

Estrogen Induces Estrogen Receptor α -Dependent cAMP Response Element-Binding Protein Phosphorylation via Mitogen Activated Protein Kinase Pathway in Basal Forebrain Cholinergic Neurons *In Vivo*

Éva M. Szegő,¹ Klaudia Barabás,¹ Júlia Balog,¹ Nóra Szilágyi,¹ Kenneth S. Korach,² Gábor Juhász,¹ and István M. Ábrahám¹

¹Neurobiology Research Group, Hungarian Academy of Sciences, Eötvös Loránd University, H-1117 Budapest, Hungary, and ²Receptor Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

In addition to classical genomic mechanisms, estrogen also exerts nonclassical effects via a signal transduction system on neurons. To study whether estrogen has a nonclassical effect on basal forebrain cholinergic system, we measured the intensity of cAMP response element-binding protein (CREB) phosphorylation (pCREB) in cholinergic neurons after administration of 17 β -estradiol to ovariectomized (OVX) mice. A significant time-dependent increase in the number of pCREB-positive cholinergic cells was detected after estrogen administration in the medial septum-diagonal band (MS-DB) and the substantia innominata (SI). The increase was first observed 15 min after estrogen administration. The role of classical estrogen receptors (ERs) was evaluated using ER knock-out mice *in vivo*. The estrogen-induced CREB phosphorylation in cholinergic neurons was present in ER β knock-out mice but completely absent in ER α knock-out mice in MS-DB and SI. A series of *in vitro* studies demonstrated that estrogen acted directly on cholinergic neurons. Selective blockade of the mitogen activated protein kinase (MAPK) pathway *in vivo* completely prevented estrogen-induced CREB phosphorylation in cholinergic neurons in MS-DB and SI. In contrast, blockade of protein kinase A (PKA) was effective only in SI. Finally, studies in intact female mice revealed levels of CREB phosphorylation within cholinergic neurons that were similar to those of estrogen-treated OVX mice. These observations demonstrate an ER α -mediated nonclassical effect of estrogen on the cholinergic neurons and that these actions are present under physiological conditions. They also reveal the role of MAPK and PKA–MAPK pathway activation in nonclassical estrogen signaling in the basal forebrain cholinergic neurons *in vivo*.

Key words: steroid; ChAT; nongenomic; transgenic mice; signaling pathways; estrogen

Introduction

The basal forebrain cholinergic system (BFC) provides a substantial source of cholinergic projections to the cortex and hippocampus and plays a pivotal role in cortical arousal, attention, and cognitive functions (Beninger et al., 1989; Zaborszky et al., 1999). Recent investigations demonstrate that estrogen regulates the function of cholinergic neurons that project to the cortex and hippocampus (McEwen, 2002; Gabor et al., 2003). Disruption of

BFC produces a deficit in attention and learning as well as a decline in reference memory, and BFC neurons are particularly vulnerable in Alzheimer's and Parkinson's diseases (Whitehouse et al., 1982; Coyle et al., 1983; Wenk et al., 1998). Among many different factors controlling the vulnerability of cholinergic neurons, the gonadal steroid estrogen is one of the essential contributors. In fact, administration of estrogen can restore synaptic connectivity in the cerebral cortex after the loss of subcortical cholinergic input without rescuing BFC cells against excitotoxic cell death (Aggarwal and Gibbs, 2000; Horvath et al., 2002). Moreover, gender differences in Alzheimer's and Parkinson's diseases suggest a possible role of gonadal steroids in cytoprotection (Leranth et al., 2000; Maccioni et al., 2001; Gibbs, 2003; Saunders-Pullman, 2003; Baum, 2005). Although estrogenic effects on the expression of cholinergic enzymes were among the first nonreproductive gonadal steroid actions disclosed in the nervous system (Gibbs et al., 1997, 2004), its molecular mechanisms in cholinergic neurons are still unknown.

Estrogen receptors (ERs) are present in cholinergic neurons, with mainly ER α found in BFC neurons (Shughrue et al., 2000;

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Correspondence should be addressed to Dr. István M. Ábrahám, Neurobiology Research Group, Hungarian Academy of Sciences, Pázmány st 1/c, H-1117 Budapest, Hungary. E-mail: abraham@dec001.geobio.elte.hu.

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Kalesnykas et al., 2005). Generally, estrogen affects neurons two different ways: either via direct DNA-binding and transcriptional activity of liganded ERs (classical effect) or via rapid intracellular signaling pathways activation through protein kinase A (PKA) and mitogen activated protein kinase (MAPK) (Carlstrom et al., 2001; Kim et al., 2002; Guerra et al., 2004; Vasudevan et al., 2005; Zhao et al., 2005). We define this action of estrogen as nonclassical effect, because this downstream process indirectly acts on gene transcription via signaling pathways. The common target of many signaling pathways is a key transcription factor, the cAMP response element (CRE)-binding protein (CREB). Cellular signaling mechanisms activate the phosphorylation of CREB (pCREB), which is required for CREB to become a transcriptional regulator (Gonzalez et al., 1989; Mayr and Montminy, 2001). Previous findings suggest a crucial role of CREB-regulated transcriptional machinery in the control of neuronal vulnerability (Tao et al., 1998; Pugazhenti et al., 2000; Lonze and Ginty, 2002; Saini et al., 2004).

A precise understanding of the effects of estrogen on any neuronal cell type requires information on the estrogen-sensitive classical and nonclassical effects. In the present study, we focused on the estrogen-induced nonclassical actions. Using CREB phosphorylation as an index of intracellular alterations, we characterized the estrogen-induced signaling in BFC neurons. Our findings demonstrate direct, estrogen-induced, ER α -mediated CREB phosphorylation via the PKA–MAPK pathway in BFC neurons.

Materials and Methods

Animals. The animal breeding and experiments were performed based on rules of Local Animal Care Committee at the Eötvös Loránd University in accordance with the European Union conform Hungarian Act of Animal Care and Experimentation. Wild-type (WT) C57BL/6J mice and estrogen receptor α or β knock-out (KO) mice [ER α KO (Lubahn et al., 1993) or ER β KO (Krege et al., 1998)] were maintained under a 12 h light/dark cycle at 20°C, and they were supplied with water and food *ad libitum*.

In vivo studies. Adult female 45- to 60-d-old wild-type (C57BL/6J) mice were bilaterally ovariectomized (OVX) under Avertin anesthesia. On postovariectomy day 14, animals received a subcutaneous injection of 33 ng/g 17 β -estradiol (E2) in 0.1 ml of ethyl-oleate vehicle (Sigma, Budapest, Hungary) or vehicle alone (V) between 8:00 and 10:00 A.M. In our previous studies, 33 ng/g E2 rapidly (<15 min) induced CREB phosphorylation in the CNS (Abraham et al., 2003); therefore, animals were killed 15 min, 1 h, or 4 h later by an overdose of Avertin (0.015 ml/g). Blood was taken for E2 radioimmunoassay (RIA), and mice were transcardially perfused with ice-cold 4% paraformaldehyde, pH 7.6 (Merck, Budapest, Hungary), in PBS solution. Brains were postfixed for 2 h at 4°C and cryoprotected in Tris-phosphate-buffered solution (TBS; pH 7.6) containing 30% sucrose (Sigma) overnight at 4°C. Coronal sections, 30 μ m thick, were cut on a freezing microtome, and four sets of sections were collected in TBS.

Based on PCR analysis, female homozygous ER α KO mice, ER β KO mice, and wild-type siblings from both strains were selected. The experimental protocol was similar to those described above, with the exception that all of these mice were killed at 15 min after E2 injection.

In a separate experiment, we administered a selective PKA inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89; Sigma) [0.67 μ g/ μ l in 10% dimethylsulfoxide (DMSO) in artificial CSF (ACSF) containing the following (in mM): 147 Na⁺, 3.5 K⁺, 2 Ca²⁺, 1 Mg²⁺, pH 7.3] or a mitogen-activated protein kinase 1/2 (MEK1/2) inhibitor 1,4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]butadiene (U0126; Cell Signaling Technology, Beverly, MA) (0.1 μ g/ μ l in 10% DMSO in ACSF) to OVX female wild-type mice. Two weeks after OVX, inhibitors were injected into the lateral ventricle (anteroposterior, -0.82; lateral, 1.50; dorsoventral, -1.50 to 1.70 from dura) under halothane (1.5% in air) anesthesia. Control

groups received 10% DMSO in ACSF. Thirty minutes after injection of inhibitors or DMSO, animals received 33 ng/g E2 or V subcutaneously, and they were killed by an overdose of Avertin (0.015 ml/g) and perfused 15 min after E2 injection. The dose of the inhibitors and timing was established previously (Cervo et al., 1997; Han and Holtzman, 2000; Rahmouni et al., 2004).

To assess the role of CREB phosphorylation in cholinergic neurons under physiological conditions, female wild-type mice were OVX or sham operated (SHAM), and they were perfused 2 weeks later. The estrous stage of SHAM mice was assessed by examination of daily vaginal smear, and mice in proestrus were selected for perfusion.

Acute brain slice preparation for assessing CREB phosphorylation in vitro. An acute brain slice protocol was performed as described previously (Abraham et al., 2003). Female wild-type 45- to 60-d-old mice were OVX. On postovariectomy day 14, animals were decapitated at ~9:00 A.M., and brains were rapidly removed and placed in oxygenated, ice-cold cutting solution for 5 min containing the following (in mM): 118 NaCl, 3 KCl, 6 MgCl₂, 0.5 CaCl₂, 25 NaHCO₃, 10 HEPES, and 11 glucose, pH 7.4, when saturated with 95% O₂ and 5% CO₂ (Sigma). Coronal slices were cut in the horizontal plane (300 μ m) with a vibratome (VT1000; Leica, Nussloch, Germany), and they were preincubated for 1 h in normal ACSF solution saturated with 95% O₂ and 5% CO₂ containing the following (in mM): 118 NaCl, 3 KCl, 10 HEPES, 1.2 MgCl₂, 2.5 CaCl₂, 25 NaHCO₃, 11 glucose, with or without 0.5 μ M tetrodotoxin (TTX; Sigma). Slices were transferred into ACSF containing 100 nM E2 in ethanol or into <0.01% ethyl-alcohol solution as vehicle. Slices pretreated with TTX also received TTX in the incubating solution. Fifteen minutes later, slices were fixed in 4% paraformaldehyde at 4°C overnight. The following day, slices were transferred into TBS containing 30% sucrose, and four sets of 30 μ m coronal sections were cut on a freezing microtome and collected into TBS.

Immunohistochemistry. Free-floating, double-labeling, peroxidase-based immunohistochemistry was performed in the same manner as we reported previously with a slight modification (Abraham et al., 2003). In brief, sections were incubated with one of the primary antibodies (pCREB, 1:100; CREB, 1:100; Cell Signaling Technology) for 48 h at 4°C. Subsequently, sections were incubated with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) for 2 h. This was followed by treatment with Vector avidin–biotin–HRP complex (Vector Standard Elite kit, 1:200; Vector Laboratories) for 2 h. Peroxidase labeling was visualized by nickel-diaminobenzidine (DAB) tetrahydrochloride, using glucose oxidase. Sections were then processed for choline acetyl-transferase (ChAT; 1:2000; Chemicon, Temecula, CA) peroxidase-based immunoreactivity (IR) (biotinylated goat anti-rabbit IgG, 1:200; avidin–biotin–HRP complex, 1:200 Vector Laboratories) and revealed using DAB only. The specificities of antibodies have been reported previously in rodent species (McNulty et al., 1998; von Gall et al., 1998) (Cell Signaling Technology data). The omission of primary antibodies resulted in a complete absence of immunoreactivity.

Estrogen RIA. Plasma estrogen concentrations were determined by RIA using a Third Generation Estradiol kit (DSL-39100; Diagnostic System Laboratories, Dallas, TX). The detection limit of estrogen was 0.6 pg/ml from 100 μ l of plasma, the intra-assay coefficient of variation was 3.5%, and the interassay coefficient of variation was 4.1%.

Analysis. The number of immunoreactive cells was counted by an Olympus BX51 (Olympus Optical, Hamburg, Germany) microscope, using 20 and 40 \times objectives. Cholinergic neurons were only assigned to be representing CREB/pCREB, if the nucleus displayed a uniform black-IR product. Using the Paxinos and Franklin (2001) atlas, the following structures were selected for analysis: medial septum–diagonal band (MS-DB; plate 23–25), substantia innominata (SI; plate 34–35), and striatum (STR; plate 23–25). Two or three sections from each structure were selected, and the numbers of single (ChAT) and double-labeled (ChAT plus CREB or ChAT plus pCREB) neurons were determined by an investigator blind to the experimental groupings. CREB and pCREB expressions in ChAT-immunoreactive neurons were calculated as the percentage of total number of ChAT-immunoreactive neurons for each structure separately.

Statistical analysis. Data are expressed as mean + SEM, and square

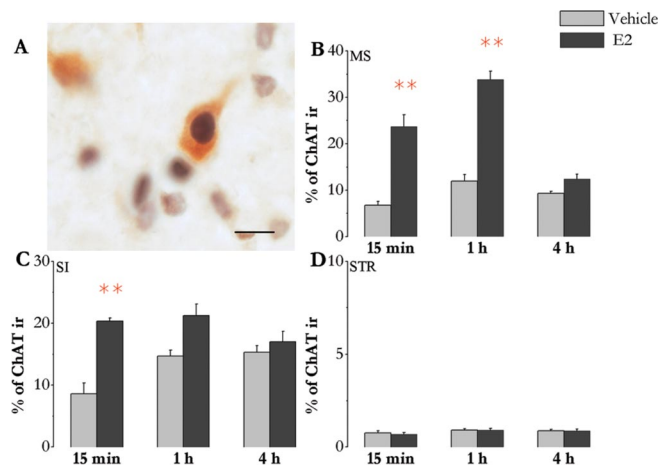


Figure 1. Estrogen rapidly induces CREB phosphorylation within cholinergic neurons in a time-dependent manner *in vivo*. The photomicrograph (A) shows clear nuclear localized CREB-IR (black) within ChAT-immunopositive neuron (brown). Scale bar, 10 μ m. Bar graphs show the percentage of cholinergic neurons expressing pCREB in the medial septum–diagonal band (B), substantia innominata (C), and striatum (D) in wild-type mice treated with vehicle or estrogen. ** $p < 0.01$; $n = 6–8$ in all groups. Data are presented as mean \pm SEM.

root was transformed for ANOVA. To examine the differences between the groups of proestrus and intact mice, one-way ANOVA was used with Tukey's *post hoc* test. Two-way ANOVA with Tukey's *post hoc* test was used to determine differences between groups in all of the other experiments (Statistica 7.0; StatSoft, Tulsa, OK).

Results

Exogenously administered estrogen or ovariectomy effectively alters the plasma estradiol concentration

The reliability of the experimental groups was verified by the fact that E2 administration or ovariectomy effectively altered the plasma estradiol concentration. Treatment of OVX female mice with 33 ng/g E2 increased the plasma estradiol concentration to a supraphysiological level at all time points: 15 min (vehicle, 3.42 ± 1.44 pg/ml; E2, 854.39 ± 197.45 pg/ml; $p < 0.05$), 1 h (vehicle, 2.37 ± 1.51 pg/ml; E2, 492.91 ± 80.89 pg/ml; $p < 0.05$), and 4 h (vehicle, 6.01 ± 0.38 pg/ml; E2, 225.35 ± 92.54 pg/ml; $p < 0.05$). The plasma estradiol concentration in OVX mice was significantly lower than that in SHAM-operated animals in the stage of proestrus (OVX, 4.5 ± 0.56 pg/ml; SHAM, 49.52 ± 5.57 pg/ml; $p < 0.05$).

Estrogen rapidly induces CREB phosphorylation in cholinergic neurons *in vivo*

CREB-IR and pCREB-IR were clearly nuclear and easily detected in cholinergic neurons with double-labeling immunocytochemistry (Fig. 1A). Administration of 33 ng/g E2 to OVX wild-type female mice resulted in a significant increase in pCREB-IR by cholinergic neurons 15 min after E2 injection ($p < 0.01$), peaked at 1 h ($p < 0.01$), and returned to the baseline at 4 h in MS-DB (Fig. 1B). In SI, E2-induced CREB phosphorylation in cholinergic neurons was significant at 15 min ($p < 0.01$), but there was no significant difference in the CREB phosphorylation in cholinergic neurons at either 1 or 4 h after treatments (Fig. 1C). E2 had no effect in STR (Fig. 1D). The expression of CREB in cholinergic neurons and the total number of cholinergic cells were not altered by E2 treatment or by time in STR, SI, and MS-DB (Table 1).

Effect of estrogen on CREB phosphorylation in cholinergic neurons is mediated by ER α

To disclose that estrogen-dependent phosphorylation of CREB in cholinergic neurons requires one of the classical ERs, we used OVX WT, ER β KO, and ER α KO mice in our additional investigations. We administered 33 ng/g E2 and evaluated the pCREB expression in cholinergic neurons at 15 min, when CREB phosphorylation in cholinergic neurons was significant in MS-DB and SI. Similarly to the WT mice, the treatment of WT littermates of the knock-out animals with 33 ng/g E2 significantly increased pCREB expression in cholinergic neurons in MS-DB and SI (Fig. 2A, B) but not in STR (Fig. 2B). Although reduced in magnitude in MS-DB (compared with WT; $p < 0.05$) (Fig. 2A), this response was maintained in ER β KO mice in MS-DB ($p < 0.01$) (Fig. 2A) and SI ($p < 0.01$) (Fig. 2B). In contrast, E2 failed to induce CREB phosphorylation in ER α KO mice in MS-DB and SI (Fig. 2A, B). Levels of pCREB in cholinergic neurons of vehicle-treated mice were not significantly different in the three animal groups (Fig. 2A–C). The level of CREB within cholinergic neurons and the total number of cholinergic neurons were not altered by estrogen treatment (data not shown).

E2-induced CREB phosphorylation in cholinergic neurons is sensitive to TTX

To confirm that E2-induced CREB phosphorylation is the result of direct action of E2 on cholinergic cells, we investigated TTX sensitivity of CREB phosphorylation in SI and MS-DB. Experiments were done on acute brain slices. The number of cholinergic neurons (MS-DB, 118.2 ± 4.2 ; SI, 66.1 ± 3.4 ; STR, 196.4 ± 1.6) and the percentage of cholinergic neurons containing CREB-IR (MS-DB, 93.3 ± 1.2 ; SI, 98.3 ± 0.6 ; STR, 90.1 ± 1.2) in slices were similar to those in the *in vivo* experiments at basal conditions. Although the basal level of pCREB was slightly higher in slices than in the *in vivo* experiments, treatment of brain slices with 100 nM E2 for 15 min significantly elevated pCREB expression within cholinergic neurons of MS-DB ($p < 0.01$) (Fig. 3B) and SI ($p < 0.01$) (Fig. 3C). E2-induced CREB phosphorylation was not affected in MS-DB ($p < 0.01$) (Fig. 3B) and SI ($p < 0.01$) (Fig. 3C) by TTX, which synaptically isolates cholinergic neurons. The number of cholinergic neurons expressing CREB was not changed by E2 or TTX (data not shown).

PKA inhibitor blocks the E2-induced CREB phosphorylation in cholinergic neurons in SI

To study the role of the cAMP–PKA pathway in E2-induced CREB phosphorylation in cholinergic neurons, we injected the selective PKA inhibitor H-89 into the lateral ventricle 30 min before E2 administration. Similarly to the previously described *in vitro* and *in vivo* experiments, pCREB-IR of cholinergic neurons was significantly higher after intracerebroventricular vehicle and subcutaneous E2 administration in MS-DB ($p < 0.01$) (Fig. 4A) and in SI ($p < 0.01$) (Fig. 4B), and there was no effect in STR (Fig. 4C) at 15 min. Intracerebroventricular administration of H-89 blocked the E2-induced CREB activation in the SI ($p < 0.01$) (Fig. 4B) but not in MS-DB (Fig. 4A). The level of CREB expression of cholinergic neurons was not altered by treatments in either of the areas examined (data not shown).

MEK1/2 inhibitor blocks the E2-induced CREB phosphorylation in cholinergic neurons in both MS-DB and SI

To demonstrate whether the E2-induced CREB phosphorylation requires MAPK pathway in cholinergic neurons, we adminis-

Table 1. Estrogen (33 ng/g) had no effect on CREB expression within cholinergic neurons and the total number of ChAT-positive neurons in STR, MS-DB, and SI of WT mice 15 min, 1 h, or 4 h after treatment

Area	Treatment	Time					
		15 min		1 h		4 h	
		%	Total	%	Total	%	Total
STR	Vehicle	91.6 ± 1.6	197.0 ± 21	90.9 ± 0.6	196.0 ± 2.9	91.8 ± 1.2	198.2 ± 2.1
	E2	89.4 ± 0.5	195.2 ± 1.2	90.1 ± 0.9	195.2 ± 2.2	89.5 ± 1.7	196.1 ± 1.8
MS-DB	Vehicle	95.2 ± 0.5	120.6 ± 3.6	90.2 ± 1.1	119.3 ± 1.9	90.6 ± 1.6	117.3 ± 1.2
	E2	92.6 ± 0.2	117.1 ± 2.2	92.6 ± 1.4	120.0 ± 2.6	91.5 ± 1.8	121.9 ± 2.9
SI	Vehicle	99.8 ± 0.1	67.2 ± 4.2	99.2 ± 0.2	70.3 ± 4.1	99.1 ± 0.2	71.2 ± 3.5
	E2	97.9 ± 0.2	69.4 ± 5.3	99.1 ± 0.1	69.6 ± 3.9	99.3 ± 0.1	72.6 ± 3.7

Data in the first column of each time point demonstrate the percentage of ChAT-immunopositive neurons expressing CREB (± SEM) (%). Data in the second column of each time point demonstrate the total number of ChAT-immunopositive neurons (± SEM) (Total); *n* = 5–7.

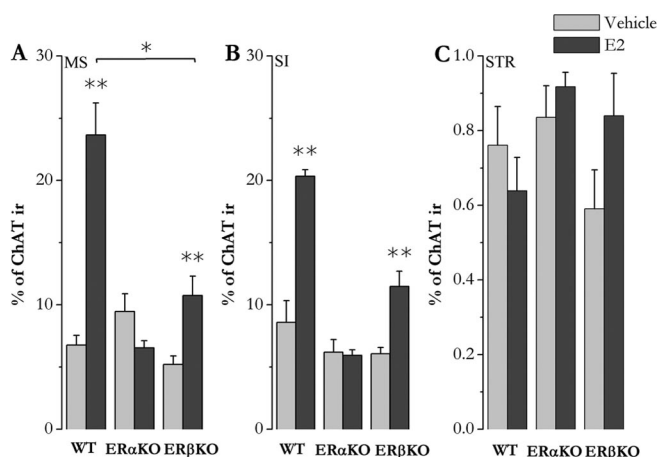


Figure 2. Estrogen requires ER α to induce CREB phosphorylation within cholinergic neurons *in vivo*. Bar graphs show the percentage of cholinergic neurons expressing pCREB in the medial septum–diagonal band (A), substantia innominata (B), or striatum (C) in wild-type, ER α KO, and ER β KO mice 15 min after vehicle or estrogen administration. ***p* < 0.01; **p* < 0.05; *n* = 4–8 in all groups. Data are presented as mean + SEM.

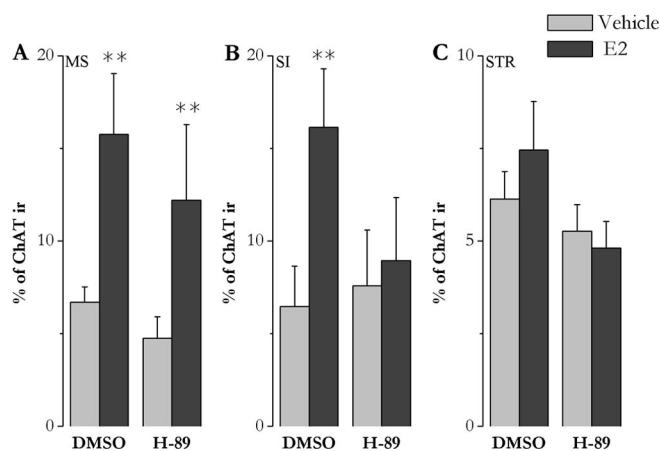


Figure 4. PKA dependence of estrogen-induced CREB phosphorylation within cholinergic cells shows a region difference *in vivo*. Bar graphs show the percentage of cholinergic neurons expressing pCREB within the medial septum–diagonal band (A), substantia innominata (B), or striatum (C) in the presence of intracerebroventricularly administered DMSO or PKA inhibitor (H-89) 15 min after vehicle or estrogen injection. ***p* < 0.01; *n* = 5–6. Data are presented as mean + SEM.

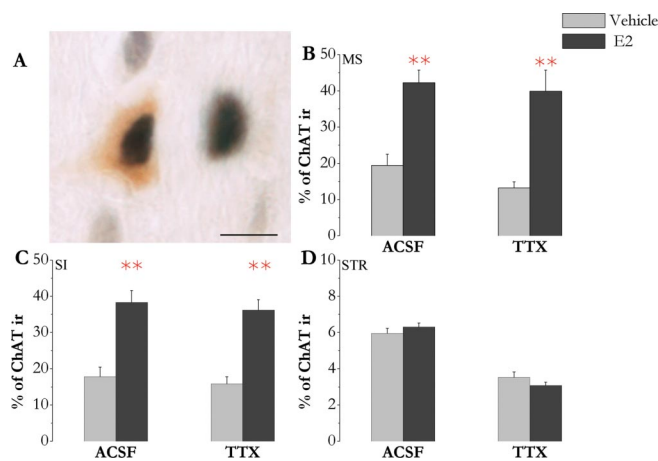


Figure 3. In the *in vitro* studies, estrogen rapidly and directly induces CREB phosphorylation within cholinergic neurons. The photomicrograph (A) presents nuclear pCREB-IR (black) within a cholinergic neuron (ChAT-IR; brown). Scale bar, 10 μ m. Bar graphs show the percentage of cholinergic neurons expressing pCREB in the medial septum–diagonal band (B), substantia innominata (C), or striatum (D) 15 min after vehicle or estrogen treatment in the presence of ACSF or 0.5 μ M TTX in ACSF (TTX). ***p* < 0.01; *n* = 6–8 in all groups. Data are presented as mean + SEM.

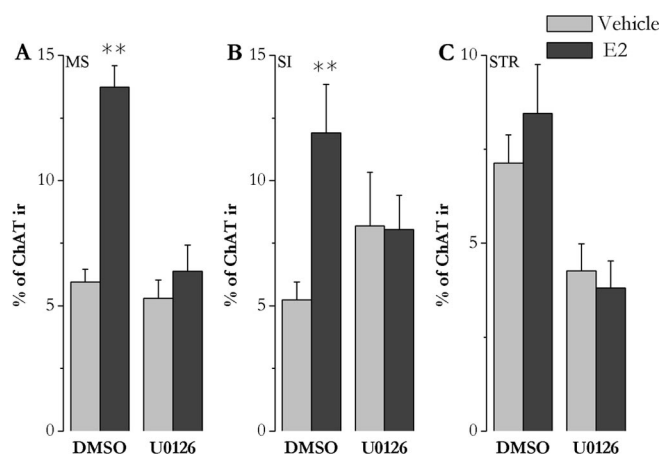


Figure 5. Estrogen-induced CREB phosphorylation within cholinergic cells is dependent on MAPK activation *in vivo*. Bar graphs show the percentage of cholinergic neurons expressing pCREB within the medial septum–diagonal band (A), substantia innominata (B), or striatum (C) in the presence of intracerebroventricularly administered DMSO or MEK1/2 inhibitor (U0126) 15 min after vehicle or estrogen injection. ***p* < 0.01; *n* = 5–6. Data are presented as mean + SEM.

tered the MEK1/2 [the activating kinase for ERK1/2 (extracellular signal-regulated kinase)] inhibitor U0126 into the lateral ventricle 30 min before E2 injection. Similarly to previously described

experiments, E2 induced CREB phosphorylation in cholinergic neurons in MS-DB (*p* < 0.01) (Fig. 5A) and SI (*p* < 0.01) (Fig. 5B), and there was no effect in STR (Fig. 5C). In contrast, intra-

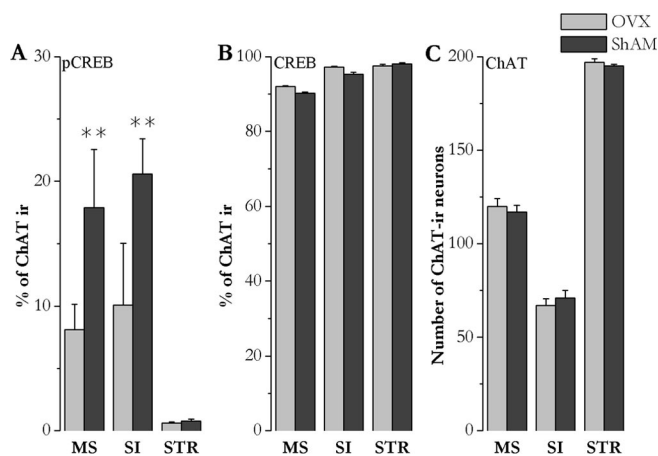


Figure 6. Phosphorylation of CREB is elevated in intact female mice during proestrus, compared with OVX control *in vivo*. Bar graphs demonstrate percentage of cholinergic neurons expressing pCREB (**A**), CREB (**B**), or the number of ChAT-immunopositive cells (**C**) in the medial septum–diagonal band, striatum, and substantia innominata. ** $p < 0.01$; $n = 5–6$ in all groups. Data are presented as mean + SEM.

cerebroventricular administration of U0126 completely blocked the E2-induced CREB phosphorylation within cholinergic neurons in both MS-DB and SI (Fig. 5*A,B*). The level of CREB expression by cholinergic neurons was not altered in this experiment (data not shown).

Levels of pCREB in cholinergic neurons remain elevated in intact female mice during proestrus

To verify that rapid increase in the E2-induced CREB phosphorylation in cholinergic neurons could occur under normal physiological elevation in estrogen level, we compared OVX mice to intact SHAM-operated proestrus females. In intact proestrus mice, the level of pCREB in MS-DB ($p < 0.01$) and SI ($p < 0.01$) was significantly higher than in OVX animals, but there was no effect in STR (Fig. 6*A*). CREB expressions within cholinergic neurons and the number of cholinergic neurons were not altered by OVX (Fig. 6*B,C*).

Discussion

We report here that E2 can rapidly (<15 min) induce CREB phosphorylation in cholinergic neurons *in vivo* in SI and MS-DB on an ER α -mediated pathway. TTX insensitivity of E2-induced CREB phosphorylation confirms that this is a direct effect of E2 on cholinergic cells. Estrogen appears to induce CREB phosphorylation via the MAPK pathway in MS-DB and SI. Blocking PKA resulted in inhibition of estrogen-induced CREB phosphorylation in SI but not in MS-DB, suggesting the existence of region-specific estrogen-induced PKA-dependent CREB phosphorylation in BFC neurons.

The possible mechanism of estrogen-induced CREB phosphorylation in basal forebrain cholinergic neurons

A great body of evidence suggests that estrogen exerts nonclassical effects in a variety of cell types. In immune cells (Karpuzoglu-Sahin et al., 2001; Grimaldi et al., 2005), smooth muscle cells (Rossi et al., 2002; Hertelendy and Zakar, 2004), and endothelial cells, estrogen can rapidly alter the availability of second messengers (Zhu and Smart, 2003; Simoncini et al., 2004). Stimulation of the second messenger system may result in activation of transcription factors such as CREB. It is important to note that the rapidity of the actions of estrogen on CREB phosphorylation

(<15 min) suggests a nonclassical mechanism, because studies demonstrated that blockade of protein synthesis or gene transcription are ineffective in altering CREB phosphorylation in such a narrow time frame (Falkenstein et al., 2000).

In the CNS, CREB can be phosphorylated via synaptic activation (Matthies et al., 1997; Lee et al., 2005). Because the cholinergic neurons are not the only ones containing classical ERs in the septal region and SI (Shughrue et al., 2000), it is likely that estrogen-induced CREB phosphorylation in cholinergic cells was initiated via transsynaptic processes. However, we demonstrated that TTX could not eliminate the estrogen-induced CREB activation, and thus our data suggest that estrogen acts directly on cholinergic neurons.

In the nervous system, quantitative immunohistochemical studies demonstrated that E2 can initiate CREB phosphorylation *in vitro* and *in vivo* as well. *In vivo* studies reveal that E2 can induce CREB phosphorylation in dorsal root ganglion cells (Purves-Tyson and Keast, 2004), anteroventral periventricular nucleus (Gu et al., 1996), bed nucleus of the stria terminalis (Zhou et al., 1996), medial preoptic area (Abraham et al., 2004), and hippocampus (Abraham and Herbison, 2005). However, little attention was given to the neuronal cell type specificity of estrogen-induced CREB phosphorylation. Previously, we demonstrated that estrogen can directly and rapidly induce the phosphorylation of CREB in gonadotropin-releasing hormone (GnRH) neurons via ER β (Abraham et al., 2003), and here we demonstrated that estrogen increases the pCREB expression in basal forebrain cholinergic neurons as well. However, there is a significant difference in E2-induced CREB phosphorylation between the two neuronal cell types. In GnRH neurons, CREB phosphorylation remained significantly elevated 4 h after E2 treatment (Abraham et al., 2003). In contrast, the time profile of estrogen-induced CREB phosphorylation in BFC cells was more transient (peaked at 15 min or 1 h), and there was no effect at 4 h. This time profile of CREB activation may reflect a substantial difference in the estrogen-sensitive signaling process, which induces CREB phosphorylation in cholinergic neurons compared with GnRH neurons. The origin of cell type differences in estrogen-induced CREB phosphorylation can be derived from the different expression of ERs between GnRH and cholinergic neurons. Although GnRH neurons predominantly express ER β (Herbison and Pape, 2001), cholinergic neurons mainly express ER α (Shughrue et al., 2000; Kalesnykas et al., 2005), and indeed we found that estrogen-induced increase in pCREB-IR in cholinergic neurons was completely blocked in ER α KO mice. Furthermore, in the striatum, where no ER α is present in cholinergic neurons (Shughrue et al., 2000), E2 failed to increase CREB phosphorylation in these neurons. Surprisingly, estrogen-induced CREB phosphorylation was partially suppressed in ER β KO compared with WT mice in MS-DB. One possible explanation is that ER α expression decreases in ER β KO mice. Recently, Bora et al. (2005) demonstrated that the level of ER α mRNA decreased in the septal region in ER β KO mice, which may implicate a partial effect of estrogen on CREB phosphorylation in MS-DB in ER β KO mice. Nevertheless, we showed that the rapid, E2-induced phosphorylation of CREB is critically dependent on ER α *in vivo*.

In terms of estrogen-induced CREB phosphorylation, we found that cholinergic neurons are different not only from GnRH neurons, but they show an area specific activation pattern and signaling in different cholinergic regions. Although estrogen induces phosphorylation of CREB in cholinergic neurons in MS-DB with an apparent maximal increase at 1 h, in the SI, the estrogen-induced CREB phosphorylation was significant only at

15 min. The region specificity suggests that different signaling mechanisms exist in cholinergic neurons on a topographical basis. Therefore, we investigated the role of PKA and MAPK pathways, because these pathways show estrogen sensitivity and are potential regulators of CREB phosphorylation. We demonstrated that estrogen-induced CREB phosphorylation in cholinergic neurons was completely blocked by the inhibition of p42/44 MAPK via blocking MEK1/2 in both SI and MS-DB, but blocking PKA was effective only in SI. Because PKA can activate the MAPK pathway and consequently CREB phosphorylation, we suggest that estrogen can induce CREB phosphorylation via a coupled PKA–MAPK pathway in SI but involves only the MAPK pathway in MS-DB. Such a difference may play a role in the different time profile of estrogen-induced CREB phosphorylation observed in cholinergic neurons of the different regions.

Our results indicate that ER α and the PKA–MAPK pathway plays an important role in estrogen-induced CREB phosphorylation in cholinergic neurons; however, the precise mechanism for activation of the PKA–MAPK signaling system via liganded ER α is unknown. Several examples of ER actions have been reported to involve phosphatidyl inositol 3-kinase, Src kinases, and G-protein-coupled receptors leading to consequent activation of PKA, MEK1/2, and CREB (Honda et al., 2000; Carlstrom et al., 2001; Zhao et al., 2005). Additional studies are needed to determine how the liganded ER α activates the PKA–MAPK pathway in cholinergic neurons.

The possible functional consequences of estrogen-induced CREB phosphorylation in basal forebrain cholinergic neurons

The most pressing question of the effects of estrogen on CREB phosphorylation in cholinergic neurons is its physiological relevance. The level of pCREB was higher in proestrus SHAM mice than in OVX animals in cholinergic neurons of MS-DB and SI, indicating that circulating, physiological concentration of estrogen can affect the cholinergic system via CREB phosphorylation.

One aspect of these data may clarify the role of BFC in the estrogen sensitivity of the hippocampal functions. Estrogen may stimulate acetylcholine release in the hippocampus via acting on cholinergic cells of MS-DB (Gibbs et al., 1997) and thereby influences GABAergic synapses in the hippocampus (Rudick et al., 2003). However, additional experiments are needed to evaluate the role of CREB in the estrogen-induced increase in acetylcholine synthesis and cholinergic functions of medial septal cholinergic neurons.

The other aspect may relate to the cytoprotective actions of estrogen. It is well known that cholinergic cells within the substantia innominata are highly vulnerable in some neurodegenerative models such as Alzheimer's disease, hypoxia, and ischemia (Whitehouse et al., 1982; Coyle et al., 1983; Brandel et al., 1991; Wenk and Willard, 1998), and estrogen has ameliorative effects in such degenerative diseases. Some findings indicate that classical ERs mediate these ameliorative actions, because they can be blocked by ER antagonists, and these are absent in ER α KO animals (Wilson et al., 2000; Dubal et al., 2001). Although the role of ER-mediated CREB activation is uncertain in estrogen-induced protective mechanisms, CREB has been linked as a key transcription factor in many neuroprotective effects (Finkbeiner et al., 1997; Walton and Dragunow, 2000). A number of gene-coding proteins with antiapoptotic effects such as Bcl₂, Bcl_{xl}, and BDNF (Tao et al., 1998; Pugazhenthii et al., 2000; Saini et al., 2004) contain CRE in its promoters, suggesting an important role for CREB-regulated transcriptional activity in neuronal survival.

It remains to be determined whether the estrogen-sensitive CREB-mediated transcription has a role in neuronal survival in the BFC system.

In summary, this study demonstrates that estrogen has a nonclassical effect in a nonreproductive neuronal population, involving cholinergic neurons, and provides the first *in vivo* evidence for the presence of functionally active ER α in nonclassical actions in cholinergic neurons. Furthermore, these findings reveal a region-specific role of PKA–MAPK signaling system in E2-induced CREB phosphorylation in cholinergic neurons. Finally, these observations suggest that estrogen-induced nonclassical actions have physiological importance in the cholinergic functions.

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