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Effects of estrogen on intracellular signaling pathways linked to activation of muscarinic acetylcholine receptors and on acetylcholinesterase activity in rat hippocampus

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ABSTRACT

The aim of the present study was to investigate the effects of estrogen lack and estrogen replacement on the production of total [^{3}H]inositol phosphate ([^{3}H]IP) induced by the activation of muscarinic acetylcholine receptors (mAChRs) and on the mechanisms for inactivation of acetylcholine. Hippocampi were obtained from rats in proestrus (PE), ovariectomized for 15 days (C15), ovariectomized for 15 days and then treated with 17β-estradiol for 7 days (E7) and ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). Ovariectomy did not change the basal level of total [^{3}H]IP in the hippocampus. 17β-Estradiol replacement (E7 and E21) reduced the basal level of total [^{3}H]IP. In all experimental groups, carbachol (CCh) caused a concentration-dependent rise in total [^{3}H]IP. The maximum effect was reached with 10^{-4} M CCh. The response to 10^{-4} M CCh in the hippocampi from C15 and E7 rats was twofold higher than in hippocampi from PE and E21 animals and was blocked by pirenzepine, but not by methoctramine. Ovariectomy or 17β-estradiol treatment for 7 days did not change neither the total acetylcholinesterase (AChE) activity nor the relative amount of mono- and dimeric G_1/G_2 and tetrameric G_4 globular forms. Conversely, hormonal treatment for 21 days induced an increase in AChE activity of G_1/G_2 and G_4 forms, indicating that 17β-estradiol stimulates both synthesis and assembly of AChE molecular forms. The present results suggest that the duration and/or a critical period with regard to the initiation of estrogen therapy are important to regulate the function of mAChRs and AChE activity in female rat hippocampus.

1. Introduction

The hippocampus is important for memory and cognition. Estrogen receptors (ERα and ERβ) are present in the dentrites of hippocampal CA1 and CA3 neurons [1–4]. *In vitro* [5,6] and *in vivo* studies [7–10; see 11, 12, for reviews] have shown that estrogen modulates synaptic function in the hippocampus. In addition to classic genomic mechanisms of estrogen action,

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mediated by ERα and ERβ, there is now convincing evidence that the steroid also exerts rapid, nongenomic actions initiated at the cell surface. These nongenomic actions activate several intracellular signaling pathways [13–15].

Acetylcholine and other neurotransmitters play an important role in cognitive processes [see 16, for review] and an interaction of these neurotransmitters, for example, dopamine, serotonin and glutamate with sex steroids have been reported. Estrogen receptors have been identified in the human basal forebrain nuclei, the major source of cholinergic innervation to the cerebral cortex, hippocampus and hypothalamus [17]. Estrogen receptors are colocalized with muscarinic acetylcholine receptors (mAChRs) and nicotinic receptors in cultured hippocampal neurons of the rat [18]. Acute treatment with estrogen or estrogen plus progesterone in ovariectomized animals can significantly affect the functional status of the cholinergic neurons in hippocampus, basal forebrain and central cortex, by modulation of choline acetyltransferase activity [19,20], high-affinity choline uptake [21,22] and acetylcholine release [23]. In addition, estrogens may affect acetylcholinesterase (AChE) activity in the hippocampus. Das et al. [24] reported that ovariectomy for 8 days reduces hippocampal AChE activity. However, estrogen replacement therapy did not reverse this effect [24], and Feng et al. [25] failed to observe changes in hippocampal AChE activity after 17 weeks of ovariectomy or after 17β-estradiol replacement for 16 weeks.

Whereas estradiol improves working memory in middle-aged rats when initiated soon after ovariectomy, it seems to have little effect after prolonged periods of estradiol deprivation (5–10 months) [26–28]. We showed that mAChRs in the rat hippocampus are upregulated 15 days after ovariectomy, compared to rats in proestrus. This effect is abolished with 17β-estradiol replacement immediately after ovariectomy, and it is slightly reduced if 17β-estradiol treatment starts 15 days after ovariectomy [29]. Taken together, these findings suggest that estrogen replacement has beneficial effects on cholinergic and cognitive function if initiated within a set period of time.

The roles of subtypes of mAChR [see 30, 31, for reviews] in hippocampal function remain to be explored. Pharmacological evidence suggests that the M_1 receptor may mediate the cognition-enhancing effects of acetylcholine [32–34] and hippocampal synaptic plasticity [35]. The stimulation of these receptors causes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), producing inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), an action mediated by the enzyme phospholipase Cβ (PLCβ) [36–38]. IP_3 receptors may mediate the propagation of Ca^{2+} waves in hippocampal dendrites induced by the activation of mAChRs [39]. Furthermore, synaptic activity can enhance IP_3 production to facilitate Ca^{2+} release in cultured hippocampal neurons [37]. These effects and the activation of extracellular signal-regulated kinases by the M_1 mAChR [40] could be important in acetylcholine-induced changes in hippocampal synaptic plasticity. In addition to producing IP_3 and DAG, mAChRs activation may directly use PIP_2 hydrolysis to regulate neuronal excitability [41].

The mechanisms underlying the effects of estrogen on intracellular signaling linked to activation of mAChRs in the hippocampus remain to be explored. The aim of this study was to investigate the effects of estrogen lack and estrogen replacement on the production of inositol phosphate induced by the stimulation of mAChRs. Our second aim was to analyze the effects of estrogen lack and estrogen replacement on the mechanisms for inactivation of acetylcholine. To do this we measured hippocampal acetylcholinesterase activity and activity of the tetrameric and mono/dimeric acetylcholinesterase forms.

2. Materials and methods

2.1. Animals and treatments

Female Wistar rats, 4 months old (204 ± 3 g, $n = 30$), were maintained on a 12-h light, 12-h dark schedule, at 22 °C, with food and water *ad libitum*. The experimental procedures were conducted according to the guidelines for the care and use of laboratory animals as approved by the Research Ethical Committee from Instituto Butantan.

Experimental groups included the following: rats in proestrus (PE, control), rats ovariectomized for 15 days (C15), rats ovariectomized for 15 days and then treated with 17β-estradiol benzoate (50 μg/kg, s.c., every other day for 7 days) (E7) and rats ovariectomized and immediately treated with 17β-estradiol benzoate (50 μg/kg, s.c., every other day for 21 days) (E21). The final injection of 17β-estradiol was given 1 day before euthanasia. These experimental groups were chosen based on previous studies [23,29,42,43]. These studies showed that estrogen replacement has beneficial effects on cholinergic and cognitive function if initiated within a set period of time.

Vaginal smears were taken for histological determination of estrous cycle [44] between 08:00 a.m. and 09:00 a.m., the animals were immediately sacrificed by decapitation and the hippocampus was isolated. Rats that showed at least two consecutive 4 or 5 days cycles were used in all subsequent experiments. In diestrus, the estradiol level gradually increases during the day, and reached peak levels at 06:00 a.m. proestrus. Progesterone level is low from 12:00 a.m. of diestrus to 02:00 p.m. of proestrus, and peak by 10:00 p.m. on proestrus [45]. Thus, rats in proestrus used in this study present high levels of 17β-estradiol and low levels of progesterone.

Rats ovariectomized for 21 days (C21) were included in the assays of total [^{3}H]inositol phosphate.

Preliminary experiments with tissues from sham-operated rats or animals injected with vehicle (ethanol and soybean oil, 1:10, v/v) were also tested as controls. Since no significant changes were observed when these two experimental groups were compared to control (rats in proestrus), all subsequent experiments were performed with tissues from rats in proestrus as control.

2.2. Measurement of total [^{3}H]inositol phosphate

Hippocampi were isolated from PE, C15, C21, E7 and E21 rats, and washed with a nutrient solution of the following composition (mM): NaCl 118.00; KCl 4.78; $CaCl_2$ 2.43; $MgSO_4$

1.16; $NaHCO_3$ 23.80; KH_2PO_4 1.17; glucose 2.92 (pH 7.4). Hippocampus slices (100 mg of tissue) were allowed to equilibrate for 10 min in nutrient solution at 37 °C under constant shaking. The slices were incubated for 40 min with 1 μCi myo[^{3}H]inositol (specific activity 47.0 Ci/mmol), and for an additional 30 min with 10 mM lithium chloride with myo[^{3}H]inositol. Tissues were then incubated in the absence (basal level) and presence of carbachol (CCh, 10^{-7} to 10^{-3}) for 40 min. Muscarinic antagonists were added 5 min prior to incubation with CCh. The antagonist concentration used was near the pK_i [46,47]. Tissues were washed three times with nutrient solution, transferred to 2 ml of methanol:chloroform (2:1, v/v) at 4 °C and homogenized with a Ultra-Turrax T25 homogenizer at 9500 rpm. Chloroform (0.62 ml) and H_2O (0.93 ml) were added to the homogenate, and the solution was centrifuged for 10 min at 2000 × *g* and 4 °C to separate the aqueous and organic phases [48,49].

Total [^{3}H]inositol phosphate was measured as previously described by Ascoli et al. [50] with the following modification. The aqueous layer was mixed with 1 ml anion-exchange resin (Dowex AG-X8, formate form, 200–400 mesh), allowed to equilibrate for 30 min at room temperature, and centrifuged at 1000 × *g* for 5 min at 4 °C. The resin was then washed sequentially with myo-inositol (4 ml) and 5 mM sodium tetraborate/60 mM sodium formate (2 ml). The resin was incubated for 30 min at room temperature with 2 ml of 0.1 M formic acid/1 M ammonium formate. The total [^{3}H] inositol phosphate was eluted and placed in scintillation vials containing OptiPhase HiSafe 3. The amount of radioactivity was determined in scintillation β-counter (LS 6500 IC, Beckman). Total [^{3}H] inositol phosphate was expressed as dpm/mg tissue.

2.3. Assay of the total AChE activity and its molecular forms

Hippocampi (n = 4) from PE, C15, E7 and E21 rats were isolated and washed in Hank's balanced salt solution. Tissues were transferred to 10 volumes of extraction buffer (containing 20 mM borate, 1 M NaCl, 5 mM EDTA, 0.5% Triton X-100, 5 mM *n*-ethylmaleimide, 2 mM benzamidine and 0.7 mM bacitracin, pH 9.0) and homogenized (Ultra-Turrax T25 homogenizer, 8000 rpm, 4 °C). The homogenates were centrifuged at 20,000 × *g* for 30 min at 4 °C [51]. The supernatant was incubated in 96-well microplates at 30 °C with the substrate acetylthiocholine [52,53] and the butyrylcholinesterase inhibitor tetraisopropyl pyrophosphoramide (Iso-OMPA, 10^{-5} M). Total AChE activity was assayed colorimetrically in quadruplicate after 40 min of incubation.

Mono- and dimeric forms of AChE (G_1/G_2) were separated from tetrameric AChE (G_4) by density gradient centrifugation [54]. Aliquots of the pooled supernatant (200 μl) from four hippocampi were layered on a 5–20% linear sucrose gradient in borate extraction buffer. Gradients were centrifuged at 300,000 × *g* for 19 h at 4 °C. Fifty fractions (20 drops) were collected and the AChE activity was determined in each fraction as described above. Enzyme activity for each molecular form was determined by measuring the area under each peak.

Protein concentration was determined with a BioRad protein assay, using BSA as standard (Bio Rad Laboratories, Hercules, CA).

2.4. Drugs, hormones and radiochemicals

Carbachol (carbamylcholine chloride), lithium chloride, myo-inositol, pirenzepine hydrochloride (pirenzepine), methoctramine tetrahydrochloride (methoctramine), acetylthiocholine, tetraisopropyl pyrophosphoramide and 17β-estradiol benzoate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Myo-[1,2-^{3}H] inositol (47.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). OptiPhase HiSafe 3 was obtained from PerkinElmer (Loughborough Leics, UK). AG® 1-X8 (200–400 mesh) resin was purchased from Bio Rad Laboratories (Richmond, CA, USA). All other drugs and reagents were obtained from Merck (Darmstadt, Germany) or Sigma Chemical Co.

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. Data were analyzed by ANOVA followed by Tukey test for multiple comparisons, or by the two-tailed Student's *t*-test to compare a response between two groups [55]. *P*-values <0.05 were accepted as significant.

3. Results

3.1. Effects of 17β-estradiol on the basal level of total [^{3}H]inositol phosphate

Ovariectomy reduced the wet weight of the uterus and estrogen replacement increased it, as expected (PE: 190.2 ± 15.4, n = 7; C15: 48.5 ± 2.7, n = 7; C21: 45.5 ± 2.8, n = 4; E7: 133.5 ± 9.6, n = 5 and E21: 216.0 ± 14.1 mg/100 g body weight, n = 6, $P < 0.05$, Tukey post-test). These values are similar to those reported by Cardoso et al. [29].

Ovariectomy for 15 and 21 days did not change the basal level of the total [^{3}H]inositol phosphate in hippocampus (106.9 ± 11.2 dpm/mg tissue, n = 19, and 78.9 ± 9.3 dpm/mg tissue, n = 4, respectively) compared to proestrus (105.5 ± 10.9 dpm/mg tissue, n = 11; $P > 0.05$, ANOVA) (Fig. 1). 17β-Estradiol replacement during the final week of a 3 weeks period of ovariectomy sharply reduced hippocampal [^{3}H]inositol phosphate levels ($P < 0.05$, Tukey post-test), compared to rats in proestrus and rats ovariectomized for 15 or 21 days (Fig. 1). Hippocampal [^{3}H]inositol phosphate levels were also low after immediate 17β-estradiol replacement (77.7 ± 6.9 dpm/mg tissue, n = 9, $P < 0.05$ compared to rats in proestrus and rats ovariectomized for 15 days, Tukey's test), but the difference with 21 days ovariectomized rats was not significant.

3.2. Effects of carbachol (CCh) and mAChR antagonists on total [^{3}H]inositol phosphate accumulation

The cholinergic agonist carbachol (CCh, 10^{-7} to 10^{-3} M) caused a dose-dependent increase of total hippocampal [^{3}H]inositol phosphate in all experimental groups (Fig. 2). The EC_{50} values

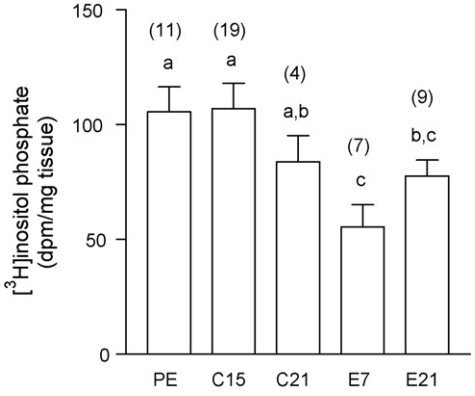

Fig. 1 – Effects of ovariectomy and 17β-estradiol treatment on basal levels of total [^{3}H]inositol phosphate in hippocampi from rats in proestrus (PE), rats ovariectomized for 15 days (C15) and 21 days (C21), rats ovariectomized for 21 days and treated with 17β-estradiol for the final 7 days (E7), and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). Error bars indicate S.E.M., number in brackets indicates number of different experiments. Different letters indicate significant differences among values obtained for different experimental groups ($P < 0.05$, ANOVA, Tukey post-test).

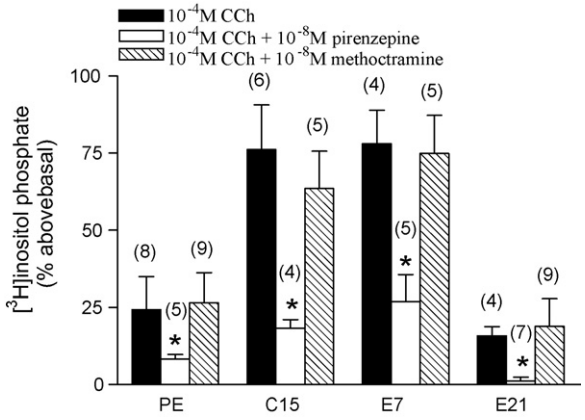

Fig. 3 – Effects of carbachol (CCh) and the mAChR antagonists pirenzepine and methoctramine on total [^{3}H]inositol phosphate accumulation in the hippocampus from rats in proestrus (PE), rats ovariectomized for 15 days (C15), rats ovariectomized for 21 days and treated with 17β-estradiol for the final 7 days (E7) and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). Error bars indicate S.E.M., number in brackets indicates number of different experiments. *Significantly different from 10^{-4} M CCh; $P < 0.05$, Student's t-test.

for CCh were 61.3, 11.9, 9.6 and 37.2 μM, respectively, in hippocampi from PE, C15, E7 and E21 animals.

Maximum inositol phosphate accumulation was obtained with 10^{-4} M CCh. Ovariectomy increased the response to the 10^{-4} M CCh (Fig. 2, 15 days: 76.1 ± 14.5% above basal, $n = 6$; 21 days: 47.8 ± 12.2% above basal, $n = 4$), compared to proestrus (24.3 ± 10.7% above basal, $n = 8$). 17β-Estradiol replacement throughout the post-ovariectomy period reverted this effect (15.8 ± 3.0% above basal, $n = 4$), but replacement limited to the last week had no effect (78.0 ± 11.0% above basal, $n = 4$). Similar results were obtained when the data were expressed as dpm/mg tissue (Fig. 2, insert).

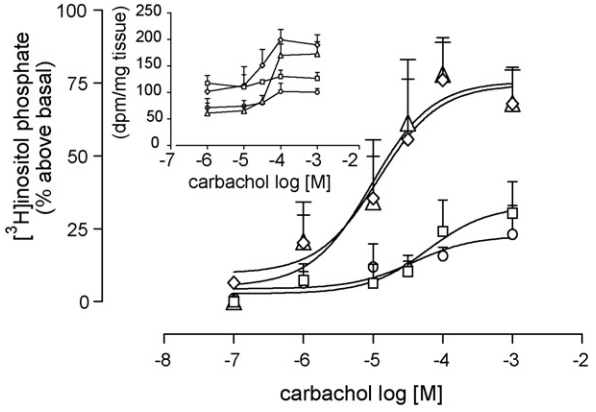

Fig. 2 – Concentration–effect curves of carbachol (CCh) on total [^{3}H]inositol phosphate accumulation in hippocampi from rats in proestrus (PE) (□), rats ovariectomized for 15 days (C15) (◇), rats ovariectomized for 21 days and treated with 17β-estradiol for the final 7 days (E7) (△) and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21) (○). Error bars indicate S.E.M., 5–19 different experiments. The results expressed as dpm/mg tissue are shown in the insert figure. The EC_{50} values for CCh were 61.3, 11.9, 9.6 and 37.2 μM, respectively, PE, C15, E7 and E21 animals.

In all experimental groups, the M_1 antagonist pirenzepine (10^{-8} M) reduced the response to carbachol (86.9 ± 9.5%, $n = 5$; 71.2 ± 12.3%, $n = 4$; 52.5 ± 15.8%, $n = 5$ and 93.6 ± 6.4%, $n = 9$, respectively), in hippocampi from PE, C15, E7 and E21 (Fig. 3). The M_2/M_4 antagonist methoctramine (10^{-8} M) failed to decrease the response to carbachol. In the absence of CCh, the mAChR antagonists did not alter the level of total [^{3}H]inositol phosphate in the hippocampus (data not shown).

3.3. *Effects of 17β-estradiol on AChE activity and molecular forms*

Total AChE activity in the hippocampus of ovariectomized rats did not differ from controls (0.92 ± 0.05 AU/mg tissue, $n = 4$ vs 0.98 ± 0.13 AU/mg tissue in proestrus, $n = 4$). 17β-Estradiol replacement throughout the post-ovariectomy period caused a sharp increase in AChE activity (1.92 ± 0.17 AU/mg tissue, $n = 4$, $P < 0.05$ compared to ovariectomized rats and rats in proestrus, Fig. 4), but replacement limited to the last week had no effect.

Globular AChE forms were separated by velocity-sedimentation analysis of aliquots of pooled hippocampus homogenates from four animals. In control, rats in proestrus, the tetrameric form (G_4) accounted for 71% of the total globular

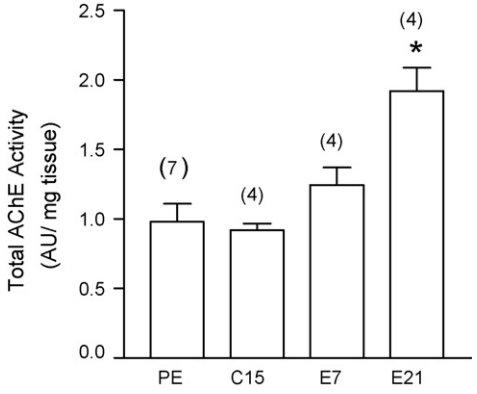

Fig. 4 – Effects of ovariectomy and 17β-estradiol treatment on total hippocampal acetylcholinesterase activity from rats in proestrus (PE), rats ovariectomized for 15 days (C15), rats ovariectomized for 21 days and treated with 17β-estradiol for the final 7 days (E7) and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). Error bars indicate S.E.M., all treatments $n = 4$ (4 hippocampi measured in quadruplicate). *Significantly different from PE; $P < 0.05$, Tukey's test.

form, and the G_1/G_2 form for 29% (Fig. 5). The relative proportion of mono- and dimeric forms (G_1/G_2) and tetrameric globular (G4) AChE forms, quantified by calculating the area under each peak, was not affected by ovariectomy (15 days) (data not shown). Administration of 17β-estradiol throughout the post-ovariectomy period increased the activity of the G_1/G_2 and G_4 forms by 61% and 78%, respectively, compared to values in proestrus (Fig. 5). This activation was not seen when 17β-estradiol replacement was limited to the final 7 days of the experiment (data not shown).

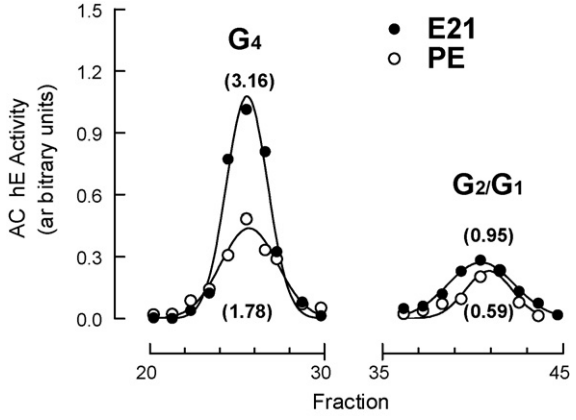

Fig. 5 – AChE oligomers in the hippocampus of intact rats in proestrus (PE) and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). AChE was extracted from pooled homogenates of the hippocampus (4 rats/treatment) and fractionated by velocity sedimentation on sucrose gradients. Results are representative of two independent experiments. Numbers in brackets indicate area under curves.

4. Discussion

The results of the present study show, for the first time, that mAChRs function is drastically affected by the duration of exposure to 17β-estradiol, since the treatment of ovariectomized rats with 17β-estradiol for 21 days was more effective than for 7 days. Furthermore, a critical period with regard to the initiation of estrogen replacement may be important to regulate the function of mAChRs.

Both genomic and nongenomic effects may contribute to the effects of estrogen on the brain. The latter involves the actions of estrogen at the cell membrane to trigger a variety of intracellular signals [see 13–15, 56, for reviews]. We found that 17β-estradiol treatment of rats ovariectomized reduced the basal level of [^{3}H]inositol phosphate in the hippocampus compared to rats in proestrus. Nevertheless, the mechanisms involved in this process remain to be explored.

Gonadal steroids may play an important role in the cholinergic neurotransmission at pre- and post-synaptic levels in the basal forebrain. The neurobiological mechanisms that underly these effects are currently unknown, but most likely reflect effects of estrogen on the survival, connectivity and function of specific neural systems in hippocampus. Previous studies from our laboratory showed that mAChRs are upregulated in hippocampus after ovariectomy (15 days) compared to rats in proestrus. This effect is abolished when 17β-estradiol is replaced immediately after ovariectomy, and it is slightly reduced if 17β-estradiol treatment begins 7 days after ovariectomy [29]. In the present study, CCh caused a dose-dependent increase of total [^{3}H]inositol phosphate. The magnitude of the response to 10^{-4} M CCh was higher in hippocampus from rats ovariectomized and ovariectomized for 15 days and then treated with 17β-estradiol for 7 days than in hippocampus from rats in proestrus and treated with 17β-estradiol immediately after ovariectomy. Taken together, these studies suggest that the duration of exposure to 17β-estradiol is important to regulate the number and function of muscarinic receptors in hippocampus. We cannot exclude the possibility that a window of opportunity, or a critical period with regard to the initiation of estrogen therapy, is important to protect cognitive function, as previously reported [26–28,57–60]. The mechanism by which the hippocampus becomes less susceptible to estradiol after 7 days of ovarian hormone deprivation remains to be determined. Several mechanisms could explain the differences between these two treatments, including cellular changes during the period of hormone deprivation and differences in estrogen receptors expression induced by extended exposure to hormone [61,62].

Various mechanisms may contribute to the effects of estrogen on mAChRs in the hippocampus. 17β-Estradiol may increase mAChR gene expression directly and/or indirectly by increasing the expression of growth factors in hippocampus [17]. In addition, progesterone deprivation could also contribute to the effects of ovariectomy on cholinergic neurotransmission [19,27].

Identification of the mAChR subtypes involved in the enhancement of synaptic plasticity may aid the treatment of memory disorders. Pharmacological evidence suggests that the M_1 receptor may mediate the cognition-enhancing effects of acetylcholine [32–34] and hippocampal synaptic plasticity

[35]. M_2 mAChRs are expressed in the hippocampus both at pre-synaptic and post-synaptic sites [30,63], but their role is not clear. Some studies suggest that blockade of brain M_2 receptors enhances learning and memory [64,65], but other studies show the opposite effect [66,67]. Functional approaches to identify which mAChR subtype is involved in each cellular response are complex, by the fact that mAChR ligands specific for each receptor subtype are not available at present [68]. However, in the present study, M_1 selective antagonist pirenzepine reduced the activation of PLC induced by carbachol, whereas M_2/M_4 selective antagonist methoctramine had no effect, suggesting the involvement of M_1 mAChR subtype in this intracellular signaling pathway. Whether estrogen plays a role in other intracellular signaling pathways coupled to mAChRs remain to be explored.

Ovariectomy reduces high-affinity choline uptake and choline acetyltransferase activity in hippocampus [20,21,69], suggesting that estradiol plays a role on cholinergic nervous system at pre-synaptic level. In addition, activation of M_1/M_3 mAChRs has been associated with increase of AChE synthesis, via PLC-dependent pathway in developing skeletal muscle cells [70]. The studies of AChE activity regulation in the hippocampus by estrogens have shown discrepancies. Das et al. [24] reported that ovariectomy for 8 days reduces hippocampal AChE activity. However, estrogen replacement therapy did not reverse this effect, and Feng et al. [25] failed to observe changes in hippocampal AChE activity after 17 weeks of ovariectomy or after 17β-estradiol replacement limited to the last 16 weeks. In the present study, ovariectomy for 15 days did not interfere with the total AChE activity in hippocampus, consistent with data previously reported after 17 weeks of ovariectomy [25]. Several possibilities could also explain these differences such as the duration of hormone deprivation, releasing dose of hormone, expression of estrogen receptors regulated by circulating levels of estrogen.

AChEs are found in several molecular forms, but in the brain the predominant isoforms expressed are the globular G_4 (tetrameric) and G_1 (monomeric) [70]. Nevertheless, the estrogen effects on AChE molecular forms had not been investigated until the present study. We showed that tetramers (G_4) represent 71% of total AChE in the hippocampus of intact female rats. Ovariectomy or 17β-estradiol replacement therapy for 7 days did not change neither the total AChE activity nor the relative amount of G_1/G_2 and tetrameric G_4 globular forms. Treatment with 17β-estradiol throughout the 21 days after ovariectomy increased the activity of both G_1/G_2 and G_4 AChE forms. Because monomers function as precursors to the oligomeric forms of the enzyme [71,72], the increased amount of G_1 induced by estrogen therapy suggests that 17β-estradiol stimulates the synthesis of AChE. Additionally, the increase of G_4 observed after estrogen therapy suggests that 17β-estradiol also influences the assembly of tetrameric AChE.

While brain G_1 AChE exists solely as a soluble enzyme, the membrane bound G_4 is the predominant form of AChE in the adult brain [see 73, for review]. G_4 may play an important role in learning and memory since changes in G_4 activity correlate with amnesia and treatment of dementia [74]. The G_4 form of AChE is reduced in several brain areas of Alzheimer patients [75], suggesting that changes in the expression of AChE molecular forms may influence the cholinergic transmission.

In conclusion, the present study indicates that estrogen modulates mAChR expression and, consequently, the intracellular signaling pathways (PLC-mediated phosphoinositide hydrolysis), and the acetylcholinesterase activity and their molecular forms. These results point out for a physiological significance and may be helpful to clarify cognitive functions in the post-menopausal women and on neurodegenerative diseases.

Acknowledgments

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REFERENCES

[1] Shughrue PJ, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. J Comp Neurol 1997;388:507–25.
[2] Shughrue PJ, Merchenthaler I. Evidence for novel estrogen binding sites in the rat hippocampus. Neuroscience 2000;99:605–12.
[3] Adams MM, Fink SE, Shah RA, Janssen WG, Hayashi S, Milner TA, et al. Estrogen and aging affect the subcellular distribution of estrogen receptor-alpha in the hippocampus of female rats. J Neurosci 2002;22:3608–14.
[4] Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashim S, et al. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology 2003;144:2055–67.
[5] Yokomaku D, Numakawa T, Numakawa Y, Suzuki S, Matsumoto T, Adachi N, et al. Estrogen enhances depolarization-induced glutamate release through activation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase in cultured hippocampal neurons. Mol Endocrinol 2003;17:831–44.
[6] Kretz O, Feste L, Wehrenberg U, Zhou L, Brauckmann S, Zhao S, et al. Hippocampal synapses depend on hippocampal estrogen synthesis. J Neurosci 2004;24:5913–21.
[7] Woolley CS, McEwen BS. Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J Neurosci 1992;12:2549–54.
[8] Woolley CS, McEwen BS. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J Comp Neurol 1993;336:293–306.
[9] Woolley CS, McEwen BS. Estradiol regulates hippocampal dendritic spine density via an *N*-methyl-D-aspartate receptor-dependent mechanism. J Neurosci 1994;14:7680–7.
[10] Day M, Good M. Ovariectomy-induced disruption of long-term synaptic depression in the hippocampal CA1 region in vivo is attenuated with chronic estrogen replacement. Neurobiol Learn Mem 2005;83:13–21.
[11] McEwen BS. Estrogen actions throughout the brain. Recent Prog Horm Res 2002;57:357–84.

[12] Toran-Allerand CD. Estrogen and the brain: beyond ER-{alpha}, ER-{beta}, and 17{beta}-estradiol. Ann NY Acad Sci 2005;1052:136–44.
[13] McEwen BS, Alves SE. Estrogen actions in the central nervous system. Endocrinol Rev 1999;20:279–307.
[14] Mehra RD, Sharma K, Nyakas C, Vij U. Estrogen receptor alpha and beta immunoreactive neurons in normal adult and aged female rat hippocampus: a qualitative and quantitative study. Brain Res 2005;1056:22–35.
[15] Cornil CA, Ball GF, Balthazart J. Functional significance of the rapid regulation of brain estrogen action: where do the estrogens come from? Brain Res 2006;1126:2–26.
[16] Friedman JI. Cholinergic targets for cognitive enhancement in schizophrenia: focus on cholinesterase inhibitors and muscarinic agonists. Psychopharmacology 2004;174:45–53.
[17] Toran-Allerand CD, Miranda RC, Bentham WD, Sohrabji F, Brown TJ, Hochberg RB, et al. Estrogen receptors colocalize with low-affinity nerve growth factor receptors in cholinergic neurons of the basal forebrain. Proc Natl Acad Sci USA 1992;89:4668–72.
[18] Hosli E, Hosli L. Cellular localization of estrogen receptors on neurones in various regions of cultured rat CNS: coexistence with cholinergic and galanin receptors. Int J Dev Neurosci 1999;17:317–30.
[19] Gibbs RB. Fluctuations in relative levels of choline acetyltransferase mRNA in different regions of the rat basal forebrain across the estrous cycle: effects of estrogen and progesterone. J Neurosci 1996;16:1049–55.
[20] Luine VN. Estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. Exp Neurol 1985;89:484–90.
[21] ÓMalley CA, Hautamaki RD, Kelley M, Meyer EM. Effects of ovariectomy and estradiol benzoate on high affinity choline uptake, Ach synthesis, and release from rat cerebral cortical synaptosomes. Brain Res 1987;403:389–92.
[22] Singh M, Meyer EM, Millard WJ, Simpkins JW. Ovarian steroid deprivation in a reversible learning impairment and compromised cholinergic function in female Sprague–Dawley rats. Brain Res 1994;644:305–12.
[23] Gibbs RB. Effects of estrogen on basal forebrain cholinergic neurons vary as a function of dose and duration of treatment. Brain Res 1997;757:10–6.
[24] Das A, Dikshit M, Srivastava SR, Srivastava UK, Nath C. Effect of ovariectomy and estrogen supplementation on brain acetylcholinesterase activity and passive-avoidance learning in rats. Can J Physiol Pharmacol 2002;80:907–14.
[25] Feng Z, Cheng Y, Zhang JT. Long-term effects of melatonin or 17 beta-estradiol on improving spatial memory performance in cognitively impaired, ovariectomized adult rats. J Pineal Res 2004;37:198–206.
[26] Markowska AL, Savonenko AV. Effectiveness of estrogen replacement in restoration of cognitive function after long-term estrogen withdrawal in aging rats. J Neurosci 2002;22:10985–9.
[27] Gibbs RB. Long-term treatment with estrogen and progesterone enhances acquisition of a spatial memory task by ovariectomized aged rats. Neurobiol Aging 2000;21:107–16.
[28] Daniel JM, Hulst JL, Berbling JL. Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. Endocrinology 2006;147:607–14.
[29] Cardoso CC, Pereira RTS, Koyama CA, Porto CS, Abdalla FMF. Effect of estrogen on muscarinic acetylcholine receptors in rat hippocampus. Neuroendocrinology 2004;80:379–86.
[30] Levey AI, Edmunds SM, Koliatsos V, Wiley RG, Heilman CJ. Expression of m1–m4 muscarinic acetylcholine receptor proteins in rat hippocampus and regulation by cholinergic innervation. J Neurosci 1995;15:4077–92.
[31] Oki T, Takagi Y, Inagaki S, Taketo MM, Manabe T, Matsui M, et al. Quantitative analysis of binding parameters of [^{3}H]N-methylscopolamine in central nervous system of muscarinic acetylcholine receptor knockout mice. Brain Res Mol Brain Res 2005;133:6–11.
[32] Coyle JT, McKinney M, Johnston MV, Hedreen JC. Synaptic neurochemistry of the basal forebrain cholinergic projection. Psychopharmacol Bull 1983;19:441–7.
[33] Mash DC, Flynn DD, Potter LT. Loss of M_2 muscarine receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. Science 1985;228:1115–7.
[34] Quirion R, Aubert I, Lapchak PA, Shaum RP, Teolis S, Gauthier S, et al. Muscarinic receptor subtypes in human neurodegenerative disorders: focus on Alzheimer's disease. Trends Pharmacol Sci 1989;(Suppl):80–4.
[35] Shinoe T, Matsui M, Taketo MM, Manabe T. Modulation of synaptic plasticity by physiological activation of M1 muscarinic acetylcholine receptors in the mouse hippocampus. J Neurosci 2005;25:11194–200.
[36] Porter AC, Bymaster FP, DeLapp NW, Yamada M, Wess J, Hamilton SE, et al. M_1 muscarinic receptor signaling in mouse hippocampus and cortex. Brain Res 2002; 944:82–9.
[37] Nash MS, Willets JM, Billups B, Challiss RA, Nahorski SR. Synaptic activity augments muscarinic acetylcholine receptor-stimulated inositol 1,4,5-trisphosphate production to facilitate Ca^{2+} release in hippocampal neurons. J Biol Chem 2004;279:49036–44.
[38] Willets JM, Nash MS, Challiss RA, Nahorski SR. Imaging of muscarinic acetylcholine receptor signaling in hippocampal neurons: evidence for phosphorylation-dependent and independent regulation by G-protein-coupled receptor kinases. J Neurosci 2004;24:4157–62.
[39] Power JM, Sah P. Nuclear calcium signalling evoked by cholinergic stimulation in hippocampal CA1 pyramidal neurons. J Neurosci 2002;22:3454–62.
[40] Berkeley JL, Levey AI. Cell-specific extracellular signal-regulated kinase activation by multiple G protein-coupled receptor families in hippocampus. Mol Pharmacol 2003;63:128–35.
[41] Young KW, Billups D, Nelson CP, Johnston N, Willets JM, Schell MJ, et al. Muscarinic acetylcholine receptor activation enhances hippocampal neuron excitability and potentiates synaptically evoked Ca(2+) signals via phosphatidylinositol 4,5-bisphosphate depletion. Mol Cell Neurosci 2005;30:48–57.
[42] Gibbs RB. Impairment of basal forebrain cholinergic neurons associated with aging and long-term loss of ovarian function. Exp Neurol 1998;151:289–302.
[43] Gibbs RB. Effects of gonadal hormone replacement on measures of basal forebrain cholinergic function. Neuroscience 2000;101:931–8.
[44] Everett JW. Progesterone and estrogen in the experimental control of ovulation time and other features of the estrous cycle in the rat. Endocrinology 1948;43:389–405.
[45] Nequin LG, Alvarez J, Schwartz NB. Measurement of serum steroid and gonadotropin levels and uterine and ovarian variables throughout 4 day and 5 day estrous cycles in the rat. Biol Reprod 1979;20:659–70.
[46] Doods HN, Willim KD, Boddeke HWGM, Entzeroth M. Characterization of muscarinic receptors in guinea-pig uterus. Eur J Pharmacol 1993;250:223–30.
[47] Dong GZ, Kameyama K, Rinken A, Haga T. Ligand binding properties of muscarinic acetylcholine receptor subtypes (m1–m5) expressed in baculovirus-infected insect cells. J Pharmacol Exp Ther 1995;274:378–84.

[48] Fox AW, Abel PW, Minneman KP. Activation of alpha 1-adrenoceptors increases [^{3}H]inositol metabolism in rat vas deferens and caudal artery. Eur J Pharmacol 1985;116:145–52.
[49] Abdalla FMF, Abreu LC, Porto CS. Effect of estrogen on intracellular signaling pathways linked to activation of M_2- and M_3-muscarinic acetylcholine receptors in the rat myometrium. Mol Cell Endocrinol 2000;160:17–24.
[50] Ascoli M, Pignataro OP, Segaloff DL. The inositol phosphate/diacylglycerol pathway in MA-10 Leydig tumor cells. Activation by arginine vasopressin and lack of effect of epidermal growth factor and human choriogonadotropin. J Biol Chem 1989;264:6674–81.
[51] Da Costa Jr VL, Lapa AJ, Godinho RO. Short- and long-term influences of calcitonin gene-related peptide on the synthesis of acetylcholinesterase in mammalian myotubes. Br J Pharmacol 2001;133:229–36.
[52] Ellman GI, Courtney KD, Andres Jr V, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
[53] Godinho RO, Lima-Landman MT, Souccar C, Lapa AJ. Trophic control of cholinesterase activity in a testosterone-dependent muscle of the rat: effects of castration and denervation. Exp Neurol 1987;96:558–68.
[54] Rotundo RL, Fambrough DM. Molecular forms of chicken embryo acetylcholinesterase in vitro and in vivo. Isolation and characterization. J Biol Chem 1979;254:4790–9.
[55] Snedecor GW, Cochran WG. Statistical methods, 7th ed., Ames, U.S.A.: Iowa State University Press; 1980. p. 89–233.
[56] Foster TC. Interaction of rapid signal transduction cascades and gene expression in mediating estrogen effects on memory over the life span. Front Neuroendocrinol 2005;26:51–64.
[57] Dohanich GP. Gonadal steroids, learning and memory. In: Pfaff DW, Arnold AP, Etgen AM, Farbach SE, Rubin RT, editors. Hormones brain and behavior, vol. 1. San Diego: Academic Press; 2002. p. 265–327.
[58] Gibbs RB, Gabor R. Estrogen and cognition: applying preclinical findings to clinical perspectives. J Neurosci Res 2003;74:637–43.
[59] Henderson VW, Guthrie JR, Dudley EC, Burger HG, Dennerstein L. Estrogen exposures and memory at midlife. Neurology 2003;60:1369–71.
[60] Daniel JM, Winsauer PJ, Brauner IN, Moerschbaecher JM. Estrogen improves response accuracy and attenuates the disruptive effects of delta9-THC in ovariectomized rats responding under a multiple schedule of repeated acquisition and performance. Behav Neurosci 2002;116:989–98.
[61] Jin M, Jin F, Zhang L, Chen Z, Huang H. Two estrogen replacement therapies differentially regulate expression of estrogen receptors alpha and beta in the hippocampus and cortex of ovariectomized rat. Brain Res Mol Brain Res 2005;142:107–14.
[62] Iivonen S, Heikkinen T, Puolivali J, Helisalmi S, Hiltunen M, Soininen H, et al. Effects of estradiol on spatial learning, hippocampal cytochrome P450 19, and estrogen alpha and beta mRNA levels in ovariectomized female mice. Neuroscience 2006;137:1143–52.
[63] Rouse ST, Thomas TM, Levey AL. Muscarinic acetylcholine receptor subtype, m2: diverse functional implications of differential synaptic localization. Life Sci 1997;60:1031–8.
[64] Quirion R, Wilson A, Rowe W, Aubert I, Richard J, Doods H, et al. Facilitation of acetylcholine release and cognitive performance by an M_2-muscarinic receptor antagonist in aged memory-impaired. J Neurosci 1995;15:1455–62.
[65] Carey GJ, Billard W, Binch III H, Cohen-Willians M, Crosby G, Grzelak M, et al. SCH 57790, a selective muscarinic M_2 receptor antagonist, releases acetylcholine and produces cognitive enhancement in laboratory animals. Eur J Pharmacol 2001;431:189–200.
[66] Messer Jr WL, Miller MD. Intrahippocampal injections of gallamine impair learning of a memory task. Neurosci Lett 1988;89:367–72.
[67] Daniel JM, Dohanich GP. Acetylcholine mediates the estrogen-induced increase in NMDA receptor binding in CA1 of the hippocampus and the associated improvement in working memory. J Neurosci 2001;21:6949–56.
[68] Caulfield MP, Birdsall NJM. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 1998;50:279–90.
[69] Wu X, Glinn MA, Ostrowski NL, Su Y, Ni B, Cole HW, et al. Raloxifene and estradiol benzoate both fully restore hippocampal choline acetyltransferase activity in ovariectomized rats. Brain Res 1999;847:98–104.
[70] Furlan I, Godinho RO. Developing skeletal muscle cells express functional muscarinic acetylcholine receptors coupled to different intracellular signaling systems. Br J Pharmacol 2005;146:389–96.
[71] Massoulie J. The origin of the molecular diversity and functional anchoring of cholinesterases. Neurosignals 2002;11:130–43.
[72] Inestrosa NC, Moreno RD, Fuentes ME. Monomeric amphiphilic forms of acetylcholinesterase appear early during brain development and may correspond to biosynthetic precursors of the amphiphilic G4 forms. Neurosci Lett 1994;173:155–8.
[73] Lane RM, Potkin SG, Enz A. Targeting acetylcholinesterase and butyrylcholinesterase in dementia. Int J Neuropsychopharmacol 2006;9:101–24.
[74] Das A, Dikshit M, Nath C. Role of molecular isoforms of acetylcholinesterase in learning and memory functions. Pharmacol Biochem Behav 2005;81:89–99.
[75] Siek GC, Katz LS, Fishman EB, Korosi TS, Marquis JK. Molecular forms of acetylcholinesterase in subcortical areas of normal and Alzheimer disease brain. Biol Psychiatry 1990;27:573–80.