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Estrogen modulation of calretinin and BDNF expression in midbrain dopaminergic neurons of ovariectomised mice

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ABSTRACT

Estrogen attenuates the loss of dopamine neurons from the substantia nigra in animal models of Parkinson's disease (PD) and excitatory amino-acid induced neurotoxicity by interactions with brain-derived neurotrophic factor (BDNF), and calretinin (CR) containing dopaminergic (DA) neurons. To examine this interaction more closely, we treated the ovariectomised (OVX) mice with estrodial for 10 days, and compared these mice to those OVX mice injected with the vehicle or control mice. Estrogen treatment in OVX mice had significantly more tyrosine hydroxylase (TH) positive neurons in the substantia nigra pars compacta (SNpc). Dopamine transporter (DAT) mRNA and BDNF mRNA levels in the midbrain were also significantly increased by estrogen treatment ($P < 0.05$). OVX markedly decreased the number of TH/CR double stained cells in the SNpc ($P < 0.05$), a trend which could be reversed by estrogen treatment. However, the number of GFAP positive cells in the substantia nigra did not show significant changes ($P > 0.05$) after vehicle or estrodial treatment. Furthermore, we found that estrogen treatment abrogated the OVX-induced decrease in the phosphorylated AKT (p-AKT), but not p-ERK. We hypothesize that short-term treatment with estrogen confers neuroprotection to DA neurons by increasing CR in the DA neurons and BDNF in the midbrain, which **possibly** related to activation of the PI3 K/Akt signaling pathway.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease commonly characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) (Jellinger, 1991). Accumulated evidence indicates that estrogen has neuroprotective effects on midbrain DA neurons. The incidence of PD in men is higher than that for women (Al Sweidi et al., 2012), and estrogen lowers the severity of early onset PD symptoms in postmenopausal women (Bourque et al., 2009). Both tyrosine hydroxylase (TH) and dopamine transporter (DAT) levels in the SNpc are related to endogenous estrogen levels, and these are higher in female animals compared with males (Miller and Cronin-Golomb, 2010a). *In vitro* and *in vivo* animal models of PD have also demonstrated that estrogens protect DA neurons from toxic compounds such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) (Leranth et al., 2000; Murray et al., 2003; Nagatsu and Sawada, 2007b; Tamás et al., 2005; Nakaso et al., 2016). On the other hand, when endogenous estrogen was removed, the number of DA neurons significantly decreased, an effect which could be reversed by estrogen replacement (Leranth et al., 2000; Miller and Cronin-Golomb, 2010a; Nagatsu and Sawada, 2007b). In addition, estrogen can also increase the number of nigral DA neurons in adult male animals, which suggest that a common mechanism is possibly shared by males and females (Murray et al., 2003).

Postmortem studies of PD patients showed that reduced levels of brain-derived neurotrophic factor (BDNF) within the SNpc were accompanied by the disease condition (Nagatsu and Sawada, 2007a). In addition, *in vitro* and *in vivo* studies have demonstrated that BDNF plays a critical role in supporting the survival and differentiation of midbrain DA neurons in normal and PD animal models (Baydyuk et al., 2011). There are widespread interactions between neurotrophic factors, such as BDNF and estrogen, in the

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central nervous system (CNS) and it seems that both estrogen and BDNF share common targets, effects, and mechanisms of action (Scharfman and MacLusky, 2006). However, it has not been clarified whether the neuroprotective effect of estrogen on the DA neurons in SNpc is through modulation BDNF level in the midbrain.

Emerging evidence indicate that astrocytes express estrogen receptors (ERs) and estrogen may protect against neuronal damage through inhibition of neurotoxic molecules level secreted by activated astrocytes (Dhandapani and Brann, 2007). On the other hand, protection of DA neurons by up-regulation of BDNF in astrocytes has been demonstrated in midbrain neuron-glia cultures (Yang et al., 2008). In organotypic slices of the mesencephalon, estrogen activation of ERs to protect DA neurons is indispensable to multiple signaling cascades, including MAPK (ERK) (Wang et al., 2011). In cultured astrocytes from the embryonic mouse midbrain, estrogen typically activate the MAPK pathway and produce neuroprective effects (Ivanova et al., 2001a, b). It will be helpful to understand the protection of DA neurons produced by estrogen through exploring the alterations in astrocytes and MAPK/ERK signaling cascade in the SNpc and its relationship with estrogen (Ivanova et al., 2001a,b).

Except for astrocytes, the GABAergic neurons containing calcium-binding proteins(CaBPs) are abundant in the substantia nigra. Calretinin (CR) belongs to the family of CaBPs and is also resistant to excitatory amino-acid-induced neurotoxicity (Lee and Tepper, 2006). DA neuro-fibers and cell bodies containing CR in the nigrostriatal pathway are reported to be less vulnerable to lesion by 6-OHDA in female rats compared to neurons that do not contain CR (Tsuboi et al., 2000). Therefore, whether the estrogen influences the amount of DA neurons containing CR in the SNpc will be explored in this study.

2. Materials and methods

2.1. Animals and surgical procedures

Female C57BL/6J mice (12–16 weeks of age, 20–25 g) were provided by Third Military University Experimental Animal Center of Chongqing (Chongqing, China). Mice were housed under standard laboratory conditions, with tap water and regular mice chow on a 12-h dark, 12-h light cycle. After anaesthetized with an i. p. injection of pentobarbital (50 mg/kg body weight)(Butler Company, Columbus, OH, USA), mice were ovariectomized (OVX mice) to remove the influence of endogenous estrogen. After two weeks post-operative recovery, mice were given a daily subcutaneous injection for 10 days that consisted of estradiol (0.1 mg/kg) dissolved in sesame oil or just the sesame oil used as vehicle. The experiment consisted of four groups; intact control females, sham operated females, OVX females plus vehicle-treatment, OVX plus estradiol-treated animals. All experimental procedures were approved by the local ethics committee for animal experiments and were performed according to the Guidelines for the Care and Use of Laboratory Animals of Third Military Medical University.

2.2. Immunohistochemistry

Twenty-four hours after the last injection, animals were anaesthetized and then perfused through the left cardiac ventricle with 100 ml 0.9% NaCl, followed by 150 ml 4% paraformaldehyde in phosphate buffer (PBS, 0.1 mol/L, pH 7.4). Brains were removed and postfixed in the same fixative for 24 h, and then stored in 30% sucrose in PB for 48 h at 4 °C. Coronal sections (35 μm) through the SNpc were cut with cryomicrotome (Leica 2100, Heidelberger Str.) and collected in 0.01 mmol/L PBS.

Sections were incubated in 0.5% H_2O_2 in PBS for 30 min at room temperature (RT) to quench endogenous peroxidase and then incubated in 0.3% Triton X-100 in PBS for 30 min. Sections were incubated in 1% BSA for 1 h at 37 °C to block nonspecific binding and then incubated with anti-TH (1:1000, Chemicon, Temecula, CA, USA), anti-CR (1:1000, Chemicon, Temecula, CA), and anti-GFAP (1:200, Zhongshan, Beijing, China) antibodies in PBS plus 1% BSA and 0.3% triton X-100 overnight at 4 °C. BSA (1%) replaced the primary antibodies in negative controls. After several washes, sections were labeled with secondary antibody for TH conjugated to F488 (Donkey anti-mouse, Jackson Immunoresearch Laboratories, West Grove, PA, USA), and a secondary antibody for CR conjugated to Cy3 (Donkey anti-rabbit, Jackson Immunoresearch Laboratories, West Grove, PA, USA). The stained cells were viewed and photographed with a Zeiss (Oberkochen, Germany) Axivert microscope equipped with a Zeiss AxioCam digital color camera connected to the Zeiss AxioVision 3.0 system.

2.3. RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the midbrain of mice (n = 4 mice/group) according to the protocol provided by the manufacturer (TIANGEN RNAprep Pure Tissue kit DP431, Tiangen Biotech, Beijing, China). RNA samples were reverse −transcribed into cDNA using PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). The cDNA was then amplified using DAT, BDNF and β-actin primers. Mouse DAT primer sequences were 5′- AAG ATC TGC CCT GTC CTG AAA G −3′ for sense and 5′- CAT CGA TCC ACA CAG ATG CCT C −3′ for antisense. Mouse BDNF primer sequences were 5′- TTA TTT CAT ACT TCG GTT GCA −3′ for sense and 5′- AGT GTC AGC CAG TGA TGT CG-3′-3′ for antisense. Mouse β-actin primer sequences were 5′-GGTCCACACCCGCCACCAGTTC-3′ for sense and 5′- GGATGCCACAG-GATTCCATACCC-3′ for antisense. Amplification was performed under the following conditions: 94 °C, 0.5 min; 58 °C, 0.5 min; and 72 °C, 1 min for 35 cycles. Conditions were chosen so that all of the amplification products obtained from the RNAs of interest were formed during the exponential phase. A 10 μl PCR product was subjected to electrophoresis on a 1% agarose gel containing ethidium bromide, at 26, 29, 32, and 35 cycles, to assure linearity of the reaction. Each set of reactions included a non-sample negative control. Amplification gels were visualized and photographed under UV light, and analyzed by computerized densitometric scanning of the images using a Bio-Rad GS-700 Imaging Densitometer(Bio-Rad, Hercules, CA,USA). All samples were normalized against the same value of expression for the β-actin.

2.4. Western blotting

Protein from the midbrain was extracted with the NucleoSpin RNA/Protein kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, and 15 μg of protein then separated by 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA).The membranes were incubated for 2 h with Tris-buffered saline containing 0.1% Tween 20 and 5% dehydrated skim milk to block nonspecific binding. The membranes were then incubated (overnight, at 4 °C) with the rabbit antibodies against p-ERK or total-ERK (1:1000, Cell signaling), p-Akt or total-Akt (1:1000, Cell signaling).After incubation with a HRP-conjugated secondary antibody(DAKO, Carpinteria, CA, USA) for 3 h at RT, the proteins were visualized by the Super Signal West Pico substrate (Pierce, Rockford, Illinois, USA), and then exposed to X-ray film. Western blots were obtained from the midbrain of three animals of each group. The relative intensities of p-ERK and p-AKT were normalized to the internal reference protein total ERK or total AKT and then normalized to the control group.