European Journal of Pharmacology 634 (2010) 192–200

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Endocrine Pharmacology

Effects of 17β-estradiol on expression of muscarinic acetylcholine receptor subtypes and estrogen receptor α in rat hippocampus

Camila Castellan Cardoso [a], Victor Proença Ricardo [c], Roberto Frussa-Filho [c], Catarina Segreti Porto [b], Fernando Maurício Francis Abdalla [a,*]

[a] Laboratory of Pharmacology, Instituto Butantan, Brazil
[b] Section of Experimental Endocrinology, Department of Pharmacology, Universidade Federal de São Paulo — Escola Paulista de Medicina, Brazil
[c] Department of Pharmacology, Universidade Federal de São Paulo — Escola Paulista de Medicina, Brazil

ARTICLE INFO

Article history:
Received 8 June 2009
Received in revised form 1 February 2010
Accepted 15 February 2010
Available online 1 March 2010

Keywords:
Muscarinic acetylcholine receptors subtype
Estrogen receptor α
17β-estradiol
Ovariectomy
Hippocampus

ABSTRACT

The aim of the present study was to investigate the effects of 17β-estradiol on expression of muscarinic acetylcholine receptor subtypes (M_1 to M_5) and estrogen receptor α, in the rat hippocampus. Hippocampi were obtained from rats in proestrus, rats ovariectomized for 15 days, rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days, and rats ovariectomized and immediately treated with 17β-estradiol for 21 days. Expression of M_1 to M_5 was increased in hippocampi of rats ovariectomized for 15 days compared to rats in proestrus. Although this effect was abolished when replacement with 17β-estradiol started immediately after ovariectomy, the increased expression of M_1, M_3 and M_5 receptor subtypes was unchanged when replacement with 17β-estradiol started only 15 days after ovariectomy. The expression of estrogen receptor α in the hippocampus was also upregulated after ovariectomy when compared to rats in proestrus. This effect was abolished when 17β-estradiol was replaced immediately after ovariectomy, and slightly reduced when the replacement started 15 days after ovariectomy. The replacement with estrogen also had beneficial effects on cognitive function, as suggested by data obtained in the plus-maze discriminative avoidance task. In conclusion, the present results provide evidence that 17β-estradiol regulates the expression of muscarinic acetylcholine receptor subtypes and estrogen receptor α. The immediate replacement with estrogen seems critical to restore the expression of these receptors after hormonal deprivation. The understanding of the regulation of expression and intracellular signaling of the muscarinic acetylcholine receptor subtype M_1 and the estrogen receptor α may be helpful to elucidate the mechanisms involved in changes of cognitive function in postmenopausal women and in neurodegenerative diseases.

1. Introduction

Muscarinic acetylcholine receptors are widely expressed throughout the central nervous system (reviewed in Levey, 1993; Levey et al., 1995; Oki et al., 2005; Langmead et al., 2008). The predominant muscarinic acetylcholine receptor in the central nervous system is the M_1 subtype, which is expressed post-synaptically in the cortex, hippocampus, striatum and thalamus (Wei et al., 1994). M_2 muscarinic acetylcholine receptors are expressed predominantly in the brainstem and thalamus, but they are also found in cholinergic synaptic terminals in the cortex, hippocampus and striatum (Rouse et al., 1997), where they control acetylcholine release. M_3 and M_5 are expressed at much lower levels than M_1 or M_2 muscarinic acetylcholine receptors in the central nervous system, and M_3 subtype is mainly found in the cortex and hippocampus (Wei et al., 1994), whereas a very discrete localization of M_5 is found in the substantia nigra (Vilaró et al., 1990; Wei et al., 1994). M_4 muscarinic acetylcholine receptor is present in several regions of the brain, including the cortex and hippocampus, but its expression is more prominent in the striatum (Wei et al., 1994), where it contributes to control dopamine release and locomotor activity.

The role of each muscarinic acetylcholine receptor in hippocampal function remains to be explored (reviewed in Levey et al., 1995; Oki et al., 2005; Langmead et al., 2008). Pharmacological evidence suggests that the M_1 muscarinic acetylcholine receptor mediates the effects of acetylcholine on cognition (Coyle et al., 1983; Mash et al., 1985; Quirion et al., 1989), hippocampal synaptic plasticity (Berkeley and Levey, 2003; Shinoe et al., 2005) and neuronal excitability (Young et al., 2005). Thus, acetylcholine and other neurotransmitters may play an important role in cognitive processes (reviewed in Friedman, 2004).

Estrogen receptors α and β (ERα and ERβ) are present in the dendrites of hippocampal CA1 and CA3 neurons (Shughrue et al.,

* Corresponding author. Laboratory of Pharmacology, Instituto Butantan, Av. Vital Brazil 1500, São Paulo, SP, 05503-900, Brazil. Tel./fax: +55 11 3726 7222.
E-mail address: fabdalla@butantan.gov.br (F.M.F. Abdalla).

0014-2999/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.02.032

1997; Shughrue and Merchenthaler, 2000; Adams et al., 2002; Mitra et al., 2003; Hojo et al., 2008). The expression of ERα in the hippocampal dendrites spines of rats in proestrus is higher than in diestrus and in males (Romeo et al., 2005). Ovariectomy (48 days) down-regulates ERα expression (Jin et al., 2005). Premarin, a conjugated equine estrogen (treatment started 12 days after ovariectomy), down-regulates ERα expression and does not affect ERβ expression in the hippocampus and cortex. On the other hand, Progynova (estradiol valerate) up-regulates ERβ and does not affect ERα expression in hippocampus and cortex (Jin et al., 2005).

In vitro (Yokomaku et al., 2003; Kretz et al., 2004) and *in vivo* studies (Woolley and McEwen, 1992, 1993, 1994; Day and Good, 2005) have shown that estrogen modulates the synaptic function in the hippocampus (reviewed in McEwen, 2002; Toran-Allerand, 2005; Cornil et al., 2006). Furthermore, brain-derived estradiol may rapidly modulate several different types of synaptic plasticity of neurons, spinogenesis and synaptic transmission, including long-term depression and long-term potentiation (reviewed in Hojo et al., 2008). Recently, a novel G-protein-coupled estrogen receptor (GPER) has been detected in hippocampal neurons (Brailoiu et al., 2007), and further investigation may reveal the contribution of this receptor to the rapid modulation of synaptic plasticity by 17β-estradiol.

Neuromodulatory actions of 17β-estradiol have been investigated in the hippocampus (Woolley and McEwen, 1992, 1994; Bi et al., 2000; Shibuya et al., 2003). Furthermore, estrogen receptors colocalize with muscarinic acetylcholine receptors and nicotinic receptors in cultured hippocampal neurons of rat (Hosli and Hosli, 1999). We have previously shown that 17β-estradiol modulates the expression and function of muscarinic acetylcholine receptors in the rat hippocampus (Cardoso et al., 2004; Pereira et al., 2008). The muscarinic acetylcholine receptors in the hippocampus of ovariectomized (15 days) rats are upregulated when compared to rats in proestrus. This effect is abolished by 17β-estradiol replacement immediately after ovariectomy, and it is slightly reduced when 17β-estradiol treatment starts 7 days after ovariectomy (Cardoso et al., 2004). Furthermore, 17β-estradiol also modulates the activation of phospholipase Cβ and the hydrolysis of phosphoinositides mediated by muscarinic acetylcholine receptor agonist (Pereira et al., 2008). Whereas treatment with17β-estradiol initiated soon after ovariectomy improves working memory in middle-aged rats, treatment initiated after prolonged periods of estradiol deprivation (5–10 months) seems to have little effect (Gibbs, 2000a; Daniel et al., 2006). Taken together, these findings suggest that there is a critical period to initiate the estrogen therapy and preserve the expression of muscarinic acetylcholine receptors and cognitive function.

Although we have previously found that 17β-estradiol regulates the expression and function of hippocampal muscarinic acetylcholine receptors (Cardoso et al., 2004; Pereira et al., 2008), the mechanisms underlying the effects of estrogen on these receptors remain to be explored. Thus, the aim of this study was to investigate the effects of estrogen deprivation and estrogen replacement on expression of each muscarinic acetylcholine receptor subtype and on expression of the estrogen receptor α. In addition, the effects of estrogen deprivation and estrogen replacement on cognition were analyzed using the plus-maze discriminative avoidance task, a behavioral paradigm that independently evaluates memory, anxiety and motor activity.

2. Materials and methods

2.1. Animals and treatments

Female Wistar rats, 4 months old, 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 approved by the Research Ethical Committee from Instituto Butantan.

Experimental groups included the following: rats in proestrus (control), rats ovariectomized for 15 days, rats ovariectomized for 15 days and then treated with 17β-estradiol benzoate (50 μg/kg, s.c., every other day for 7 days), and rats ovariectomized and immediately treated with 17β-estradiol benzoate (50 μg/kg, s.c. every other day for 21 days). The final injection of 17β-estradiol was given one day before euthanasia. These experimental groups were chosen based on a previous study from our laboratory, which demonstrated a time-dependent increase in the number of muscarinic acetylcholine receptors in the hippocampus after ovariectomy when compared to control rats in proestrus, with a maximum increase after 15 days of ovariectomy (Cardoso et al., 2004). The replacement with estradiol for 21 days immediately after ovariectomy avoided this effect, but treatment with estradiol for 7 days after 15 days of ovariectomy only slightly changed the number of muscarinic acetylcholine receptors in the hippocampus. Our results are in accordance to others in the literature, which show that the beneficial effects of estrogen replacement on cholinergic and cognitive function are only observed if initiated within a set period of time (Gibbs, 1997, 1998, 2000b; Daniel et al., 2006).

Vaginal smears were taken for histological determination of estrous cycle (Everett, 1948) between 08:00 a.m. to 09:00 a.m., and the animals were immediately sacrificed by decapitation to isolate the hippocampus. Rats that showed at least two consecutive 4 or 5 day cycles were used in all subsequent experiments. In diestrus, the estradiol level gradually increases during the day, and reaches 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 (Nequin et al., 1979). Thus, rats in proestrus used in this study present high levels of 17β-estradiol and low levels of progesterone.

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. Immunoprecipitation assays for detection of mAChR subtypes

2.2.1. Membrane preparation

Hippocampi were isolated from all experimental groups, minced and homogenized in 25 mM Tris–HCl, pH 7.4 (containing 0.3 M sucrose, 5 mM $MgCl_2$, 1 mM EDTA and 1 mM PMSF) at 4 °C, with an Ultra-Turrax homogenizer. Membrane preparation was obtained as previously described (Maróstica et al., 2001; Cardoso et al., 2004). Protein concentration was determined according to Bradford (1976). Uterus, heart, submandibular gland and striatum membranes were also obtained from all experimental groups and the membrane preparations were obtained as described above.

2.2.2. Receptor labeling, solubilization and immunoprecipitation

Muscarinic acetylcholine receptors present in the membranes were radiolabeled with 2 nM of [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB) (specific activity 43 Ci/mmol), a subtype-nonselective antagonist with a high affinity and very slow dissociation. Briefly, membrane preparation (100 μg, in duplicate) was incubated with 2 nM [^{3}H]QNB (maximum binding obtained in saturation binding experiments, reported by Cardoso et al., 2004) in the absence (total binding) and presence of 1 μM atropine (nonspecific binding) for 1 h at 30 C (Cardoso et al., 2004). The specific binding was calculated as the difference between the total and nonspecific binding. After incubation, the binding reaction was stopped by cooling on ice and rapid filtration through GF/B glass fiber filter (Whatman International Ltd, Maidstone, UK) under vacuum. The filters were washed three times with the ice-cold buffer, partially dried under vacuum, and placed in

scintillation vials containing OptiPhase HiSafe 3 (Perkin Elmer, Loughborough Leics., UK). The amount of radioactivity was determined in scintillation β-counter (LS 6500 IC, Beckmann, USA).

In another series of experiments, membrane preparation (100 μg, in duplicate) was incubated with 2 nM [^{3}H]QNB in the absence (total binding) and presence of 1 μM atropine (nonspecific binding) for 1 h at 30 °C. [^{3}H]QNB-receptors were solubilized with digitonin and sodium deoxycholate (Shiozaki et al., 1999; Myslivecek et al., 2008) in 25 mM Tris–HCl, pH 7.4 (containing 5 mM $MgCl_2$, 0.1% sodium deoxycholate, 1 mM EDTA and 1 mM PMSF) for 1 h at 4 °C. After that, 1.0% digitonin was added. The sample was incubated for 15 min at 4 °C and centrifuged at 15,000×*g* for 20 min at 4 °C. The supernatant, containing the solubilized receptors, was collected and the amount of radioactivity was determined.

The supernatant, containing the solubilized receptors, was also immunoprecipitated by subtype-specific antibodies. The supernatant (100 μl), containing solubilized receptors, was incubated with 0.5 μg of polyclonal antibodies raised against rabbit muscarinic acetylcholine receptor subtypes [M_1 (H-120, sc 9106), M_2 (H170, sc 9107), M_3 (H210, sc 9108) M_4 (H175, sc 9109) and M_5 (H-197, sc 9110), Santa Cruz Biotechnology, CA, USA] (specific) or with IgG (nonspecific) (Sigma Co, MO, USA) in 25 mM Tris–HCl, pH 7.4 (containing 5 mM $MgCl_2$, 0.1% sodium deoxycholate, 1 mM EDTA and 1 mM PMSF) for 4 h at 4 °C. After this incubation, 20 μl of Pansorbin (Calbiochem, CA, USA) was added and incubated under agitation for 1 h at 4 °C and then centrifuged at 15,000×*g* for 10 min (Shiozaki et al., 1999). The pellet was washed with 200 μl Tris–HCl, pH 7.4 (containing 5 mM $MgCl_2$, 1 mM EDTA and 1 mM PMSF), and centrifuged again at 15,000×*g* for 10 min. The amount of radioactivity was determined in 50 μl Pansorbin deposit ([^{3}H]QNB-subtype-specific antibodies) (total) or Pansorbin deposit ([^{3}H]QNB-IgG) (nonspecific). The specific immunoprecipitation (difference between the total and nonspecific) was determined and the results were expressed as fmol/mg protein.

The percentage of specific immunoprecipitation for each muscarinic acetylcholine receptor subtype was calculated by the ratio between the [^{3}H]QNB counting in the Pansorbin pellet and the total counting (counting of supernatant and Pansorbin pellet) (Shiozaki et al., 1999).

2.3. Western blot for detection of estrogen receptor α

The expression of the estrogen receptor α was determined by Western blot as previously described by Lucas et al. (2008), with the following modifications. The entire hippocampus was minced and homogenized in ice-cold lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% sodium deoxycholate, 1% NP-40, 1 mM EDTA, 0.1% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF, Sigma) with an Ultra-Turrax homogenizer. The homogenate was centrifuged at 3000×*g* for 30 min at 4 ° C. Aliquots of the supernatant (50 μg protein/lane) were incubated with sample buffer containing 10%β-mercaptoethanol and subjected to 7.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred onto nitrocellulose membranes (0.45 μm pore size, Hybond ECL, Amersham Biosciences, Freiburg, Germany), 1 h, 100 V at 4 °C. Membranes were blocked in phosphate-buffered saline (PBS, 0.14 mM NaCl, 80 mM Na_2HPO_4, 140 mM $Na_2HPO_4 \cdot H_2O$, pH 8.0) containing 0.2% Tween 20, 10% glycerol and 5% nonfat dry milk, for 1 h at room temperature. Estrogen receptor α was detected by probing membranes with rabbit anti-estrogen receptor α antibody (MC-20, sc 542, Santa Cruz Biotechnology, CA, USA) diluted 1:200 in blocking solution, for 3 h at 4 °C. Membranes were washed in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) (TBS) and the antigens bound to primary antibodies were detected with the horseradish peroxidase (HRP)-conjugated anti-IgG secondary antibodies (goat anti-rabbit, 1:20,000, Pierce Biotechnology, IL, USA) for 1 h at room temperature. Thereafter, the membranes were washed with TBS-T and immunoreactive bands were visualized onto pre-flashed Biomax XAR film (Eastman Kodak Co., NY, USA) by enhanced chemiluminescence reagent (Luminol, PerkinElmer, MA, USA). β-actin levels were monitored on the same blot to ensure equal protein loading, using anti-β-actin antibody (1:1000, Sigma) for 2 h at room temperature. In negative controls, primary antibodies were preadsorbed with the respective peptide immunogen and processed as described above. Apparent molecular weights of protein bands were determined from molecular weight standards (Prestained protein marker, low range 14–90 kDa, Bio-Rad Laboratories, CA, USA).

Band intensities of estrogen receptor α and β-actin from individual experiments were quantified by densitometric analysis of linear-range autoradiograms using Epson Expression 1680 scanner (Epson America Inc., CA, USA) and Quick Scan 2000 WIN software (Helena Laboratories, TX, USA). Results were normalized to the β-actin expression in each experiment and plotted (mean ± S.E.M.) as % of control (rats in proestrus).

2.4. Plus-maze discriminative avoidance task

Plus-maze discriminative avoidance task was determined as previously described by Silva et al. (1997). Briefly, twelve rats obtained from each experimental group were submitted to the discriminative avoidance conditioning, which was performed in a modified elevated plus-maze, made of wood, containing two open arms (50×15 cm) opposite to two enclosed arms (50×15×40 cm). A 100 W lamp was placed exactly over the middle of one of the enclosed arms (aversive enclosed arm). The floor of this arm was covered with black rubber. Each animal was placed into the center of the apparatus and, over a period of 10 min, every time the animal entered the enclosed arm containing the lamp, an aversive situation was produced until the animal left the arm. The aversive stimuli were the 100 W light and a frontal hot air blow produced by a 700 W hair drier placed above the end of the aversive enclosed arm. On each side of the plus-maze discriminative avoidance apparatus, there were different extramaze visual cues (door, window, cupboard and observer) that rats could use to distinguish the location of the different arms of the maze. Test was performed 1 day after conditioning. In this test, the animals were again placed in the apparatus for 3 min without receiving the aversive stimulation. The time spent in the aversive and in the non-aversive enclosed arms, as well as the time spent in the open arms was scored during the training and testing sessions. In this new discriminative avoidance test, learning/memory was evaluated by the time spent in the aversive vs non-aversive enclosed arms, and anxiety was evaluated by the percent of time spent in the open arms of the apparatus.

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. Data were analyzed by ANOVA followed by Newman–Keuls test for multiple comparisons or by Duncan's test in the plus-maze discriminative avoidance task (Snedecor and Cochran, 1980). P values <0.05 were accepted as significant.

3. Results

3.1. Specific binding of [^{3}H]QNB to hippocampal membranes and solubilized receptors

Ovariectomy for 15 days increased the specific binding of [^{3}H]QNB to hippocampal membranes by 2-fold, compared to rats in proestrus (control) ($P<0.05$). 17β-estradiol replacement starting 2 weeks after ovariectomy did not affect the increase in [^{3}H]QNB binding observed after ovariectomy ($P>0.05$). However, immediate replacement with 17β-estradiol throughout the post-ovariectomy period prevented the

effect of ovariectomy on [^{3}H]QNB binding ($P<0.05$) (Fig. 1A). A similar profile of [^{3}H]QNB binding was obtained using solubilized receptors from the hippocampal membranes (Fig. 1B).

3.2. Effects of 17β-estradiol on the expression of muscarinic acetylcholine receptor subtypes

The immunoprecipitation with specific antibodies allowed to estimate the relative amounts of each muscarinic acetylcholine receptor (M_1 to M_5) in the hippocampus of rats in proestrus (control), rats ovariectomized for 15 days, rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days, and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (Table 1).The uterus was used as a negative control for M_1 receptors, and heart, submandibular gland and striatum were used as positive controls for M_2, M_3 and M_4 receptors, respectively. These results confirmed that the M_1 muscarinic acetylcholine receptor is not the only subtype present in the hippocampus, but it largely predominates over the other muscarinic acetylcholine receptors in the hippocampus obtained from rats in proestrus (control). A similar amount of precipitated muscarinic acetylcholine receptors was also obtained in the hippocampus from rats ovariectomized for 15 days, rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (Table 1).

The quantification of each muscarinic acetylcholine receptor subtype in the hippocampus from all experimental groups is shown in Table 2. Ovariectomy for 15 days induced an increase in the expression of all muscarinic acetylcholine receptor subtypes (M_1 to M_5) compared to control ($P<0.05$). 17β-estradiol replacement during the final week of a 3 week period of ovariectomy did not affect the effects of ovariectomy ($P>0.05$). On the other hand replacement with 17β-estradiol throughout the post-ovariectomy period prevented the effect of ovariectomy ($P<0.05$) (Table 2).

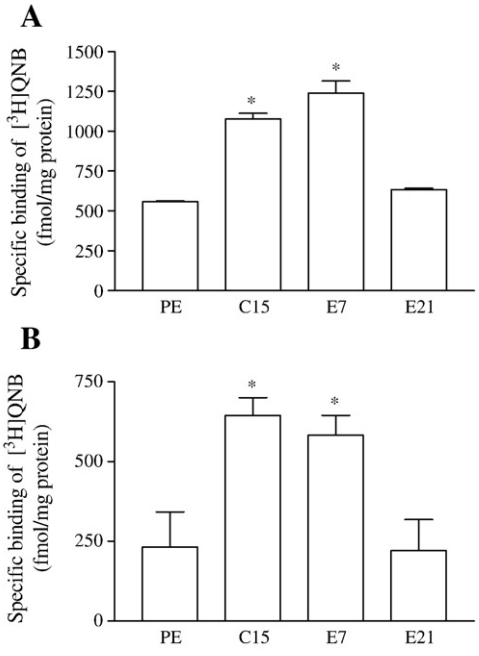

Fig. 1. Effects of ovariectomy and 17β-estradiol on expression of muscarinic acetylcholine receptors in rat hippocampus. Specific binding of [^{3}H]QNB in hippocampus membrane (A) and solubilized receptor (B) obtained from rats in proestrus (PE), rats ovariectomized for 15 days (C15), rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days (E7), and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). Hippocampus membranes and solubilized receptor were incubated with 2 nM [^{3}H]QNB in the absence (total binding) and presence (nonspecific binding) of 1 μM atropine. The specific binding was calculated as the difference between the total and nonspecific binding. Values are expressed as mean ± S.E.M. of 5 experiments performed in duplicate. *$P<0.05$ vs PE or E21 groups.

Table 1
Proportion of precipitated muscarinic acetylcholine receptor subtypes in hippocampus obtained from rats in proestrus (PE), rats ovariectomized for 15 days (C15), rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days (E7) and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). Data are mean ± S.E.M. of number of experiments in parenthesis, performed in duplicate.

	PE	C15	E7	E21
M_1	61.16 ± 5.75	62.25 ± 3.22	60.40 ± 0.93	54.90 ± 2.83
(%)	(6)	(4)	(5)	(7)
M_2	23.83 ± 1.70	18.66 ± 2.18	23.50 ± 3.61	24.50 ± 3.50
(%)	(6)	(6)	(4)	(3)
M_3	8.48 ± 0.95	6.90 ± 1.00	7.50 ± 0.93	8.30 ± 0.26
(%)	(5)	(6)	(5)	(4)
M_4	6.06 ± 1.01	6.67 ± 0.48	7.76 ± 0.30	5.33 ± 0.43
(%)	(5)	(4)	(5)	(6)
M_5	0.58 ± 0.02	0.47 ± 0.04	0.48 ± 0.03	0.57 ± 0.02
(%)	(3)	(3)	(3)	(3)
Uterus	0.75 ± 0.10	1.05 ± 0.35	0.58 ± 0.20	0.76 ± 0.15
(%)	(3)	(3)	(3)	(3)
Heart	89.30 ± 3.37	87.00 ± 5.00	85.00 ± 2.00	83.00 ± 2.00
(%)	(4)	(3)	(3)	(3)
Submandibular gland	78.00 ± 7.50	65.50 ± 13.50	73.00 ± 2.00	76.50 ± 2.50
(%)	(3)	(3)	(3)	(3)
Striatum	55.75 ± 10.60	56.56 ± 9.50	49.00 ± 3.50	52.55 ± 6.02
(%)	(4)	(3)	(3)	(3)

No significant difference was detected for each muscarinic acetylcholine receptor subtypes among the different experimental groups (ANOVA; $P>0.05$). Uterus was used as negative control for M_1 muscarinic acetylcholine receptors and heart, submandibular gland and striatum were used as positive control for M_2, M_3 and M_4 muscarinic acetylcholine receptors, respectively.

3.3. Effects of 17β-estradiol on the expression of estrogen receptor α

Ovariectomy for 15 days increased the expression of estrogen receptor α in hippocampus (81.50 ± 22.44%, $n=6$, $P<0.05$) compared to control (rats in proestrus). 17β-estradiol replacement during the final week of a 3 weeks period of ovariectomy only partially prevented the effect of ovariectomy (23.75 ± 9.80%, $n=6$, $P<0.05$). On the other hand, replacement with 17β-estradiol throughout the post-ovariectomy period completely prevented the effect of ovariectomy on estrogen receptor α expression ($P<0.05$) (Fig. 2).

3.4. Effects of 17β-estradiol on plus-maze discriminative avoidance task

3.4.1. Time spent in the aversive × non-aversive enclosed arms

In the training session (Fig. 3A), two-way ANOVA revealed significant effects among experimental groups [$F(3,92)=4.34$; $P<0.05$] and arm types [$F(1,92)=2047.60$; $P<0.001$], and experimental group × arm type interaction [$F(3,92)=4.60$; $P<0.001$]. Indeed, post hoc analysis showed that all experimental groups spent less time in the aversive enclosed arm than in the non-aversive enclosed arm. All experimental groups spent more time in the non-aversive enclosed arm compared to control group (rats in proestrus). In the test session (Fig. 3B), two-way ANOVA revealed a significant effect only between the arm types [$F(1,92)=60.81$; $P<0.001$], but not among experimental groups [$F(3,92)=0.14$; $P>0.05$] or experimental group × arm type interaction [$F(3,92)=0.37$; $P>0.05$]. Post hoc analysis showed that all experimental groups spent less time in the aversive enclosed arm than in the non-aversive enclosed arm.

3.4.2. Percent of time spent in the open arms

In the training session (Fig. 4A), one-way ANOVA revealed significant differences among experimental groups [$F(3,46)=4.65$; $P<0.01$]. Indeed, post hoc analysis showed a significant decrease in the time percentage spent in the open arms by all experimental groups

Table 2
Muscarinic acetylcholine receptor subtypes expression in hippocampus obtained from rats in proestrus (PE), ovariectomized for 15 days (C15), rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days (E7) and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21).

	M_1 (fmol/mg protein)	M_2 (fmol/mg protein)	M_3 (fmol/mg protein)	M_4 (fmol/mg protein)	M_5 (fmol/mg protein)
PE	61.00 ± 4.66^a (5)	25.04 ± 3.00^a (5)	11.43 ± 1.52^a (5)	6.70 ± 0.72^a (5)	0.58 ± 0.08^a (3)
C15	174.03 ± 9.74^b (4)	54.32 ± 1.63^b (5)	21.06 ± 1.15^b (5)	17.45 ± 0.42^b (5)	1.53 ± 0.11^b (3)
E7	180.01 ± 6.92^b (5)	69.92 ± 3.10^c (5)	19.26 ± 0.95^b (5)	21.84 ± 0.50^c (5)	1.35 ± 0.05^b (3)
E21	84.47 ± 1.07^a (7)	35.73 ± 3.53^a (5)	14.81 ± 1.03^a (4)	7.34 ± 0.75^a (5)	0.83 ± 0.03^a (3)

Data are mean ± S.E.M. of number of experiments in parenthesis, performed in duplicate.
Different letters indicate significant differences among values obtained for different experimental groups ($P<0.05$, Newman–Keuls test).

compared to control group (rats in proestrus). No significant differences were observed in the test session (Fig. 4B) [$F(3,46) = 1.20$; $P>0.05$].

3.4.3. Total number of entries

In the training session (Fig. 5A), one-way ANOVA revealed significant differences among experimental groups [$F(3,46) = 4.28$; $P<0.05$]. Indeed, post hoc analysis revealed that all experimental groups presented a significant decrease in the total number of entries compared to control group (rats in proestrus). In the test session (Fig. 5B), one-way ANOVA revealed significant differences among experimental groups [$F(3,46) = 4.68$; $P<0.01$]. Indeed, post hoc analysis revealed that all experimental groups presented a significant decrease in the total number of entries compared to control group.

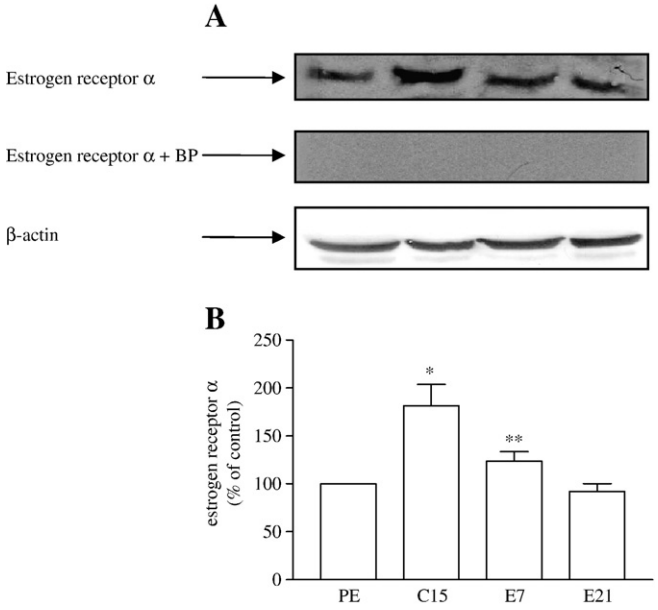

Fig. 2. Effects of ovariectomy and 17β-estradiol replacement on estrogen receptor α expression in rat hippocampus. Total protein extracts (50 μg protein/lane) obtained from hippocampus of rats in proestrus (PE), rats ovariectomized for 15 days (C15), rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days (E7), and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21) were subjected to 10% SDS-PAGE. A. Western blot using the anti-estrogen receptor α revealed specific bands (top panel). Negative controls were performed using the primary antibody preadsorbed with the respective blocking peptide (BP) (middle panel). β-actin levels were monitored on the same blot to ensure equal protein loading (bottom panel). The relative positions of estrogen receptor α and β-actin proteins were determined from molecular weight standards. The data shown are representative of at least 6 independent experiments. B. The bars represent the densitometric analysis of the Western blot. Results were normalized to β-actin expression in each sample and plotted (mean ± S.E.M.) in relation to control (rat in proestrus, PE = 100%). *$P<0.05$ vs PE, E7 or E21 groups; **$P<0.05$ vs PE, C15 or E21 groups.

4. Discussion

The results of the present study show that muscarinic acetylcholine receptor subtypes and estrogen receptor α are regulated by 17β-estradiol. A critical period to start estrogen replacement after hormonal deprivation is important to regulate the expression and consequently the function of these receptors. Further experimental approaches will be important to clarify whether the duration of 17β-estradiol treatment is also important for this regulation.

Gonadal steroids may play an important role on cholinergic neurotransmission in the basal forebrain at pre- and post-synaptic levels (Toran-Allerand et al., 1992; Gibbs, 1996; Daniel et al., 2006; Towart et al., 2003; Milner et al., 2005). The neurobiological mechanisms underlying these effects are currently unknown, but most likely involve effects of estrogen on the survival, connectivity and function of specific neural systems in the hippocampus. Previous studies from our laboratory showed that muscarinic acetylcholine receptors are upregulated in the hippocampus after ovariectomy (15 days), compared to rats in proestrus. This effect is abolished when

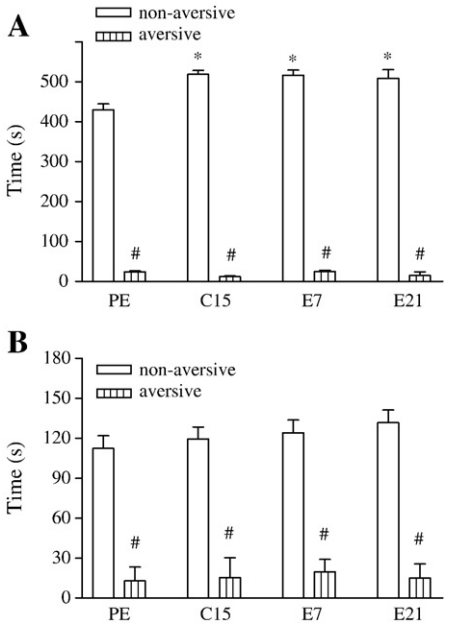

Fig. 3. Effects of ovariectomy and 17β-estradiol replacement on rats tested in the plus-maze discriminative avoidance task. Time (s) spent in the aversive and non-aversive enclosed arm in the training (A) and test sessions (B) presented by rats in proestrus (PE), rats ovariectomized for 15 days (C15), rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days (E7), and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). Values are expressed as mean ± S.E.M. *$P<0.05$ vs PE group; #$P<0.05$ vs time spent in non-aversive enclosed arm.

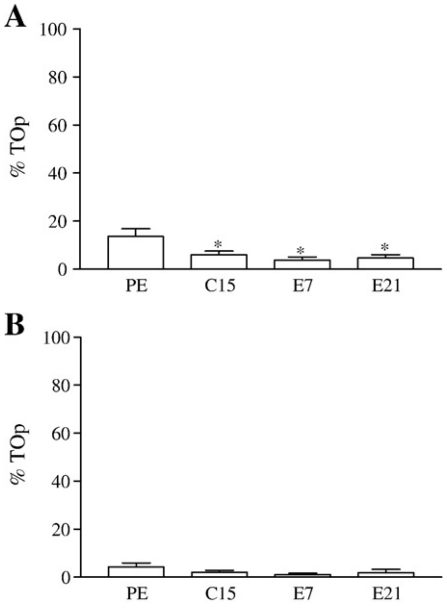

Fig. 4. Effects of ovariectomy and 17β-estradiol on rats tested in the plus-maze discriminative avoidance task. Percent time spent in the open arms (%TOp) in the training (A) and test sessions (B) presented by rats in proestrus (PE), rats ovariectomized for 15 days (C15), rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days (E7), and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). Values are expressed as mean ± S.E.M. *$P<0.05$ vs PE group.

17β-estradiol is replaced immediately after ovariectomy, and it is slightly reduced when treatment with 17β-estradiol begins 7 days after ovariectomy (Cardoso et al., 2004). As shown in the present study, the same results are found either using hippocampus membranes or solubilized receptors.

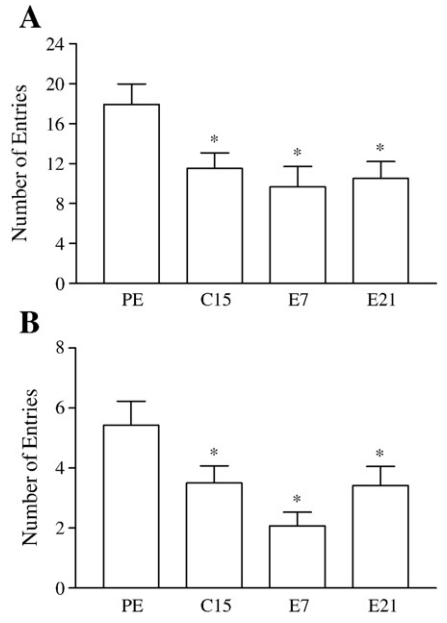

Fig. 5. Effects of ovariectomy and 17β-estradiol on rats tested in the plus-maze discriminative avoidance task. Number of entries in any arm in the training (A) and test sessions (B) presented by rats in proestrus (PE), rats ovariectomized for 15 days (C15), rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days (E7), and rats ovariectomized and immediately treated with 17β-estradiol for 21 days (E21). Values are expressed as mean ± S.E.M. *$P<0.05$ vs PE group.

Our immunoprecipitation studies confirmed that although hippocampus expresses all muscarinic receptor subtypes, the population of M_1 receptors is predominant. In addition, the amount of M_2 in the hippocampus is higher than the amount of M_3, M_4 and M_5 muscarinic acetylcholine receptors. These results are in accordance with other reports in the literature. In hippocampus of male rats immunoprecipitation studies also indicated a predominance of M_1 subtype (55%) and low expression of M_2 (17%), M_3 (10%) and M_4 (15%) (Levey, 1993). Similarly, binding studies using [^{3}H]methyl-scopolamine showed 69% expression of M_1, 30% of M_2, 17% of M_3, 9% of M_4 and 1% of M_5 receptors in the hippocampus of male rats (Oki et al., 2005). It has been suggested that M_1 receptors in the cerebral cortex and hippocampus may play an important role in higher cognitive processes, such as learning and memory (reviewed in Friedman, 2004). The prevalence of M_1 receptors in the hippocampus reinforces this idea. To validate the immunoprecipitation assay, the uterus obtained from rats in proestrus was used as a negative control for M_1 subtype (Doods et al., 1993; Abdalla et al., 2004). The immunoprecipitation values for M_1 muscarinic acetylcholine receptors (0.75 ± 0.10%) obtained in the present study are similar to those reported in the literature in membranes of the rabbit uterus (2.50% and 0.70%) (Dörje et al., 1991; Brandes and Ruggieri, 1995). Heart, submandibular gland and striatum obtained from rats in proestrus were used as positive controls for M_2, M_3 and M_4 muscarinic acetylcholine receptor subtypes, respectively (Doods et al., 1993; Levey, 1993). The expression of M_2 in heart (89.30 ± 3.37%), M_3 in submandibular gland (78.00 ± 7.50%) and M_4 in striatum (55.75 ± 10.16%) are similar to those found in the literature for M_2 in rabbit heart (88 and 89%) (Dörje et al., 1991; Brandes and Ruggieri, 1995), M_3 in rabbit submandibular gland (65%) (Dörje et al., 1991) and M_4 muscarinic acetylcholine receptors in rat striatum (46%) (Levey, 1993). In the present study, similar results were obtained in uterus, heart, submandibular gland and striatum obtained from rats ovariectomized for 15 days, rats ovariectomized for 15 days and then treated with 17β-estradiol for 7 days and rats ovariectomized and immediately treated with 17β-estradiol for 21 days.

With the development of specific antibodies targeted to specific peptide sequences in each of the five subtypes of muscarinic acetylcholine receptors (Luthin et al., 1988; Levey, 1990; Levey et al., 1991; Wall et al., 1991, Li et al., 1991; Mayanil et al., 1991, Wall et al., 1992) it was possible to immunoprecipitate and determine the expression of these receptors in a number of tissues of several species. This method is often more sensitive than Western blot, providing a valuable tool to evaluate the amount and proportion of muscarinic receptor subtypes in biological samples. Using this method we presently determined that ovariectomy for 15 days induced an upregulation of M_1 to M_5 muscarinic acetylcholine receptor expression compared to rats in proestrus. This effect was abolished when 17β-estradiol was replaced immediately after ovariectomy. On the other hand, 17β-estradiol replacement during the final week of a 3 week period of ovariectomy had no effect on M_1, M_3 and M_5 muscarinic acetylcholine receptors expression and upregulated M_2 and M_4 muscarinic acetylcholine receptors. Taken together, these results suggest that a critical period with regard to the initiation of estrogen replacement and/or the duration of exposure to 17β-estradiol may be important to regulate the expression of muscarinic acetylcholine receptor subtypes. Similarly, El-Bakri et al. (2002) reported that ovariectomy for 10 weeks upregulated M_4 receptor in rat hippocampus. 17β-estradiol treatment for 10 weeks, starting 2 days after ovariectomy, prevented this effect. Further experimental approaches will be important to clarify the mechanisms involved and the functional significance of the upregulation of M_2 and M_4 muscarinic acetylcholine receptor subtypes. Several mechanisms may contribute to the effects of estrogen on the hippocampus. First, 17β-estradiol may directly increase muscarinic acetylcholine receptor gene expression in the hippocampus. Alternatively, 17β-estradiol may indirectly stimulate muscarinic acetylcholine receptors gene expression by increasing the expression of growth factors in hippocampus (Toran-Allerand et al., 1992).

Previous studies have mapped the distributions of estrogen receptors α and β in rat brain. Both subtypes are present in the hippocampus, a region specifically related with cognition (Osterlund et al., 2000a,b; Towart et al., 2003; Milner et al., 2005). Considering that the expression of estrogen receptor α is higher than estrogen receptor β in rat hippocampus (Mitra et al., 2003; Jin et al., 2005), in the present study we determined the effect of 17β-estradiol on estrogen receptor α expression. Estrogen receptor α was upregulated in the hippocampus after ovariectomy (15 days) compared to rats in proestrus. This effect was abolished when 17β-estradiol was replaced immediately after ovariectomy, and it was slightly reduced when 17β-estradiol treatment began 7 days after ovariectomy.

Estrogen receptors are colocalized with muscarinic acetylcholine receptors in cultured hippocampal neurons of the rat (Hosli and Hosli, 1999), and in the present study both receptors were modulated by 17β-estradiol. Further investigation on the crosstalking between ERα and M_1 muscarinic acetylcholine receptor signaling will provide new insight into the hippocampus function and neuronal diseases. The stimulation of both receptors causes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), producing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, an action mediated by the enzyme phospholipase Cβ (Marino et al., 2001; Porter et al., 2002; Nash et al., 2004; Willets et al., 2004). Recent studies have demonstrated that estrogens induce calcium influx via L-type voltage-gated channels in hippocampal neurons and play an important role in synaptic plasticity, neuroprotection, and memory formation (Sarkar et al., 2008). Muscarinic acetylcholine receptors also modulate specific ionic conductances in different cells (reviewed in Lanzafame et al., 2003). Furthermore, both receptors can activate extracellular signal-regulated kinases (reviewed in Lucas et al., 2008; Manavathi and Kumar, 2006; Berkeley and Levey, 2003).

To gain insight into the role of estrogen in cognition, we used the plus-maze discriminative avoidance task, a behavioral paradigm which independently and concomitantly evaluates memory, anxiety and motor activity. In this animal model, the avoidance of the aversive enclosed arm upon testing (evaluated by the comparison between the time spent in the aversive and non-aversive enclosed arms) is considered a measurement of retention, since amnesic manipulations decrease (Silva and Frussa-Filho, 2000; Silva et al., 2002; Calzavara et al., 2004; Patti et al., 2005) and memory-improving treatments increase this parameter (Silva et al., 2002). In addition, as mentioned elsewhere, this behavioral model has been shown to concomitantly evaluate anxiety-related behaviors (Silva and Frussa-Filho, 2000; Silva et al., 2002; Calzavara et al., 2004; Silva et al., 2004) and locomotor activity (Silva et al., 2002; Castro et al., 2005; Carvalho et al., 2006; Kameda et al., 2007).

In the present study, all experimental groups presented a similar pattern of decrease in the time spent in the aversive compared to the non-aversive enclosed arm in the test session, indicating similar levels of memory retention. On the other hand, the expression of muscarinic acetylcholine receptor in the ovariectomized rats was higher than in all experimental groups. The gonadal steroids play an important role in the cholinergic neurotransmission at pre- and post-synaptic levels (Toran-Allerand et al., 1992; Gibbs, 1996; Daniel et al., 2006; Towart et al., 2003; Milner et al., 2005). Thus, we hypothesize that a reduction of estrogen levels would affect survival, connectivity and function of cholinergic systems in the hippocampus, which in turn could reduce the release of acetylcholine and induce a compensatory upregulation of muscarinic acetylcholine receptors in order to avoid possible cognitive impairments. On the other hand, the immediate estrogen replacement for 21 days may prevent the effect of ovariectomy on upregulation of muscarinic acetylcholine receptors and on memory impairment. These results support the idea that estrogen replacement has beneficial effects on cholinergic and cognitive function if initiated within a set period of time.

Although beyond the scope of the present study, an interesting finding was observed concerning the anxiety levels of the animals. Ovariectomy induced an increase in anxiety-like behavior (revealed by the decrease in the time spent in the open arms of the plus-maze discriminative avoidance task), supporting previous data (de Chaves et al., 2009). This increase in anxiety may have lead to a reduction in the exploratory behavior of the rodents, reflected by the reduction in the number of entries in any of the arms of the apparatus. Similar results have already been described using different behavioral paradigms, such as the open-field (Bouwknecht et al., 2007). In parallel, the increase in anxiety-like behavior induced by ovariectomy may also have increased the reactivity to aversive stimuli, since all ovariectomized animals spent more time in the non-aversive arm of the apparatus. Estrogen replacement did not affect these behavioral alterations. Thus, further studies are required in order to better address the possible mechanisms underlying these processes.

In conclusion, the present results provide evidence that the expression of muscarinic acetylcholine receptor subtypes and estrogen receptor α are regulated by 17β-estradiol. A critical period with regard to the initiation of estrogen replacement may play a role in this regulation. The understanding of the regulation of the intracellular signaling of M_1 muscarinic acetylcholine receptor subtype and estrogen receptor α by estrogen may be helpful to clarify the role of estrogen in the physiological cognitive function and in alterations observed in postmenopausal women and neurodegenerative diseases.

Acknowledgements

This study was supported by grant from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant 06/53285-9), Brazil. Researcher fellowship (C.S. Porto) was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Master fellowship supported by CNPq (C.C. Cardoso).

References

Abdalla, F.M.F., Maróstica, E., Picarelli, Z.P., Abreu, L.C., Avellar, M.C., Porto, C.S., 2004. Effect of estrogen on muscarinic acetylcholine receptor expression in rat myometrium. Mol. Cell. Endocrinol. 213, 139–148.

Adams, M.M., Fink, S.E., Shah, R.A., Janssen, W.G., Hayashi, S., Milner, T.A., McEwen, B.S., Morrison, J.H., 2002. Estrogen and aging affect the subcellular distribution of estrogen receptor-alpha in the hippocampus of female rats. J. Neurosci. 22, 3608–3614.

Berkeley, J.L., Levey, A., 2003. Cell-specific extracellular signal-regulated kinase activation by multiple G protein-coupled receptor families in hippocampus. Mol. Pharmacol. 63, 128–135.

Bi, R., Broutman, G., Foy, M.R., Thompson, R.F., Baudry, M., 2000. The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus. Proc. Natl. Acad. Sci. U.S.A. 97, 3602–3607.

Bouwknecht, J.A., Spiga, F., Staub, D.R., Hale, M.W., Shekhar, A., Lowry, C.A., 2007. Differential effects of exposure to low-light or high-light open-field on anxiety-related behaviors: relationship to c-Fos expression in serotonergic and non-serotonergic neurons in the dorsal raphe nucleus. Brain Res. Bull. 72, 32–43.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brailoiu, E., Dun, S.L., Brailoiu, G.C., Mizuo, K., Sklar, L.A., Oprea, T.I., Prossnitz, E.R., Dun, N.J., 2007. Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. J. Endocrinol. 193, 311–321.

Brandes, S.B., Ruggieri, M.R., 1995. Muscarinic receptor subtypes in normal, fetal, and gravid rabbit bladder, heart and uterus. Adv. Exp. Med. Biol. 385, 241–249.

Calzavara, M.B., Lopez, G.B., Abílio, V.C., Silva, R.H., Frussa-Filho, R., 2004. Role of anxiety levels in memory performance of spontaneously hypertensive rats. Behav Pharmacol 15, 545–553.

Cardoso, C.C., Pereira, R.T.S., Koyama, C.A., Porto, C.S., Abdalla, F.M.F., 2004. Effect of estrogen on muscarinic acetylcholine receptors in rat hippocampus. Neuroendocrinology 80, 379–386.

Carvalho, R.C., Patti, C.L., Takatsu-Coleman, A.L., Kameda, S.R., Souza, C.F., Garcez do Carmo, L., Abílio, V.C, Frussa-Filho, R., Silva, R.H., 2006. Effects of reserpine on the plus-maze discriminative avoidance task: dissociation between memory and motor impairments. Brain Res. 1122, 179–183.

Castro, J.P., Frussa-Flho, R., Fukushiro, D.F., Chinen, C.C., Abilio, V.C., Silva, R.H., 2005. Effects of long-term continuous exposure to light on memory and anxiety. Physiol. Behav. 86, 218–223.

Cornil, C.A., Ball, G.F., Balthazart, J., 2006. Functional significance of the rapid regulation of brain estrogen action: where do the estrogens come from? Brain Res. 1126, 2–26.

Coyle, J.T., McKinney, M., Johnston, M.V., Hedreen, J.C., 1983. Synaptic neurochemistry of the basal forebrain cholinergic projection. Psychopharmacol. Bull. 19, 441–447.

Daniel, J.M., Hulst, J.L., Berbling, J.L., 2006. 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 147, 607–614.
Day, M., Good, M., 2005. Ovariectomy-induced disruption of long-term synaptic depression in the hippocampal CA1 region *in vivo* is attenuated with chronic estrogen replacement. Neurobiol. Learn. Mem. 83, 13–21.
de Chaves, G., Moretti, M., Castro, A.A., Dagostin, W., da Silva, G.G., Boeck, C.R., Quevedo, J., Gavioli, E.C., 2009. Effects of long-term ovariectomy on anxiety and behavioral despair in rats. Physiol. Behav. 97, 420–425.
Doods, H.N., Willim, K.D., Boddeke, H.W.G.M., Entzeroth, M., 1993. Characterization of muscarinic receptors in guinea-pig uterus. Eur. J. Pharmacol. 250, 223–230.
Dörje, F., Levey, A.I., Brann, M.R., 1991. Immunological detection of muscarinic receptor subtype proteins (m1–m5) in rabbit peripheral tissues. Mol. Pharmacol. 40, 459–462.
El-Bakri, N.K., Adem, A., Suliman, I.A., Mulugeta, E., Karlsson, E., Lindgren, J.U., Winblad, B., Islam, A., 2002. Estrogen and progesterone treatment: effects on muscarinic M_4 receptor subtype in the rat brain. Brain Res. 948, 131–137.
Everett, J.W., 1948. Progesterone and estrogen in the experimental control of ovulation time and other features of the estrous cycle in the rat. Endocrinology 43, 389–405.
Friedman, J.I., 2004. Cholinergic targets for cognitive enhancement in schizophrenia: focus on cholinesterase inhibitors and muscarinic agonists. Psychopharmacology 174, 45–53.
Gibbs, R.B., 1996. 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. 16, 1049–1055.
Gibbs, R.B., 1997. Effects of estrogen on basal forebrain cholinergic neurons vary as a function of dose and duration of treatment. Brain Res. 757, 10–16.
Gibbs, R.B., 1998. Impairment of basal forebrain cholinergic neurons associated with aging and long-term loss of ovarian function. Exp. Neurol. 151, 289–302.
Gibbs, R.B., 2000a. Effects of gonadal hormone replacement on measures of basal forebrain cholinergic function. Neuroscience 101, 931–938.
Gibbs, R.B., 2000b. Long-term treatment with estrogen and progesterone enhances acquisition of a spatial memory task by ovariectomized aged rats. Neurobiol. Aging 21, 107–116.
Hojo, Y., Murakami, G., Mukai, H., Higo, S., Hatanaka, Y., Ogiue-Ikeda, M., Ishii, H., Kimoto, T., Kawato, S., 2008. Estrogen synthesis in the brain: role in synaptic plasticity and memory. Mol. Cell. Endocrinol. 290, 31–43.
Hosli, E., Hosli, L., 1999. Cellular localization of estrogen receptors on neurones in various regions of cultured rat CNS: coexistence with cholinergic and galanin receptors. Int. J. Dev. Neurosci. 17, 317–330.
Jin, M., Jin, F., Zhang, L., Chen, Z., Huang, H., 2005. 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. 142, 107–114.
Kameda, S.R., Frussa-Filho, R., Carvalho, R.C., Takatsu-Coleman, A.L., Ricardo, V.P., Patti, C.L., Calzavara, M.B., Lopez, G.B., Araujo, N.P., Abílio, V.C., de Ribeiro, R.A., D'Almeida, V., Silva, R.H., 2007. Dissociation of the effects of ethanol on memory, anxiety and motor behavior in mice tested in the plus-maze discriminative avoidance task. Psychopharmacology (Berl) 192, 39–48.
Kretz, O., Feste, L., Wehrenberg, U., Zhou, L., Brauckmann, S., Zhao, S., Prange-Kiel, J., Naumann, T., Jarry, H., Frotscher, M., Rune, G.M., 2004. Hippocampal synapses depend on hippocampal estrogen synthesis. J. Neurosci. 24, 5913–5921.
Langmead, C.J., Watson, J., Reavill, C., 2008. Muscarinic acetylcholine receptors as CNS drug targets. Pharmacol. Ther. 117, 232–243.
Lanzafame, A.A., Christopoulos, A., Mitchelson, F., 2003. Cellular signaling mechanisms for muscarinic acetylcholine receptors. Receptors Channels 9, 241–260.
Levey, A.I., Stormann, T.M., Brann, M.R., 1990. Bacterial expression of human muscarinic receptor fusion proteins and generation of subtype-specific antisera. FEBS Lett. 275, 65–69.
Levey, A.I., 1993. Immunological localization of m1–m5 muscarinic acetylcholine receptors in peripheral tissues and brain. Life Sci. 52, 441–448.
Levey, A.I., Kitt, C.A., Simonds, W.F., Price, D.L., Brann, M.R., 1991. Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J. Neurosci. 11, 3218–3226.
Levey, A.I., Edmunds, S.M., Koliatsos, V., Wiley, R.G., Heilman, C.J., 1995. Expression of m1–m4 muscarinic acetylcholine receptor proteins in rat hippocampus and regulation by cholinergic innervation. J. Neurosci. 15, 4077–4092.
Li, M., Yasuda, R.P., Wall, S.J., Wellstein, A., Wolfe, B.B., 1991. Distribution of m2 muscarinic receptors in rat brain using antisera selective for m2 receptors. Mol. Pharmacol. 40, 28–35.
Lucas, T.F., Siu, E.R., Esteves, C.A., Monteiro, H.P., Oliveira, C.A., Porto, C.S., Lazari, M.F., 2008. 17beta-estradiol induces the translocation of the estrogen receptors ESR1 and ESR2 to the cell membrane, MAPK3/1 phosphorylation and proliferation of cultured immature rat Sertoli cells. Biol. Reprod. 78, 101–114.
Luthin, G.R., Harkness, J., Artymyshyn, R.P., Wolfe, B.B., 1988. Antibodies to a synthetic peptide can be used to distinguish between muscarinic acetylcholine receptor binding sites in brain and heart. Mol. Pharmacol. 34, 327–333.
Manavathi, B., Kumar, R., 2006. Steering estrogen signals from the plasma membrane to the nucleus: two sides of the coin. J. Cell. Physiol. 207, 594–604.
Marino, M., Distefano, E., Trentalance, A., Smith, C.L., 2001. Estradiol-induced IP(3) mediates the estrogen receptor activity expressed in human cells. Mol. Cell. Endocrinol. 182, 19–26.
Maróstica, E., Guaze, E.F., Avellar, M.C., Porto, C.S., 2001. Characterization of muscarinic acetylcholine receptors in the rat epididymis. Biol. Reprod. 65, 1120–1126.
Mash, D.C., Flynn, D.D., Potter, L.T., 1985. Loss of M_2 muscarine receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. Science 228, 1115–1117.
Mayanil, C.S., Richardson, R.M., Hosey, M.M., 1991. Subtype-specific antibodies for muscarinic cholinergic receptors. I. Characterization using transfected cells and avian and mammalian cardiac membranes. Mol. Pharmacol. 40, 900–907.
McEwen, B.S., 2002. Estrogen actions throughout the brain. Recent Prog. Horm. Res. 57, 357–384.
Milner, T.A., Ayoola, K., Drake, C.T., Herrick, S.P., Tabori, N.E., McEwen, B.S., Warrier, S., Alves, S.E., 2005. Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation. J. Comp; Neurol. 491, 81–95.
Mitra, S.W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H.A., Hayashim, S., Pfaff, D.W., Ogawa, S., Rohrer, S.P., Scheffer, J.M., McEwen, B.S., Alves, S.E., 2003. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology 144, 2055–2067.
Myslivecek, J., Klein, M., Novakova, M., Ricny, J., 2008. The detection of the non-M_2 muscarinic receptor subtype in the rat heart atria and ventricles. Naunyn Schmiedebergs Arch. Pharmacol. 378, 103–116.
Nash, M.S., Willets, J.M., Billups, B., Challiss, R.A., Nahorski, S.R., 2004. Synaptic activity augments muscarinic acetylcholine receptor-stimulated inositol 1, 4, 5-trisphosphate production to facilitate Ca2+ release in hippocampal neurons. J. Biol. Chem. 279, 49036–49044.
Nequin, L.G., Alvarez, J., Schwartz, N.B., 1979. 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. 20, 659–670.
Oki, T., Takagi, Y., Inagaki, S., Taketo, M.M., Manabe, T., Matsui, M., Yamada, S., 2005. 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. 133, 6–11.
Osterlund, M.K., Grandien, K., Keller, E., Hurd, Y.L., 2000a. The human brain has distinct regional expression patterns of estrogen receptor alpha mRNA isoforms derived from alternative promoters. J. Neurochem. 75, 1390–1397.
Osterlund, M.K., Gustafsson, J.A., Keller, E., Hurd, Y.L., 2000b. Estrogen receptor beta (ERbeta) messenger ribonucleic acid (mRNA) expression within the human forebrain: distinct distribution pattern to ERalpha mRNA. J. Clin. Endocrinol. Metab. 85, 3840–3846.
Patti, C.L., Kameda, S.R., Carvalho, R.C., Takatsu-Coleman, A.L., Lopez, G.B., Niigaki, S.T., Abílio, V.C., Frussa-Filho, R., Silva, R.H., 2005. Effects of morphine on the plus-maze discriminative avoidance task: role of state-dependent learning. Psychopharmacology (Berl) 184, 1–12.
Pereira, R.T.S., Porto, C.S., Godinho, R.O., Abdalla, F.M.F., 2008. Effects of estrogen on intracellular signaling pathways linked to activation of muscarinic acetylcholine receptors and on acetylcholinesterase activity in rat hippocampus. Biochem. Pharmacol. 75, 1827–1834.
Porter, A.C., Bymaster, F.P., DeLapp, N.W., Yamada, M., Wess, J., Hamilton, S.E., Nathanson, N.M., Felder, C.C., 2002. M_1 muscarinic receptor signaling in mouse hippocampus and cortex. Brain Res. 944, 82–89.
Quirion, R., Aubert, I., Lapchak, P.A., Shaum, R.P., Teolis, S., Gauthier, S., Araújo, D.M., 1989. Muscarinic receptor subtypes in human neurodegenerative disorders: focus on Alzheimer's disease. Trends Pharmacol. Sci. Suppl. 80–84.
Romeo, R.D., McCarthy, J.B., Wang, A., Milner, T.A., McEwen, B.S., 2005. Sex differences in hippocampal estradiol-induced N-methyl-D-aspartic acid binding and ultrastructural localization of estrogen receptor-alpha. Neuroendocrinology 81, 391–399.
Rouse, S.T., Thomas, T.M., Levey, A.L., 1997. Muscarinic acetylcholine receptor subtype, m2: diverse functional implications of differential synaptic localization. Life Sci. 60, 1031–1038.
Sarkar, S.N., Huang, R.Q., Logan, S.M., Yi, K.D., Dillon, G.H., Simpkins, J.W., 2008. Estrogens directly potentiate neuronal L-type Ca2+ channels. Proc. Natl. Acad. Sci. U.S.A. 105, 15148–15153.
Shibuya, K., Takata, N., Hojo, Y., Furukawa, A., Yasumatsu, N., Kimoto, T., Enami, T., Suzuki, K., Tanabe, N., Ishii, H., Mukai, H., Takahashi, T., Hattori, T.A., Kawato, S., 2003. Hippocampal cytochrome P450s synthesize brain neurosteroids which are paracrine neuromodulators of synaptic signal transduction. Biochim. Biophys. Acta 1619, 301–316.
Shinoe, T., Matsui, M., Taketo, M.M., Manabe, T., 2005. Modulation of synaptic plasticity by physiological activation of M_1 muscarinic acetylcholine receptors in the mouse hippocampus. J. Neurosci. 25, 11194–11200.
Shiozaki, K., Iseki, E., Uchiyama, H., Watanabe, Y., Haga, T., Kameyama, K., Ikeda, T., Yamamoto, T., Kosaka, K., 1999. Alterations of muscarinic acetylcholine receptor subtypes in diffuse Lewy body disease: relation to Alzheimer's disease. J Neurol. Neurosurg. Psychiatry 67, 209–213.
Shughrue, P.J., Merchenthaler, I., 2000. Evidence for novel estrogen binding sites in the rat hippocampus. Neuroscience 99, 605–612.
Shughrue, P.J., Lane, M.V., Merchenthaler, I., 1997. Comparative distribuition of estrogen receptor-alpha and-beta mRNA in the rat central nervous system. J. Comp. Neurol. 388, 507–525.
Silva, R.H., Frussa-Filho, R., 2000. The plus-maze discriminative avoidance task: a new model to study memory-anxiety interactions. Effects of chlordiazepoxide and caffeine. J. Neurosci. Methods 102, 117–125.
Silva, R.H., Bellot, R.G., Vital, M.A., Frussa-Filho, R., 1997. Effects of long-term ganglioside GM1 administration on a new discriminative avoidance test in normal adult mice. Psychopharmacology (Berl) 129, 322–328.
Silva, R.H., Abilio, V.C., Torres-Leite, D., Bergamo, M., Chinen, C.C., Claro, F.T., Carvalho, R. C., Frussa-Filho, R., 2002. Concomitant development of oral dyskinesia and memory deficits in reserpine-treated male and female mice. Behav. Brain Res. 132, 171–177.
Silva, R.H., Chehin, A.B., Kameda, S.R., Takatsu-Coleman, A.L., Abilio, V.C., Tufik, S., Frussa-Filho, R., 2004. Effects of pre- or post-training paradoxical sleep deprivation on two animal models of learning and memory in mice. Neurobiol. Learn. Mem. 82, 90–98.
Snedecor, G.W., Cochran, W.G., 1980. Statistical Methods, 7th ed. The Iowa State University Press Ames, U.S.A, pp. 89–233.

Toran-Allerand, C.D., 2005. Estrogen and the brain: beyond ER-{alpha}, ER-{beta}, and 17{beta}-estradiol. Ann. N. Y. Acad. Sci. 1052, 136–144.

Toran-Allerand, C.D., Miranda, R.C., Bentham, W.D., Sohrabji, F., Brown, T.J., Hochberg, R. B., MacLusky, N.J., 1992. Estrogen receptors colocalize with low-affinity nerve growth factor receptors in cholinergic neurons of the basal forebrain. Proc. Natl. Acad. Sci. U.S.A. 89, 4668–4672.

Towart, L.A., Alves, S.E., Znamensky, V., Hayashi, S., McEwen, B.S., Milner, T.A., 2003. Subcellular relationships between cholinergic terminals and estrogen receptor-alpha in the dorsal hippocampus. J. Comp. Neurol. 463, 390–401.

Vilaró, M.T., Palacios, J.M., Mengod, G., 1990. Localization of m5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. Neurosci. Lett. 114, 154–159.

Wall, S.J., Yasuda, R.P., Hory, F., Flagg, S., Martin, B.M., Ginns, E.I., Wolfe, B.B., 1991. Production of antisera selective for m1 muscarinic receptors using fusion proteins: distribution of m1 receptors in rat brain. Mol. Pharmacol. 39, 643–649.

Wall, S.J., Yasuda, R.P., Li, M., Ciesla, W., Wolfe, B.B., 1992. The ontogeny of m1–m5 muscarinic receptor subtypes in rat forebrain. Brain Res. Dev. Brain Res. 66, 181–185.

Wei, J., Walton, E.A., Milici, A., Buccafusco, J.J., 1994. m1–m5 muscarinic receptor distribution in rat CNS by RT-PCR and HPLC. J. Neurochem. 63, 815–821.

Willets, J.M., Nash, M.S., Challiss, R.A., Nahorski, S.R., 2004. Imaging of muscarinic acetylcholine receptor signaling in hippocampal neurons: evidence for phosphorylation-dependent and independent regulation by G-protein-coupled receptor kinases. J. Neurosci. 24, 4157–4162.

Woolley, C.S., McEwen, B.S., 1992. Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J. Neurosci. 12, 2549–2554.

Woolley, C.S., McEwen, B.S., 1993. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J. Comp. Neurol. 336, 293–306.

Woolley, C.S., McEwen, B.S., 1994. Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism. J. Neurosci. 14, 7680–7687.

Yokomaku, D., Numakawa, T., Numakawa, Y., Suzuki, S., Matsumoto, T., Adachi, N., Nishio, C., Taguchi, T., Hatanaka, H., 2003. Estrogen enhances depolarization-induced glutamate release through activation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase in cultured hippocampal neurons. Mol. Endocrinol. 17, 831–844.

Young, K.W., Billups, D., Nelson, C.P., Johnston, N., Willets, J.M., Schell, M.J., Challiss, R.A., Nahorski, S.R., 2005. Muscarinic acetylcholine receptor activation enhances hippocampal neuron excitability and potentiates synaptically evoked Ca(2+) signals via phosphatidylinositol 4, 5-bisphosphate depletion. Mol. Cell. Neurosci. 30, 48–57.