovered by ICI 182,780 in the HY region (Fig. 4c, d).

The expressions of TRPV6, NCX1, and PMCA1 proteins were significantly increased by P4, but TRPV5 protein expression showed no significant change in the CT region. The levels of TRPV6, NCX1, and PMCA1 proteins were recovered by RU486 in the CT region (Fig. 4e, f). In the HY region, TRPV5 and TRPV6 levels were decreased by P4, but NCX1 and PMCA1 levels were increased. The levels of TRPV5, TRPV6, NCX1, and PMCA1 proteins were recovered by RU486 in the HY region (Fig. 4g, h).

The levels of NCX1 and PMCA1 proteins were increased by DEX in the CT and HY regions, and those levels were recovered by RU486 in both regions (Fig. 4i-l). The levels of TRPV5 and TRPV6 proteins did not significantly change in the CT region. However, TRPV5 protein expression was decreased by DEX and

was recovered by RU486 in the HY region. TRPV6 protein expression did not significantly change in the HY region.

Overall, calcium-processing proteins were affected by E2, P4, and DEX treatments, and those effects were blocked by ICI 182,780 or RU486 treatments. The mRNA levels of ERa, PR, and GR showed no significant differences between the vehicle group and the E2-, P4-, and DEX-treated groups. Taken together, these results suggest that steroid hormone regulation of TRPV5, TRPV6, NCX1, and PMCA1 proteins may be a function of ER-, PR- and GR-dependent regulation in the CT and HY regions.

Discussion

Calcium-processing proteins, such as TRPV5, TRPV6, NCX1, and PMCA1, regulate intracellular calcium homeostasis in epithelial cells. Previous reports have also shown a role of calcium-processing proteins in calcium homeostasis in duodenum, heart, kidney, lung, and uterus [11, 16]. However, expression patterns and regulation of calcium-processing proteins in brain regions have not been described. The present study demonstrated the expression patterns of TRPV5, TRPV6, NCX1, and PMCA1 proteins in a variety of brain regions such as OB, CT, HY, CB, and BS. The TRPV5, TRPV6, NCX1, and PMCA1 proteins were detected in all brain regions. We focused on the CT and HY brain regions because many proteins and receptors are expressed more highly in those regions than in other brain regions [22-24].

In the brain, TRPV5 and TRPV6 have important roles in the activity of calcium ion channels in neurons, with calcium regulation signaling increasing or decreasing the intracellular calcium level. TRPV5 and TRPV6 are expressed in several discrete nuclei and cells within the brain, and they have a role in neuroendocrine regulation [11, 25-27]. *NCX1* is expressed in brain and is present in neurons, astrocytes, oligodendrocytes, and microglia, in which they have an important role in maintaining Na^+ and Ca^{2+} homeostasis. In addition, *NCX1* exerts a neuroprotective effect by promoting Ca^{2+} influx and refilling in primary cortical neurons [28]. PMCA1 protein has an essential role in the regulation of intracellular calcium levels in neurons and in maintaining proper neuronal function and survival [3]. Therefore, this study was undertaken to demonstrate whether TRPV5, TRPV6, NCX1, and PMCA1 can maintain cytosolic calcium homeostasis in the immature rat brain.

Many studies have suggested that calcium-processing proteins, depending on tissue specificity, are regulated by E2, P4, and DEX steroid hormones. For example, TRPV6 is upregulated by E2 and P4 in mouse duodenum [8], while NCX1 and PMCA1 are downregulated by E2 in rat esophagus [10] but upregulated by DEX in mouse lung [16]. However, the regulation of calcium-processing proteins in the brain by these steroid hormones has not been described. This study has successfully demonstrated the regulation of calcium-processing proteins by E2, P4, and DEX in the CT and HY.

There were significant changes in the expressions of calcium-processing proteins following E2, P4, and DEX treatment, and these proteins were co-expressed with the ERα, PR, and GR proteins. Intracellular calcium concentration is altered in neonatal astrocytes through ER by the action of E2 [29]. Moreover, E2

modulation of intracellular calcium concentration is dependent on ERα/β proteins associated with the plasma membrane [29]. E2 protects against mitochondrial oxidative stress in the hippocampus, cerebral cortex, and hypothalamus in ovariectomized female rats. In addition, E2 is involved in the regulation of calcium concentration via TRPV1 in the hippocampus and dorsal root ganglion of rats [30]. Also, E2 regulates calcium levels through the L-type calcium channel Cav1.2 protein in rat primary cortical astrocytes [13]. E2 rapidly increases the free cytosolic calcium spike, mediating ERα-dependent pathway and intracellular calcium levels in hypothalamic astrocytes [31]. The TRPV6 protein expression is increased during the proestrus cycle in the mediobasal hypothalamus of mice, whereas TRPV5 protein expression is increased during metestrus and diestrus in several discrete nuclei and glial cells; thus, TRPV5 and TRPV6 have emerged as potentially E2-regulated via ERα [26, 27]. Therefore, the roles of calcium-processing proteins following E2, P4, and DEX treatment are tissue-specific and are associated with ERα, PR, and GR, respectively.

E2 acts in concert with P4 to control several non-reproductive brain functions, such as cognition and neuroprotection [23]. P4 has been shown to have a pleiotropic action in the brain and has neuroprotective effects in the brain [32]. PR has been found in several brain regions such as hippocampus, cortex, hypothalamus, and cerebellum.[23]. The wide distribution of PR in the brain suggests that this receptor may regulate neuroprotection, cognition, and motor and sensory functions. P4 regulation of calcium signaling through inhibition of voltage-gated calcium channels has the neuroprotective effects [33]. Glucocorticoids can protect or support the normal functions of organs under stress from the injury of the CNS, and a high dose DEX treatment can decrease intracellular calcium in hypothalamic neurons [17]. DEX produces its biochemical function mainly by binding to the GR, which is expressed in nearly all cell types but has varying effects in different cell types. In cortical neurons, DEX can protect against glutamate-induced cell death by decreasing calcium signaling [18].

The present study showed that the expressions of TRPV5, TRPV6, NCX1, and PMCA1 proteins were regulated by E2, P4, and DEX via their mediation of the ER-, PR-, and GR-dependent pathways in CT and HY, suggesting that the regulation of intracellular calcium concentration via TRPV5, TRPV6, NCX1, and PMCA1 proteins by E2, P4, and DEX may provide important neuroprotective effects in the brain. In the future, we will address the concentration of cytosolic calcium or calcium signaling pathway after treated with steroid hormones in the brain.

Conclusions

In summary, the results of this study have shown that TRPV5-, TRPV6-, NCX1-, and PMCA1-positive cells were highly expressed and widely distributed in the rat brain. The expressions of these proteins were observed in the OB, CT, dentate gyrus, thalamus, HY, VTA, CB, and BS. In addition, TRPV5, TRPV6, NCX1, and PMCA1 were expressed in the brain, and their expressions were regulated by E2, P4, and DEX via ER-, PR- and GR-dependent pathways in the CT and HY. Together, our data suggest that calcium-processing proteins in the brain are regulated by steroid hormones, which may serve as an important regulatory mechanism for cytosolic calcium absorption and release in the brain.

Abbreviations

E2, estrogen; P4, progesterone; DEX, dexamethasone; RU486, mifepristone; VE, vehicle; OB, olfactory bulb; CT, cerebral cortex; HY, hypothalamus; CB, cerebellum; BS, brain stem

Declarations

Ethics approval and consent to participate

All procedures for animal care were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the Chungbuk National University (CBNUA-1113-18-02).

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are included in this published article and are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

S.Y.P., E.-M.J. and E.-B.J. designed, performed and analyzed the experiments and wrote the paper. Y.-M.Y. analyzed the experiments. E.-B.J. conceived and supervised the study.

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Not applicable.

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Figures

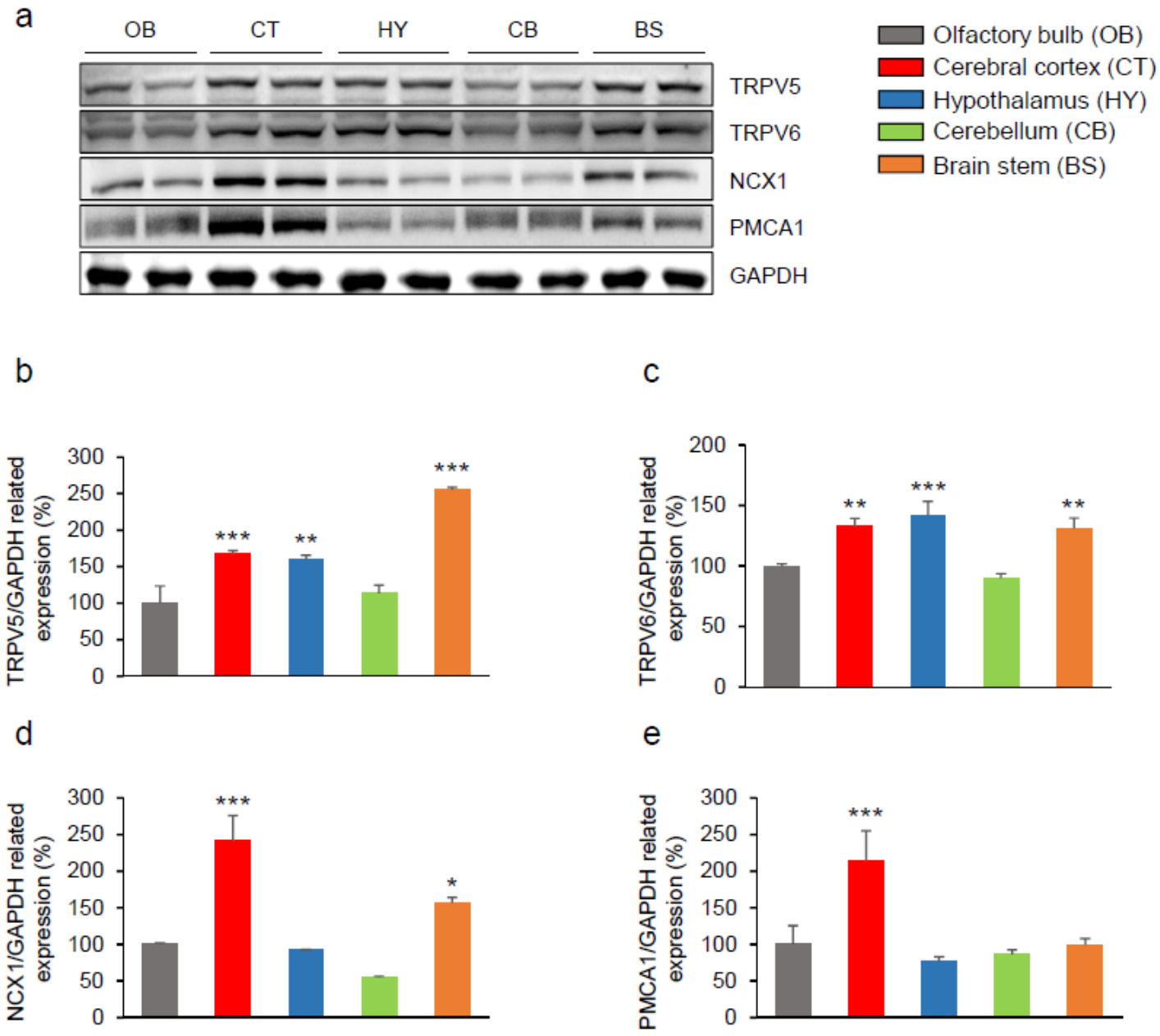

Figure 1

Expression levels of calcium-processing proteins in immature rat brain regions. (a) Samples from different regions of the olfactory bulb (OB), cerebral cortex (CT), hypothalamus (HY), cerebellum (CB), and brain stem (BS) in the immature rat brain were analyzed by western blot method. (b) TRPV5, (c) TRPV6, (d) NCX1, and (e) PMCA1 results are presented as bar graphs. Expressions of TRPV5, TRPV6, NCX1, and PMCA1 proteins were normalized to that of the internal control gene (GAPDH). Data expressed as means ± S.E.M. Statistical significance was determined by using one-way ANOVA with Tukey's correction test. *P < 0.05 versus OB, **p < 0.01 versus OB, ***p < 0.001 versus OB.

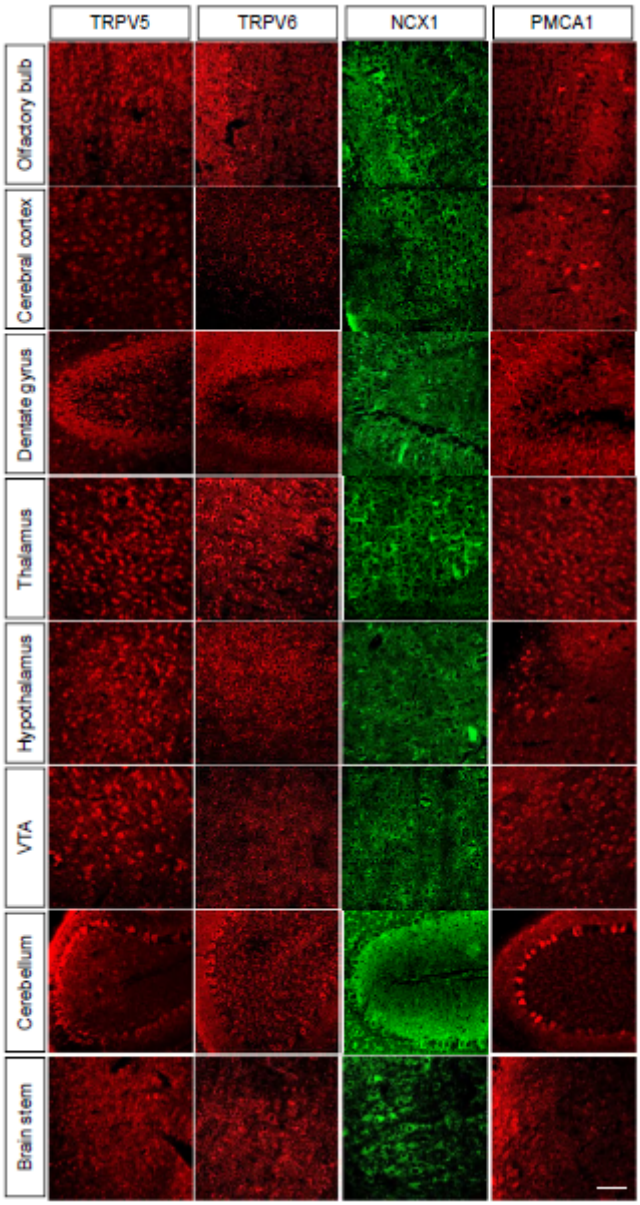

Figure 2

Distribution of calcium-processing proteins in immature rat brain regions. TRPV5, TRPV6, NCX1, and PMCA1 proteins were identified in the immature rat brain by immunofluorescence assays. Localization

was performed with the indicated antibodies in the OB, CT, dentate gyrus, thalamus, HY, ventral tegmental area (VTA), CB, and BS. Scale bar, 200 □m.

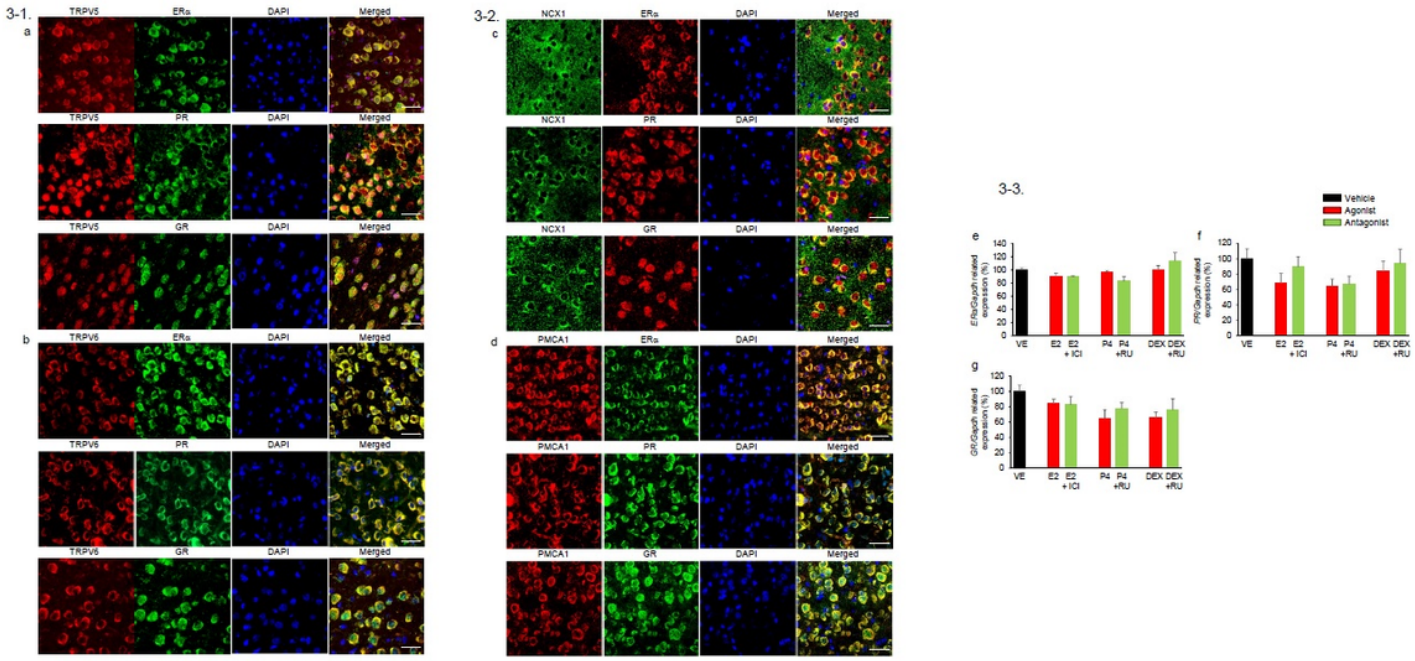

Figure 3

Colocalization of TRPV5, TRPV6, NCX1, and PMCA1 with ER□, PR, and GR in the immature rat brain. Double immunofluorescence staining of (a) TRPV5, (b) TRPV6, (c) NCX1, and (d) PMCA1 with ER□, PR, and GR. TRPV5, TRPV6, NCX1, and PMCA1 proteins co-expressed with ER□, PR, and GR□ The mRNA levels of ER□, PR, and GR were determined by using real-time PCR. The TRPV5, TRPV6, NCX1, and PMCA1 were regulated via ER-, PR- and GR-dependent pathways in the immature rat brain. Scale bar, 100□□m. (e-g) The mRNA expression of ER□, PR□ and GR were analyzed by real-time PCR. The expression of all genes was normalized to that of the internal control gene (GAPDH). Data expressed as means ± S.E.M. Statistical significance was determined by using one-way ANOVA with Tukey's correction test.

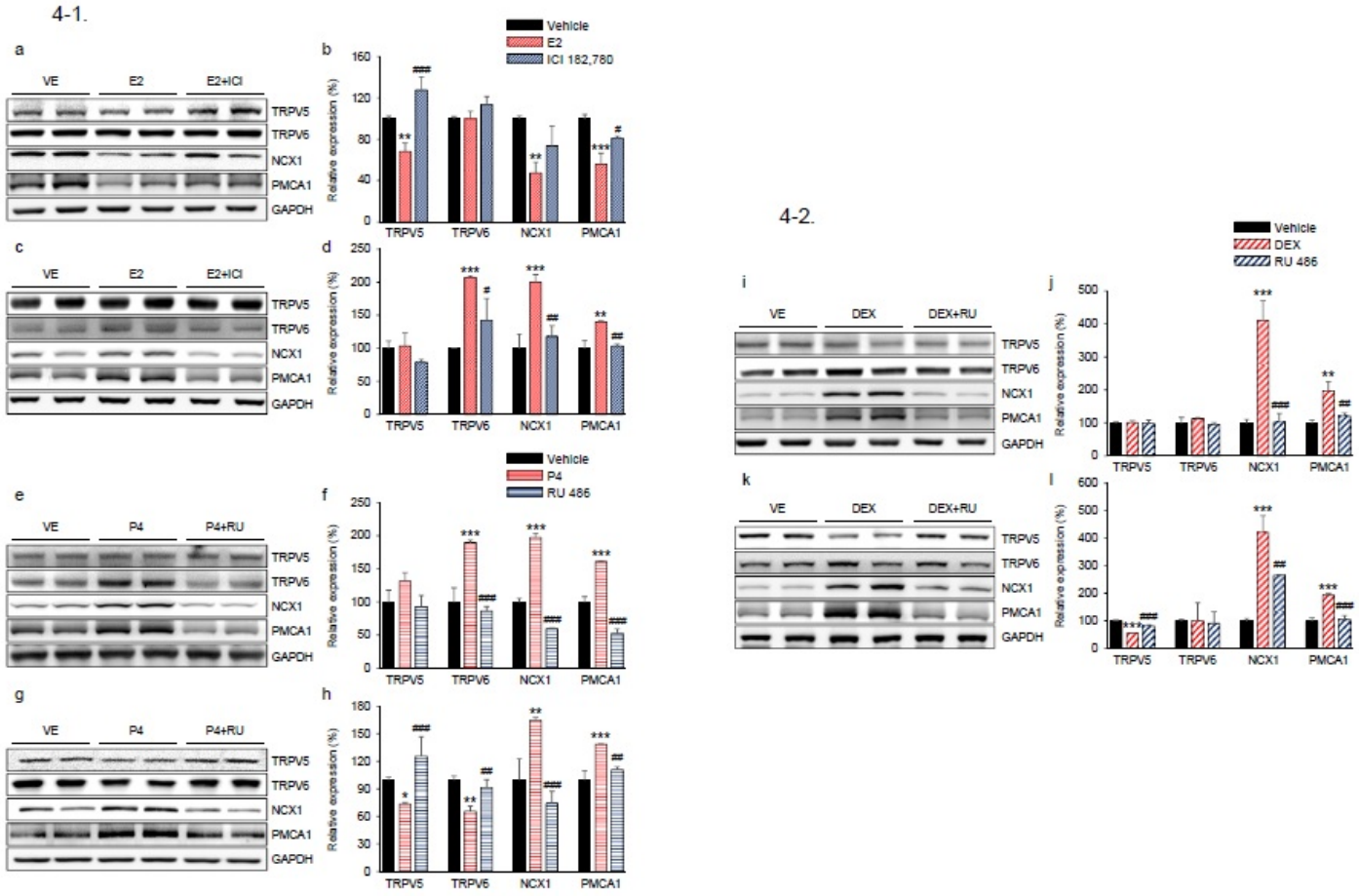

Figure 4

Effect of steroid hormones on expressions of calcium-processing proteins in the immature rat brain. The expressions of TRPV5, TRPV6, NCX1, and PMCA1 proteins were regulated by E2 (a, b) in CT and (c, d) HY, regulated by P4 (e, f) in CT and (g, h) HY, and regulated by DEX (i, j) in CT and (k, l) HY. The expression levels of TRPV5, TRPV6, NCX1, and PMCA1 proteins were regulated by steroid hormone in the CT and HY. The expressions of TRPV5, TRPV6, NCX1, and PMCA1 proteins were normalized to that of the internal control gene (GAPDH). Data expressed as means ± S.E.M. Statistical significance was determined by one-way ANOVA with Tukey's correction test. *P < 0.05 versus vehicle (VE), **p < 0.01 versus VE, ***p < 0.001 versus VE, #p < 0.05 versus agonist, ##p < 0.01 versus agonist, and ###p < 0.001 versus agonist.

Supplementary Files

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