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Estrogen levels modify scopolamine-induced amnesia in gonadally intact rats

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ABSTRACT

Previous studies suggested that estrogen plays a role in cognitive function by modulating the cholinergic transmission. However, most of the studies dealing with this subject have been conducted using ovariectomized rats. In the present study we evaluated the effects of physiological and supra-physiological variation of estrogen levels on scopolamine-induced amnesia in gonadally intact female rats. We used the plus-maze discriminative avoidance task (PMDAT) in order to evaluate anxiety levels and motor activity concomitantly to the memory performance. In experiment 1, female Wistar rats in each estrous cycle phase received scopolamine (1 mg/kg) or saline i.p. 20 min before the training session in the PMDAT. In experiment 2, rats in diestrus received estradiol valerate (1 mg/kg) or sesame oil i.m., and scopolamine (1 mg/kg) or saline i.p., 45 min and 20 min before the training, respectively. In experiment 3, rats in diestrus received scopolamine (1 mg/kg) or saline i.p. 20 min before the training, and estradiol valerate (1 mg/kg) or sesame oil i.m. immediately after the training session. In all experiments, a test session was performed 24 h later. The main results showed that: (1) scopolamine impaired retrieval and induced anxiolytic and hyperlocomotor effects in all experiments; (2) this cholinergic antagonist impaired acquisition only in animals in diestrus; (3) acute administration of estradiol valerate prevented the learning impairment induced by scopolamine and (4) interfered with memory consolidation process. The results suggest that endogenous variations in estrogen levels across the estrous cycle modulate some aspects of memory mediated by the cholinergic system. Indeed, specifically in diestrus, a stage with low estrogen levels, the impairment produced by scopolamine on the acquisition was counteracted by exogenous administration of the hormone, whereas the posttraining treatment potentiated the negative effects of scopolamine during the consolidation phase of memory.

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1. Introduction

In the last decades, numerous studies have addressed the role of estrogen and its replacement in some neurodegenerative disorders, mainly Alzheimer's disease (Panidis et al., 2001; Zec and Trivedi, 2002). Estrogen has been shown to reduce cognitive impairments through different mechanisms, such as modulation of neurotransmitters, synaptic plasticity, increase of cerebral blood flow, protection against apoptosis, anti-inflammatory actions and antioxidant properties (for review, see Cholerton et al., 2002). In the brain, the estrogen receptors are located in several structures, including the hippocampus and the amygdala of both humans (Osterlund et al., 2000) and rodents (McEwen and Alves, 1999).

Estrogens are a group of steroid hormones that includes three biologically significant compounds: estrone, estradiol and estriol. Estradiol valerate (EV), an analog of estradiol, was shown to improve some types of memory (Hosseini et al., 2010; Vázquez-Pereyra et al., 1995). Besides their classical genomic actions, estrogens also activate non-genomic mechanisms through intracellular signaling pathways that are critical

Abbreviations: PMDAT, plus-maze discriminative avoidance task; SCO, scopolamine; EV, estradiol valerate; SAL, saline; VEH, vehicle sesame oil; MET, metestrus; DIE, diestrus; PRO, proestrus; EST, estrus; %TAV, percentage of time in aversive arm; %TOA, percentage of time in open arm; NO, nitric oxide; ER, estrogen receptor; WHI, Women's Health Initiative; NAV, non-aversive arm; AV, aversive arm; OA, open arm; RIA, radioimmunoassay; MW, molecular weight.

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for memory processes (Fitzpatrick et al., 2002; Kuroki et al., 2000; Mannella and Brinton, 2006; Sawai et al., 2002). These mechanisms are probably involved with the short-term beneficial effects of estradiol on memory acquisition (Levin, 2005; Walf and Frye, 2008). This rapid effect of the hormone on memory can be achieved during a two-hour time window (Luine et al., 2003; Packard, 1998).

Many cellular and behavioral acute effects appear to require physiological concentrations of estrogen higher than normal (Cornil, 2009). Behavioral studies have also revealed that rapid changes in estrogen bioavailability, resulting from a single injection of a high dose of estradiol, modify the expression of some behaviors (sexual behavior and pain threshold) within less than 1 h. These data indicate that the action of estrogen in the brain can occur in different time ranges (short to long-term), possibly combining non-genomic and genomic actions (Balthazart et al., 2006).

In animal models, sexual hormones have shown to influence memory mainly through neuronal pathways related to the hippocampus (McEwen, 2002). Particularly, estrogen influences learning and memory evaluated in hippocampus-dependent tasks (McEwen, 2002; Zec and Trivedi, 2002). In addition, this effect is possibly associated with increased acetylcholine release in the hippocampus (Dumas et al., 2006, 2008). Other investigations have suggested that a long-term deprivation of estrogen decreases the functional status of the basal forebrain cholinergic projections to the cortex and hippocampus (Gibbs, 1998). Further, both the activity of choline acetyltransferase and the release of acetylcholine in the neocortex, hippocampus and basal forebrain are enhanced by estrogen (Gibbs, 2000; Marriott and Korol, 2003). Lastly, studies showed that estrogen increases the acetylcholine release stimulated by potassium in the hippocampus (Gibbs et al., 1997) and potentiates the effects of cholinergic agonists in avoidance tasks when administered directly into the hippocampus (Farr et al., 2000). All these findings indicate that estrogen may mediate memory processes through modulation of the cholinergic system.

It has been suggested that a previous impairment has to be present for the estrogen's beneficial effects to occur (Gibbs and Aggarwal, 1998; Tinkler and Voytko, 2005). In this respect, scopolamine (SCO) is a muscarinic receptor antagonist that classically induces cognitive impairments in behavioral tests (Silva et al., 1999; Wallenstein and Vago, 2001). Wink and coworkers described that the loss of cholinergic stimuli produced by the administration of SCO is comparable to the one underlying the aging processes (Wink et al., 2006). Furthermore, the replacement with estrogen in ovariectomized rats enhances memory acquisition and counteracts the negative effects of SCO on working and spatial memory tasks (Fader et al., 1999; Gibbs, 1999). In this respect, studies in animal models are usually conducted with ovariectomized animals, and do not take into account the natural hormonal changes in gonadally intact females (Frick, 2009).

Estrogen has also been shown to influence fear and anxiety-related behaviors. For example, endogenous changes in estrogen levels may increase women's susceptibility to anxiety disorders (Walf and Frye, 2006). In this respect, the literature shows that memory can be dependent on affective contents, such as anxiety level (Silva and Frussa-Filho, 2000) and the emotional valence (negative or positive) of the events (Labar and Cabeza, 2006). Further, the neural circuit for emotional reinforcement includes brain regions that are also related to memory formation, i.e. amygdala, hippocampus and prefrontal cortex (Labar and Cabeza, 2006; Mathews, 1990). Thus, the hypothesis that estrogen can influence female rats' performance in cognitive tasks with emotional contexts by modulating anxiety-like behavior should be considered (Frye and Walf, 2004).

The aim of the present study was to investigate the effects of physiological and supra-physiological variations of estrogen levels on SCO-induced amnesia in gonadally intact female rats. We used the plus-maze discriminative avoidance task (PMDAT; Silva et al., 1999; Silva and Frussa-Filho, 2000) in order to evaluate anxiety levels and motor activity concomitantly to the learning and memory performances.

2. Methods

2.1. Animals

Three-month-old female Wistar rats (250–300 g) were housed with free access to food and water, in a number of 4 or 5, under controlled conditions of temperature (23–24 °C) and a 12 h light/12 h dark cycle (lights on 06:30 am). The rats were handled according to the Brazilian law for the use of animals in scientific research (Law Number 11.794) and all procedures described were approved by the local ethical committee (CEUA/UFRN no. 036/2010). Prior to the procedures all the animals were gently handled for 10 min/day for 3 days.

2.2. Drugs

Scopolamine hydrobromide (SCO, Sigma, USA) was diluted in saline solution and given i.p. at 1 mg/kg. This dose choice was based on previous studies showing scopolamine-induced impairment in rodent memory models (Barbosa et al., 2010; Claro et al., 2006), including the task used here (Claro et al., 1999; Silva et al., 1999). Estradiol valerate (EV, Sigma, USA) was dissolved with absolute ethanol and sesame oil and left overnight for evaporation. Afterward, the compound was diluted to the correct concentration with sesame oil and given i.m. at 1 mg/kg. Estradiol levels are considered supra-physiological at doses above 0.5 mg/ml in rodents (Cornil et al., 2006). Saline solution (SAL) (0.9% NaCl) or vehicle sesame oil (VEH) in a volume of 1 ml/kg were administered to controls for SCO or EV treatments, respectively.

2.3. Estrous cycle

Before the beginning of the experiments, the estrous cycle was monitored by the analysis of vaginal smears under a light microscopy for three complete cycles of 4–5 days. Vaginal secretions were collected by the gentle introduction of plastic pipettes with distillated water in a volume of 0.1 ml. Only animals with regular cycling were included in the experiments (approximately 95%). The four stages of estrous cycle were determined according to the following characteristics: estrus (EST) — predominance of cornified cells; proestrus (PRO) — predominance of epithelial nucleated cells; diestrus (DIE) — predominance of leucocytes and metestrus (MET) — similar proportion of nucleated cells, leucocytes and cornified cells (Marcondes et al., 2001; Pompili et al., 2010).

2.4. Plus-maze discriminative avoidance task (PMDAT)

The apparatus employed was a modified elevated plus-maze, made of wood, containing two enclosed arms (50 × 15 × 40 cm), an aversive (AV) and a non-aversive (NAV), opposite to two open arms (OA; 50 × 15 cm). The task consisted of two sessions: training and testing, each lasting 10 min. In the training session, each rat was placed in the center of the apparatus with body orientation toward the intersection between the open arms. Every time the animal entered with the four paws into the aversive enclosed arm, aversive stimuli were turned on until the animal left the arm. The aversive stimuli were a 100-W light and an 80 dB noise produced by a lamp and speakers placed over the aversive enclosed arm. In the test session, the animals were again placed in the apparatus, but without presentation of the aversive stimulation (Fig. 1). All behavior experiments were performed between 1:00 and 5:00 p.m. The sessions were recorded with a digital camera placed over the apparatus and the behavioral quantification was performed by a video-tracking software (Anymaze®, Stoelting, USA).

The percent time spent in aversive arm [%TAV = time in NAV / (time in NAV + AV) × 100] was used to assess aversive memory (%TAV overall, when the entire session was considered, and %TAV in time blocks, when the variable was analyzed throughout the sessions). The whole sessions durations were divided into three blocks: initial (from start to third minute), middle (fourth to seventh minute) and final (eighth to

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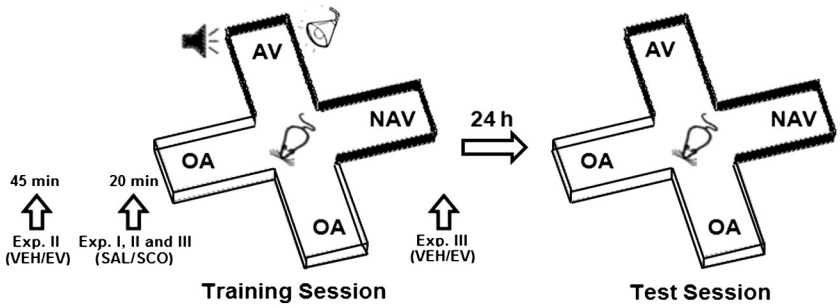

Fig. 1. Schematic diagram of the behavioral task and experiments I, II and III. VEH, oil vehicle; SAL, saline; SCO, scopolamine; EV, estradiol valerate; OA, open arms; AV, aversive enclosed arm; NAV, non-aversive enclosed arm.

tenth minute) in order to evaluate learning in the training session, as well as retrieval (first block) and extinction in the test session. Anxiety-like behavior was evaluated by the percent time spent in open arms [%TOA = time in OA / (time in OA + NAV + AV) × 100] and motor activity was evaluated by total distance traveled (m) in the whole apparatus in both sessions. The analysis of the habituation index [distance in training session / distance in test session (TR / TE)] was used to evaluate the habituation to the apparatus (nonassociative learning).

2.5. Experimental procedure

A summary of the behavioral task and experimental procedure can be seen in Fig. 1.

2.5.1. Experiment I: Influence of estrous cycle on the scopolamine-induced memory deficit

Female rats in each phase of the estrous cycle (n = 9–13 per phase) received 1 mg/kg SCO or SAL (0.9% NaCl) 20 min before the training session in the PMDAT. The test session was performed 24 h later, without pharmacological treatment.

2.5.2. Experiment II: Effects of pre-training estrogen on the scopolamine-induced memory deficit

In this experiment, all animals were in DIE phase. Forty-five minutes before the training in the PMDAT, the animals received a pre-treatment with 1 mg/kg EV or VEH. Twenty-five minutes later, the animals received the same treatment described in experiment I. The experimental groups (n = 8–9) were: VEH/SAL, VEH/SCO, EV/SAL and EV/SCO. The training was performed after 20 min, and the test session was conducted 24 h later.

2.5.3. Experiment III: Effects of posttraining estrogen on the scopolamine-induced memory deficit

In experiment III, all animals were in DIE phase. Female rats received the same treatment as describe in experiment I. Immediately after the training session, 1 mg/kg EV or VEH were administered. The experimental groups (n = 7–8) were: SAL/VEH, SCO/VEH, SAL/EV and SCO/EV. The test session was performed 24 h later.

2.6. Determination of estradiol levels

Female rats were euthanized to assess bioavailability of estradiol at different time points of the experimental protocol. All animals were in DIE phase. Control group received treatment with VEH (n = 5) and experimental groups received treatment with 1 mg/kg EV. Blood samples were collected by decapitation in tubes containing EDTA immediately (control group) and 45 min (n = 8), 6 h (n = 8) or 24 h (n = 9) after the hormone injection (experimental group).

The samples were placed in tubes containing EDTA and centrifuged at 3000 rpm for 10 min. Plasma was collected and stored at −20 °C until the assays. The concentration of plasma estradiol was determined by double antibody radioimmunoassay (RIA) using MP Biomedicals kits (Orangeburg, NY, USA). The lower limits of detection were 5.0 pg/ml. The intra-assay coefficients of variation were 4.3%. All samples were measured in duplicate and, when needed, at different dilutions. To prevent inter-assay variation, all samples of the same experiment were assayed in the same RIA. Data from estradiol concentration were adjusted by the dilution factor and expressed in pg/ml.

2.7. Statistical analysis

All data were checked for normality using the Kolmogorov–Smirnov test. Multivariate analyses of variance (two-way ANOVA) for the treatment × cycle phase (experiment I), pre-treatment × treatment (experiment II) and post-treatment × treatment (experiment III) were applied to %TAV, %TOA, as well as distance traveled in the apparatus in each behavioral session. Moreover, one-way ANOVA was performed to analyze estrogen levels at different time points after administration. Duncan's post hoc was run for group comparisons.

The %TAV throughout the sessions was compared by ANOVA with repeated measures (three-way ANOVA) for time blocks × phase × treatment (experiment I), time blocks × pre-treatment × treatment (experiment II) and time blocks × post-treatment × treatment (experiment III). In experiment I, further analysis was performed using two-way ANOVA (time blocks × treatment for each cycle phase). We used Bonferroni's for post hoc analysis.

Comparisons from habituation index were performed using Kruskal–Wallis one-way analysis of variance, followed by Mann–Whitney's *U*-test. All results were considered significant at $p \leq 0.05$.

3. Results

3.1. Experiment I: Influence of estrous cycle on the scopolamine-induced memory deficit

3.1.1. Learning and memory

In the training session, two-way ANOVA for %TAV during the whole session did not reveal treatment [$F(1,78) = 1.72$; $p = 0.19$], cycle phase [$F(3,78) = 2.02$; $p = 0.11$] or interaction [$F(3,78) = 0.54$; $p = 0.65$] effects (Fig. 2A). Three-way ANOVA for %TAV in time blocks showed a significant within-subject time effect [$F(2,156) = 19.68$; $p < 0.001$], no interaction effects and a marginally significant between-subject treatment effect [$F(1,78) = 3.39$; $p = 0.069$]. Further, a two-way ANOVA for %TAV in time blocks was performed to each cycle phase, revealing significant within-subject time effects in: MET [$F(2,38) = 21.39$; $p < 0.001$], DIE [$F(2,40) = 4.15$; $p = 0.023$], PRO [$F(2,40) = 3.53$; $p = 0.038$] and EST [$F(2,38) = 3.41$; $p = 0.046$]. Bonferroni's post

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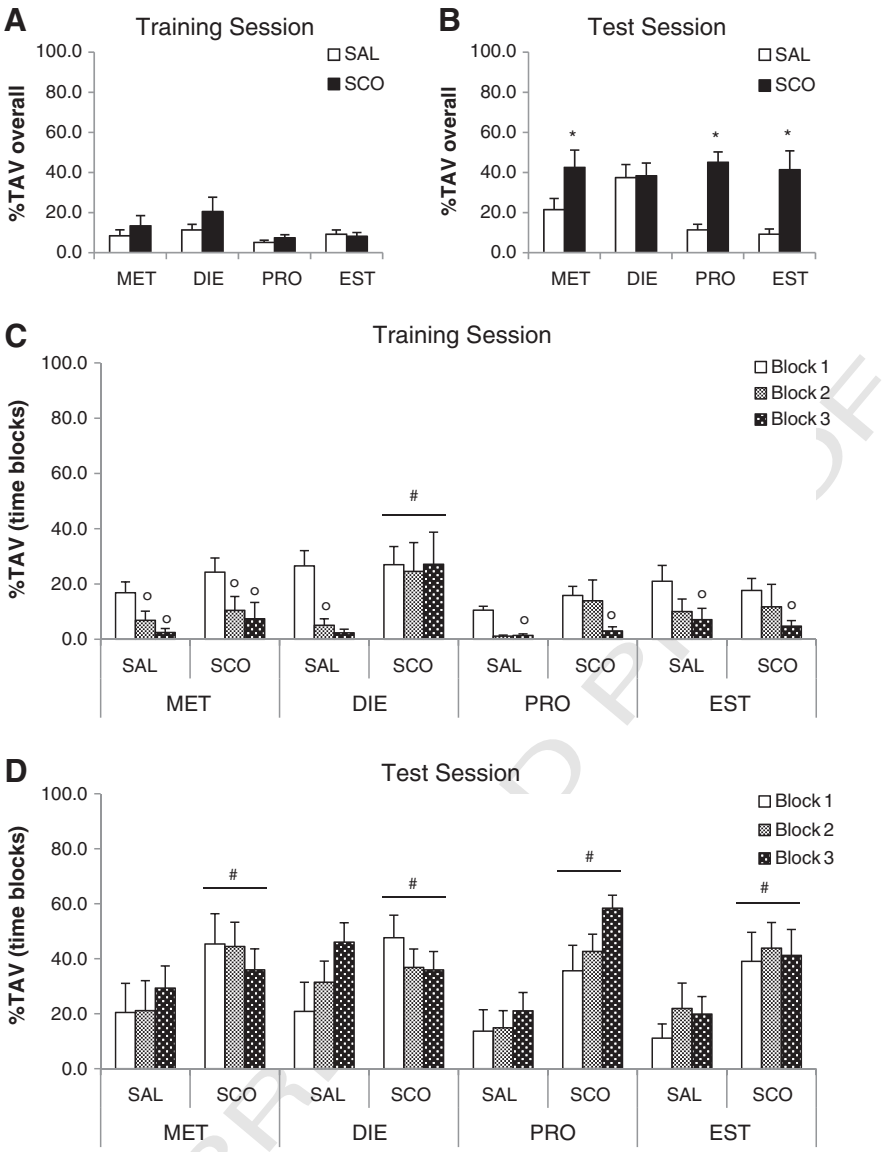

Fig. 2. Effects of saline (SAL) or 1 mg/kg scopolamine (SCO) treatment across estrous cycle phases (MET: metestrus; DIE: diestrus; PRO: proestrus; EST: estrus) on learning and memory in the plus-maze discriminative avoidance task. Mean ± SE for percent time spent in aversive enclosed arm (%TAV) overall (A, B) and in time blocks (C, D) in the training and test sessions. *p < 0.05 compared to SAL (two-way ANOVA followed by Duncan's post hoc); °p < 0.05 compared to the first time block (repeated measures ANOVA followed by Bonferroni's post hoc); #p < 0.05 compared to SAL (repeated measures ANOVA).

hoc showed that %TAV was significantly decreased in the second or in the third blocks when compared to the initial block for both treatments (SAL or SCO) in MET, PRO and EST phases. An interaction between time and treatment [$F(2,40) = 3.55$; $p = 0.038$] was detected only for the DIE phase, indicating a learning deficit induced by SCO treatment in this cycle phase (Fig. 2C).

In the test session, two-way ANOVA for %TAV during the whole session revealed a significant effect of treatment [$F(1,78) = 20.53$; $p < 0.001$]. Duncan's post hoc showed a significant increase in %TAV for SCO compared to SAL animals in MET, PRO and EST phases, but not in DIE (Fig. 2B). When %TAV was analyzed in time blocks, three-way ANOVA indicated significant within-subject time effect [$F(2,156) = 3.06$; $p = 0.05$], interaction (time block × phase × treatment) [$F(6,156) = 2.16$; $p = 0.049$] and a between-subject treatment effect [$F(1,78) = 14.59$; $p < 0.001$]. Bonferroni's post hoc revealed a marginally significant increase in the third block compared to the initial block between groups. The fact that no significant difference for treatment was found in %TAV during the whole session in DIE is due to increased values of SAL animals in this phase, which can be explained by the gradual increase of %TAV across blocks in this group (Fig. 2D).

When the habituation index was analyzed, Kruskal–Wallis ANOVA detected a significant phase effect [$H(3) = 7.96$; $p = 0.047$]. PRO/SAL animals showed increased habituation compared to the MET/SAL ($U = 17.5$; $p = 0.041$; Table 1) and DIE/SAL ($U = 13.5$; $p = 0.017$; Table 1) groups, but not to the EST/SAL group ($U = 27.0$; $p = 0.228$; Table 1). Further, PRO/SAL habituation index was also increased compared to the PRO/SCO group ($U = 14.5$; $p = 0.003$; Table 1).

3.1.2. Anxiety-like behavior and locomotion

A significant treatment effect for %TOA was revealed by two-way ANOVA in the training session [$F(1,78) = 5.31$; $p = 0.024$], indicating an increase of open arm exploration by SCO animals in all cycle phases. However, Duncan's post hoc did not detect differences between groups (Table 2). A marginally treatment effect was pointed out in the test session by two-way ANOVA [$F(1,78) = 3.76$; $p = 0.056$].

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Table 1
Effects of estrous cycle, treatment with SCO (1 mg/kg), pre- and post-treatment with EV (1 mg/kg) in plus-maze discriminative avoidance task. Median (minimum; maximum values) for habituation index (distance in training / distance in test sessions — TR / TE).

Experiments	Habituation index
I. Estrous cycle	
MET-SAL	1.0 (0.7; 1.4)
MET-SCO	1.25 (0.6; 3.3)
DIE-SAL	0.7 (0.2; 2.0)
DIE-SCO	0.9 (0.2; 2.6)
PRO-SAL	1.5 (0.8; 10.8)*,#
PRO-SCO	1.0 (0.4; 1.1)
EST-SAL	1.0 (0.8; 11.3)
EST-SCO	0.9 (0.4; 10.2)
II. Pre-training	
VEH-SAL	1.0 (0.7; 2.0)
VEH-SCO	1.0 (0.3; 1.8)
EV-SAL	1.2 (0.4; 8.6)
EV-SCO	1.1 (0.6; 1.7)
III. Posttraining	
SAL-VEH	3.3 (1.2; 4.3)
SCO-VEH	1.8 (1.0; 6.2)
SAL-EV	2.05 (1.1; 3.9)
SCO-EV	1.45 (0.5; 1.8)

* $p < 0.05$ compared to MET/SAL ($U = 17.5$; $p = 0.041$) and DIE/SAL ($U = 13.5$; $p = 0.017$) groups (Kruskal–Wallis ANOVA followed by Mann–Whitney's *U*-test).
# $p < 0.05$ compared to PRO/SCO group ($U = 14.5$; $p = 0.003$; Mann–Whitney's *U*-test).

Table 2
Effects of estrous cycle, treatment with SCO (1 mg/kg), pre- and post-treatment with EV (1 mg/kg) in plus-maze discriminative avoidance task. Mean ± SE for percent time spent in open arms (%TOA) and distance traveled in both training and test sessions.

Experiments	%TOA	Distance (m)
I. Estrous cycle		
Training session		
MET-SAL	8.0 ± 2.1	16.2 ± 2.4
MET-SCO	30.3 ± 8.9	26.3 ± 2.5*
DIE-SAL	8.9 ± 1.8	20.6 ± 2.0
DIE-SCO	30.6 ± 10.1	24.0 ± 3.9
PRO-SAL	12.9 ± 5.4	14.7 ± 1.9
PRO-SCO	16.0 ± 6.6	18.8 ± 1.7*
EST-SAL	16.7 ± 4.1	16.9 ± 2.9
EST-SCO	18.5 ± 8.2	21.5 ± 2.8
Test session		
MET-SAL	17.2 ± 9.2	15.1 ± 1.9
MET-SCO	6.6 ± 1.6	23.1 ± 3.2
DIE-SAL	9.6 ± 2.0	32.9 ± 6.8#
DIE-SCO	12.6 ± 2.1	26.5 ± 4.5#
PRO-SAL	9.7 ± 4.8	10.2 ± 1.9
PRO-SCO	9.4 ± 2.4	22.6 ± 1.8
EST-SAL	20.1 ± 7.2	15.4 ± 3.4
EST-SCO	5.4 ± 1.4	21.4 ± 2.6
II. Pre-training		
Training session		
VEH-SAL	14.8 ± 3.1	18.8 ± 1.9
VEH-SCO	59.1 ± 0.9*	26.5 ± 4.9
EV-SAL	22.5 ± 6.8	18.0 ± 2.2
EV-SCO	48.2 ± 0.7*	28.0 ± 3.4
Test session		
VEH-SAL	19.5 ± 5.0	18.9 ± 2.8
VEH-SCO	28.4 ± 8.1	27.7 ± 1.7*
EV-SAL	15.7 ± 5.2	13.3 ± 3.3
EV-SCO	27.9 ± 6.3	24.8 ± 1.5*
III. Posttraining		
Training session		
SAL-VEH	5.6 ± 2.4	10.9 ± 2.4
SCO-VEH	71.3 ± 1.1*	30.8 ± 5.0*
SAL-EV	16.6 ± 5.2	19.1 ± 2.2
SCO-EV	51.6 ± 2.1*	25.5 ± 5.4*
Test session		
SAL-VEH	2.4 ± 1.0	5.2 ± 2.0
SCO-VEH	12.4 ± 5.0	17.0 ± 3.8*
SAL-EV	8.1 ± 4.3	11.5 ± 3.0
SCO-EV	12.4 ± 3.8	18.3 ± 3.3*

* $p < 0.05$ compared to SAL control groups (two-way ANOVA followed by Duncan's post hoc).
# $p < 0.05$ compared to other phases (two-way ANOVA and Duncan's post hoc).

The analysis of distance traveled by two-way ANOVA displayed significant treatment effect in the training session [$F(1,78) = 7.93$; $p = 0.006$]. Duncan's post hoc show increase in motor activity of SCO animals compared to SAL in MET and PRO phases (Table 2). A significant estrous cycle phase effect was detected for distance traveled in the test session [$F(3,78) = 5.58$; $p = 0.002$]. Duncan's post hoc showed that locomotion is higher in DIE than all other phases (Table 2).

3.2. Experiment II: Effects of pre-training estrogen effects on scopolamine-induced memory deficit

3.2.1. Learning and memory

Two-way ANOVA for %TAV during the whole training session revealed a significant effect of treatment [$F(1,30) = 10.54$; $p = 0.003$]. Duncan's post hoc showed that %TAV was increased in VEH/SCO compared to VEH/SAL animals, while the EV/SCO group did not differ from controls (Fig. 3A). The analyses for %TAV in time blocks were in accordance to the analysis for the whole session (data not shown).

In the test session, two-way ANOVA detected a significant effect of treatment [$F(1,30) = 5.56$; $p = 0.025$] in %TAV during the whole session, indicating that the effects of SCO on retrieval occurs regardless pre-treatment. Duncan's post hoc did not detect differences between groups for this parameter (Fig. 3B), which was corroborated by the analyses of %TAV in time blocks (data not shown).

Kruskal–Wallis ANOVA did not show significant effects [$H(3) = 1.82$, $p = 0.60$] for the habituation index (Table 1).

3.2.2. Anxiety-like behavior and locomotion

Two-way ANOVA revealed a significant effect of treatment for %TOA in both training [$F(1,30) = 17.13$; $p < 0.001$] and test sessions [$F(1,30) = 4.99$; $p = 0.033$]. Additionally, in the training session, Duncan's post hoc showed significant increase in %TOA for VEH/SCO and EV/SCO groups compared to VEH/SAL and EV/SAL, respectively (Table 2).

The analysis of distance traveled by two-way ANOVA revealed significant effect of treatment in the training [$F(1,30) = 7.36$; $p = 0.011$] and test [$F(1,30) = 14.47$; $p = 0.001$] sessions. Duncan's post hoc showed increase in motor activity of VEH/SCO and EV/SCO animals compared to VEH/SAL and EV/SAL, respectively, in the test session (Table 2).

3.3. Experiment III: Effects of posttraining estrogen effects on scopolamine-induced memory deficits

3.3.1. Learning and memory

In the training session, a significant effect of treatment [$F(1,26) = 12.85$; $p = 0.001$] was detected for %TAV during the whole session by two-way ANOVA. Duncan's post hoc showed that %TAV was increased in SCO/VEH and SCO/EV compared to SAL/VEH and SAL/EV groups, respectively (Fig. 4A), which corroborated the analyses of time blocks (data not shown).

Two-way ANOVA showed significant effects of treatment [$F(1,26) = 21.01$; $p < 0.001$] and post-treatment [$F(1,26) = 5.30$; $p = 0.029$] for %TAV during the whole test session. Duncan's post hoc revealed that SCO/VEH presented increased %TAV compared to SAL/VEH and SAL/EV. Also, SCO/EV showed increased %TAV compared to all other groups, suggesting that animals post-treated with EV presented impaired performance (Fig. 4B). These effects were also found when %TAV was analyzed in time blocks (data not shown).

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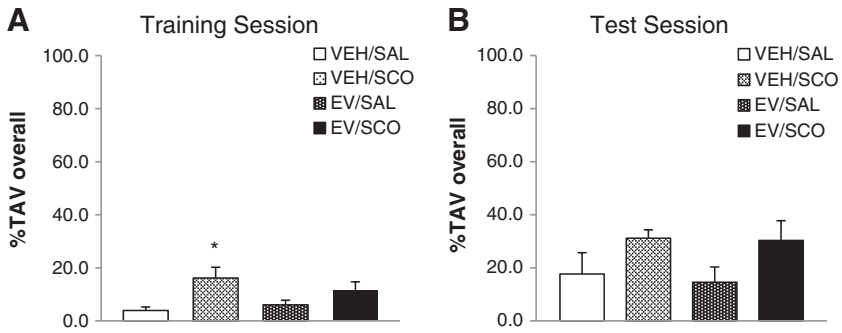

Fig. 3. Effects of the pre-treatment with oil vehicle (VEH) or 1 mg/kg estradiol valerate (EV) and treatment with saline (SAL) or 1 mg/kg scopolamine (SCO) on learning and memory in the plus-maze discriminative avoidance task. Mean ± SE for percent time spent in aversive enclosed arm (%TAV) overall in the training (A) and test (B) sessions. *$p < 0.05$ compared to VEH/SAL (two-way ANOVA followed by Duncan's post hoc).

The analysis of habituation index by Kruskal–Wallis ANOVA did not show significant differences between groups [$H(3) = 5.55$; $p = 0.135$; Table 1].

3.3.2. Anxiety-like behavior and locomotion

A significant effect of treatment was detected for %TOA by two-way ANOVA in the training [$F(1,26) = 29.82$; $p < 0.001$] but not in the test session [$F(1,26) = 3.29$; $p = 0.081$], corroborating the increase of open arm exploration induced by SCO showed in the other experiments. Duncan's post hoc showed significant increase in %TOA for SCO/VEH and SCO/EV compared to the SAL/VEH and SAL/EV groups, respectively (Table 2).

Two-way ANOVA for distance traveled revealed a significant effect of treatment in the training [$F(1,26) = 9.83$; $p = 0.004$] and test [$F(1,26) = 8.52$; $p = 0.007$] sessions. Duncan's post hoc showed increase in motor activity of SCO/VEH and SCO/EV animals compared to SAL/VEH in both sessions (Table 2).

3.4. Estradiol levels

One-way ANOVA detected a between-subject effect [$F(3,29) = 10.05$; $p < 0.001$] to the estradiol levels at different time ranges. Duncan's post hoc showed an increase of the estradiol concentration after 45 min, which remained for 24 h, with a peak after 6 h, compared to control group in DIE phase (Fig. 5).

4. Discussion

Our results showed that the treatment with SCO impaired retrieval, which corroborates the well-known amnesic effect of this substance. Indeed, in the test session, animals at different stages of the estrous cycle failed to avoid the aversive enclosed arm. Treatment with SCO also impaired acquisition, but only in animals in DIE phase. In addition, SCO induced anxiolytic effects and increased motor activity. Finally, EV pre-training prevented the SCO-induced acquisition impairment in DIE phase, whereas posttraining EV potentiated the SCO-induced anterograde amnesia. An overview of the results is displayed in Table 3.

In the present study, learning of the task in the different phases of the cycle in females was investigated by the analysis of %TAV in time blocks along the training session. Saline groups in all cycle phases learned the task by showing a decrease of %TAV throughout the session. SCO did not provoke deficits in the task acquisition in MET, PRO and EST animals, but this impairment was observed in DIE animals (Fig. 2C). It should be noted that DIE is a phase with low estrogen levels (Becker et al., 2005; Marcondes et al., 2001). In line with our results, Wallenstein and Vago (2001) showed that the acquisition and consolidation of contextual fear conditioning was impaired by intrahippocampal administration of SCO in female rats. However, in that study, the estrous cycle was not analyzed. When the estrous cycle is taken into account in different types of tasks, fluctuating endogenous levels of estrogen (Korol et al., 2004) or sex (Schmidt et al., 2009) can influence learning by modulating the choice or shift of strategy during acquisition of the task.

Previous studies have shown an effect of estrous cycle per se on memory performance (Frick and Berger-Sweeney, 2001; Korol et al., 2004; Milad et al., 2009). However, this is not unequivocal because there are also some studies indicating lack of differences in the performance across the cycle (Frye, 1995; Healy et al., 1999; Warren and Juraska, 1997). Although in our study we did not observe a general significant effect of estrous cycle, differences between stages do appear

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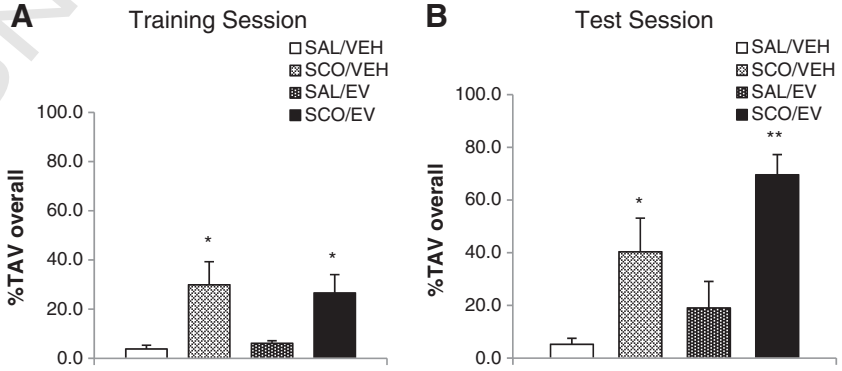

Fig. 4. Effects of the treatment with saline (SAL) or 1 mg/kg scopolamine (SCO) and posttraining treatment with oil vehicle (VEH) or 1 mg/kg estradiol valerate (EV) on learning and memory in the plus-maze discriminative avoidance task. Mean ± SE for percent time spent in aversive enclosed arm (%TAV) overall in the training (A) and test (B) sessions. $p < 0.05$ *compared to SAL/VEH and SAL/EV and **compared to all other groups (two-way ANOVA followed by Duncan's post hoc).

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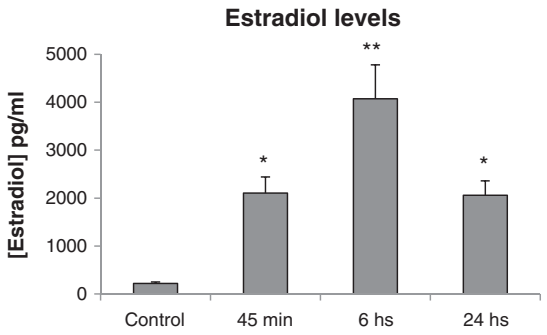

Fig. 5. Estradiol concentration at different time ranges in plasma after injection of 1 mg/kg estradiol valerate (EV). Mean ± SE for estradiol concentration adjusted by dilution factor (1:10). $p < 0.001$ *compared to control group in DIE phase and **compared to all other groups (one-way ANOVA followed by Duncan's post hoc).

when the stages are compared separately, as discussed above. Further, the analysis of the habituation index showed better habituation of the control animals in the proestrus phase, i.e. a faster response to the contextual recognition of the apparatus (Table 1). This result could be related to the estrogen peak typical of this phase during the acquisition of the task. It should be considered that the hormone levels would modulate aspects of learning and memory related to hippocampus-dependent or independent tasks (habit memory, stimulus–response, spatial and working memory, attention levels). Thus, these effects could involve various structures (hippocampus, dorsal striatum and amygdala) and convey the value of multiple memory systems that could be involved in the behavioral changes observed (see Packard and Goodman, 2012 for review).

Regarding the effects of SCO, previous studies using this paradigm did not evaluate learning across the training session (Claro et al., 1999; Silva et al., 1999). However, some studies with other behavioral tasks suggested that SCO affects more specifically attention than learning and memory (Mirza and Stolerman, 2000; Sarter et al., 2003). In addition, most researches assessed the impairment induced by this drug especially on working and short-term memories (Buresová and Bures, 1982; Stevens, 1981). Hence, the impaired acquisition promoted by the cholinergic antagonist could be a result of its effects on attention, working memory, or short-term memory, all aspects of the learning process. Conversely, at the same dose range which was used here, SCO did not impair the acquisition in spatial and contextual tasks (Decker et al., 1990). However, it is important to point out that all these previous studies used male rats. Further, the action of SCO is probably dependent on the type of task used, the time of injection, training experience, dose ranges and sex.

In the test session of the first experiment, SCO increased %TAV when compared to saline-treated animals, indicating amnesic effects. In agreement with our data, previous studies using male rodents in this task showed that SCO also induced amnesia (Silva et al., 1999). Although a general treatment effect was revealed (regardless of the stage of estrous cycle), the difference between treatments was not shown for rats in DIE when %TAV overall was analyzed (see Fig. 2B). This would raise the possibility that SCO was not effective in inducing amnesia during this cycle stage. However, while all SCO-treated groups seem to show the same amount of aversive arm exploration, saline-treated rats in DIE show higher %TAV than rats in other phases. When the %TAV was analyzed in time blocks throughout the test session (Fig. 2D), a progressive increase in aversive arm exploration was shown by DIE animals. This suggests that these animals retrieved the task (low levels of exploration in the first block), but presented memory extinction across the session. Conversely, SCO-treated animals in DIE show high levels of aversive arm exploration from the beginning of the session onward, which corroborates the presence of the amnesic effect of SCO in the other cycle phases.

In a recent study, in which estrous cycle was not considered, we observed that females present lower extinction in the PMDAT when compared to males (Ribeiro et al., 2010). As shown by Milad et al. (2009), the extinction of an aversive task by female rats seems to depend on the estrous cycle phase in which the subject is being tested. In the present work, DIE females have shown extinction of the task, unlike the rats in the other phases. This suggests that the lack of extinction by this gender (observed in the study of Ribeiro et al., 2010) is related to the hormonal status.

In the second experiment, the impairing effect of SCO on acquisition was shown again. All groups (except VEH/SCO) avoided the aversive arm in the training session (Fig. 3A), indicating that the acute administration of EV was able to prevent the SCO-induced impaired acquisition in animals with lower endogenous hormone levels. Gibbs et al. (1998) made a point of the ability for estrogen to attenuate the effect of SCO only when the analysis was limited to animals with serum levels of estradiol less than 200 pg/ml, whereas higher levels of estradiol were ineffective. This is not what was seen in our study, in which estrogen levels reached ten times that amount (i.e. 2000 pg/ml, see Fig. 5). Nevertheless, these results are in line with the hypothesis that there is a relationship between cholinergic transmission, estrogen and mnemonic system. Some studies have addressed the importance of both endogenous (Gibbs, 1996) and exogenous (Fader et al., 1999) estrogen–cholinergic interaction. Most of them focused on spatial tasks, and showed that this hormone is able to prevent SCO-induced deficits, including those on the acquisition (Gibbs, 1999). Other studies have mentioned this interaction in areas related to emotion, such as the amygdala (Luine et al., 1975). Previous studies from our lab demonstrated that the performance of animals in this task can be influenced by pharmacological manipulations in the amygdala (Munguba et al., 2011; Ribeiro et al., 2011), which corroborate a possible role of this area in the effects described here.

The learning impairment induced by SCO occurred once again in the training session of the third experiment (Fig. 4A). In the test session, an effect of posttraining treatment with EV was detected, indicating that there was an increase in %TAV (Fig. 4B), mainly in the EV/SCO group. In contrast to what was found in experiment II, in which estrogen precluded the deleterious action of the SCO on the acquisition, posttraining treatment with estrogen potentiated the retrieval deficit caused by SCO. This result is probably the outcome of hormonal influence during memory consolidation. It has been demonstrated that estradiol influences the memory storage in a time-dependent manner and that the memory enhancing effect of peripheral posttraining injection of estradiol in ovariectomized rats is blocked by SCO (Packard, 1998).

Regarding the possible mechanisms involved in the results showed here, Hasselmo (2006) presented the role of acetylcholine in learning and memory on a cortical dynamics perspective. In his model, during memory codification (learning), high levels of acetylcholine would be necessary in order to (1) enhance the magnitude of afferent input to cortex through the action at nicotinic receptors and (2) concomitantly suppress the magnitude of feedback excitation (through glutamate release) in the cortex via presynaptic inhibition by muscarinic receptors (which would prevent the interference by retrieval of previous events).

Table 3
Overview of the estrous cycle stages (MET: metestrus; DIE: diestrus; PRO: proestrus; EST: estrus) and estradiol valerate (EV; 1 mg/kg) effects on scopolamine-induced amnesia, anxiolytic and stimulant motor actions in the plus-maze discriminative avoidance task. Arrows represent increase (↑) or decrease (↓) in each behavioral parameter.

	Scopolamine (1 mg/kg)			
Experiments	Learning	Memory	Anxiety	Activity
I. Estrous cycle	MET↑	MET↓	MET↓	MET↑
	DIE↓	DIE↓	DIE↓	DIE↑
	PRO↑	PRO↓	PRO↓	PRO↑
	EST↑	EST↓	EST↓	EST↑
II. Pre-training EV	DIE↑	DIE↓	DIE↓	DIE↑
III. Posttraining EV	DIE↓	DIE↓↓	DIE↓	DIE↑

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Conversely, during memory consolidation, low acetylcholine levels would result in a weaker influence of afferent inputs relative to the strength of excitatory feedback, which allows a longer lasting memory. Considering this hypothesis and the fact that estrogen is able to increase acetylcholine release and choline acetyltransferase activity in the neocortex (Gibbs, 2000; Marriott and Korol, 2003), the high levels of acetylcholine during learning could offset the negative action of SCO, while this increase during consolidation would surpass the amnesic effect of the drug.

From another standpoint, the WHI (Women's Health Initiative) showed an increased risk of stroke and dementia in women on hormone therapy and predicted that estrogen treatment would not be capable of preventing cognitive impairment in elderly patients. Thus, it is important to consider the possibility that administration of estrogen may have a negative impact on the brain (Azcoitia et al., 2011; Shumaker et al., 2003). As mentioned above, the dose of estradiol is a critical factor for the direction of their effects on behavior. Indeed, high doses (as used in the present study) can impair performance on various tasks that assess learning and memory (Barha et al., 2010; Galea et al., 2001; Holmes et al., 2002). Besides a possible role of the interaction with the cholinergic system (discussed above), recent studies bring out the role of nitric oxide (NO) system (Sadeghian et al., 2012) and brain tissue oxidative damage (Khodabandehloo et al., 2013) in the involvement of the memory deficits caused by high doses of EV.

The variation of the effects of estrogen on cognitive function could be related to the mechanism of action. Studies showed that EV is able to up-regulate ERβ and does not affect ERα expression in the hippocampus and cortex (Jin et al., 2005). In another study, ERα and the muscarinic acetylcholine receptors were up-regulated in the hippocampus after ovariectomized animals compared to rats in proestrus. This effect was abolished when estradiol was chronically replaced immediately after the ovariectomy (Cardoso et al., 2004, 2010). In this sense, ERα knockout mice were less vulnerable to the negative effects of a large dose of estradiol. Thus, it is suggested that in wild type females the damage triggered by high hormone levels would be mediated via this receptor (Rissman, 2008).

There is evidence that high concentrations of estrogen are presumably produced in the brain by local aromatase activity (see Cornil et al., 2006 for review). Based on the fact that exogenous high doses would mimic the natural situation in the target brain areas during behavioral studies, Cornil et al. (2006) proposed that supra-physiological circulating concentrations would be necessary to assess the rapid effects of steroid hormones. Thus, to analyze hormone bioavailability, we measured estradiol levels at different time points after injections (Fig. 5). The concentrations reached are equivalent to 5.61 (45 min and 24 h) and 11.22 nM (6 h), based on the molecular weight of EV (MW = 356.5). These concentrations are required to induce effective activation of several non-genomic signaling pathways. However, as some genomic effects can be initiated within 30 min, it is not possible to determine the mode of action of the effects observed (Cornil et al., 2006).

Concerning anxiety-like behavior, although %TOA was increased by the administration of SCO (Table 2), estrogen administration did not modify this anxiolytic effect. This suggests that the modulation of cholinergic function by this hormone is specific to learning (Exp. II) and memory (Exp. III) processes. It is known that changes in the endogenous and exogenous levels of both estrogen and progesterone can alter anxiety behavior in rodents (Becker et al., 2005; Frye et al., 2000; Marcondes et al., 2001; Marvan et al., 1996; Mora et al., 1996; Pompili et al., 2010). Further, Aguiar et al. (2006) observed a decrease of anxiety in adult female rats treated with EV. In the case of the present study, although no significant differences between the estrous cycle phases were observed (Table 2), a gradual increase in %TOA during training session can be observed in the phases with higher hormone levels (i.e. proestrus and estrus).

As far as the effects of SCO per se on anxiety are concerned, a reproducible anxiolytic effect was observed across experiments. In this respect, while few studies have also reported that SCO has anxiolytic activity (De-Mello and Carobrez, 2002), others have suggested anxiogenic effects (Hughes et al., 2004; Smythe et al., 1996; Zarrindast et al., 2011). On the other hand, it has been also reported that SCO affects visual and auditory discrimination as well as interferes with the sensory processing (Klinkenberg and Blokland, 2010). A research addressed this question using light/dark preference in the elevated plus-maze (de Araújo Godinho et al., 2007). The results showed that the effects on anxiety may be confused for visual discrimination deficits caused by the muscarinic antagonist, establishing false positives. Thus, it is still controversial if cholinergic transmission effectively participates in the reduction of anxiety-related behaviors. In addition, most experiments (except the one from Hughes et al., 2004) were conducted using males and do not bring the matter of hormonal influence on such behavior (Toufexis et al., 2006). To our knowledge, we report for the first time that SCO promotes an anxiolytic effect in female rats. In a human study comparing genders, women presented larger antianxiety and antidepressant response following SCO treatment than men. The authors proposed that the interaction among cholinergic, glutamatergic transmissions and estrogen could be involved in the regulation of this response (Furey et al., 2010). In our study, the anxiolytic effect of SCO appeared more prominent (although not significantly different) in the metestrus and diestrus (see Table 2), suggesting a possible interaction of the SCO with the hormonal status.

Another general consequence of SCO treatment was the increase in motor activity, evaluated by the distance traveled (Table 2). Overall, SCO has been shown to increase motor activity in several studies (Renfro et al., 1972; Sipos et al., 1999). Some brain areas such as the hippocampus, striatum, prefrontal cortex (Day et al., 1991) and amygdala (Nomura et al., 1994) appear to be correlated with the motor enhancement produced by cholinergic blockage. In contrast, experiments using working memory tasks have reported no effect, or even decreased motor activity (Anisman and Kokkinidis, 1975; Feigley, 1974). Again, the function of SCO on exploratory behavior seems to depend on experimental factors (type of task, drug dose, injection time etc.).

In addition, we found an effect of estrous cycle phases on the motor activity. Rats in DIE phase explored more the apparatus during the test session, regardless of treatment. A possible explanation would be that the animals did not habituate to the apparatus (displayed by a lowest habituation index, see Table 1), lacking a natural reduction of exploratory drive in a known environment.

Regarding a methodological issue, it is relevant to verify if non-specific alterations (in anxiety levels or motor behavior, for example) are interfering with measurements related to learning and memory shown in the present study. In this sense, we have not found any statistical correlations between the parameters that measure different behaviors (data not shown), which suggests dissociation of memory outcomes and other non-specific effects. However, the interpretation of these effects as one-dimensional is not unequivocal. As recent reviewed by Klinkenberg and Blokland (2010), SCO is a well-known amnesic drug producing cognitive impairments in several behavior tasks. However, the cognitive–behavioral effect of systemic administration of this muscarinic antagonist is not very selective and may not result from a specific effect on learning and memory processes. Thus, it is likely that effects on learning and memory performance which are observed after higher doses are mediated by (1) primary effects on attention and sensory/stimulus discrimination, (2) non-specific effects on behavior (e.g., motor activity, anxiety), and (3) peripheral side-effects (e.g., pupil dilation, salivation). Nevertheless, in the present study, neither the anxiolytic effect nor the motor increase induced by SCO were modified by estrogen administration, suggesting that the modulation of cholinergic function by this hormone was specific to learning (Exp. II) and memory (Exp. III) processes. Further, the possibility of confounding effects compromising the interpretation of memory investigations highlights the relevance of the use of the plus-maze discriminative avoidance task to concomitantly dissociate cognitive and non-cognitive

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aspects of several pharmacological manipulations (Carvalho et al., 2006; Kameda et al., 2007; Melo et al., 2012; Niigaki et al., 2010).

5. Conclusion

The results suggest endogenous variations in estrogen levels across the estrous cycle modulate aspects of cognitive function mediated by the cholinergic system. Indeed, SCO induced acquisition impairment specifically in DIE, a stage with low estrogen levels, and this impairment was counteracted by previous exogenous administration of the hormone. Moreover, the posttraining treatment with estrogen potentiated the memory impairment induced by the cholinergic antagonist. We suggest different time courses and balance between non-genomic and genomic actions influence the regulation and functioning of cholinergic system during the stages of memory. Nevertheless, there is evidence of the interaction between estradiol and other neurotransmitter systems, and a participation of these systems in the results reported here cannot be ruled out.

Contributors

AMM wrote the paper; AMM, GSI, AMR and RHS designed the experiments; AMM, DSS, PTM, AFS and VKMS performed the experiments; RHS, AC and AMR contributed to writing and theoretical discussions; and RHS coordinated the study.

Q5 Uncited references

Contreras et al., 2000
Johnston and File, 1991
Pellow et al., 1985
Zimmerberg and Farley, 1993

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