

Interaction between estradiol and 5-HT_{1A} receptors in the median raphe nucleus on acquisition of aversive information and association to the context in ovariectomized rats



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ABSTRACT

The median raphe nucleus (MRN) is related to stress resistance and defensive responses, a crucial source of serotonergic neurons that project to prosencephalic structures related to stress and anxiety. Estrogen receptors were identified in this mesencephalic structure. It is possible that the estrogen action is related to serotonin effect on somatodendritic 5-HT_{1A} receptors, inhibiting the function of serotonergic neurons and thus preventing of the stress effect and inducing anxiolysis. So, in order to evaluate these aspects, female *Wistar* rats were ovariectomized and 21 days later were given a direct microinjection of estradiol benzoate (EB) (1200 ng) into the MRN, preceded by microinjections of saline or WAY100.635 (100 ng), a 5-HT_{1A} receptor antagonist. Immediately after the two microinjections, the ovariectomized rats were conditioned with an aversive event (foot shock) session in a Skinner box. Twenty-four hours later, they were exposed to the same context in a test session for 5 min for behavioral assessment: freezing, rearing, locomotion, grooming, and autonomic responses (fecal boluses and micturition). EB microinjection in the MRN prior to the exposure of animals to the foot shocks in the conditioning session did not alter their behavior in this session, but neutralized the association of the aversive experience to the context: there was a decrease in the expression of freezing and an increased rearing activity in the test session. This effect was reversed by prior microinjection of WAY100.635. In conclusion, EB acted on serotonergic neurons in the MRN of the ovariectomized rats, impairing the association of the aversive experience to the context, by co-modulating the functionality of somatodendritic 5-HT_{1A}.

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1. Introduction

Compelling evidence indicates that females are more susceptible to anxiety than males (Leach et al., 2008; McLean and Anderson, 2009; NIMH, 2012), mainly in phases of the hormonal cycle when serum levels of estrogen are low, such as during premenstrual and peri-menopausal periods, or after bilateral oophorectomy (Altemus, 2006; Bekker and van Mens-Verhulst, 2007; McHenry et al., 2014; Rocca et al., 2008; Seeman, 1997). During these periods, there appears to be an increase in sensitivity to stressors (Andreano and Cahill, 2010; Kask et al., 2008; Ossewaarde et al., 2010). Experimental and clinical studies (in mice, rats, and humans) have

demonstrated that estrogen replacement treatment minimizes the effect of stressors, decreases anxiety (Walf and Frye, 2010; Walf et al., 2009; Wharton et al., 2013), and affects the functionality of the serotonergic system (Amin et al., 2005; Borrow and Cameron, 2014; Genazzani et al., 2007; Lasiuk and Hegadoren, 2007; McEwen, 2002).

The median raphe nucleus (MRN) is considered to be a crucial source of serotonergic neurons that project to prosencephalic structures related to stress and anxiety, such as the dorsal hippocampus (Azmitia and Segal, 1978; McKenna and Vertes, 2001). These anatomic analyses support theoretical assumptions that the MRN-dorsal hippocampus pathway is a critical component in stress resistance (Deakin and Graeff, 1991) and in angiogenesis (Andrade et al., 2013) by integrating Gray's "behavioral inhibition system" (Gray and McNaughton, 2000).

Several studies have demonstrated the effects of stimulation or

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inactivation of the MRN on anxiety (for review see Andrade et al., 2013). More specifically, serotonergic neurons from the MRN were involved in the manifestation of freezing in contextual conditioning (Andrade et al., 2005; Avanzi and Brandão, 2001; Avanzi et al., 1998; Fendt and Fanselow, 1999; Luyten et al., 2011; Silva et al., 2002, 2004), an anxiety test which involves aversive conditioning and spatial context (Fanselow, 2000; Orsini et al., 2013). In addition, there is a wealth of experimental evidence indicating that the dorsal hippocampus, the main projection of the MRN, facilitates learning contextual characteristics in which conditioning occurred (Fanselow, 2000; Gewirtz et al., 2000; Gupta et al., 2001; Maren et al., 1997; Maren and Holt, 2000).

Estrogen receptors have been identified in neurons located in the MRN (Leranth et al., 1999; Alves et al., 2000), indicating a role for estrogen in this location. It is possible this role is to potentiate the action of serotonin on somatodendritic 5-HT_{1A} receptors, inhibiting the function of serotonergic neurons (McEwen, 2002; Inoue et al., 2014), and causing anxiolysis (Andrade et al., 2005, 2009). One study showed that the microinjection of estradiol benzoate (EB: β -estradiol 3 benzoate) into the MRN of ovariectomized rats decreased the manifestation of freezing in the same context as that in which the animals received foot shocks (Andrade et al., 2009). This effect was reversed by prior injection of WAY100.635, a 5-HT_{1A} receptor antagonist. In this case, the pharmacological manipulations were conducted 24 h after the aversive conditioning session, immediately before exposure to the context (test session). Thus, EB blocked the association between the aversive experience and the context.

The present study aimed to evaluate whether the estradiol microinjected into the MRN before exposure to an aversive stimulus (foot shocks) would impair the acquisition of aversive information and the association with context, and if the 5-HT_{1A} receptors would be involved in this effect. The hypothesis of this investigation was that estradiol could contribute for the decrease of the aversive conditioning process, minimizing anxiety-like behavior in ovariectomized rats by modulating the function of the serotonergic neurons in MRN.

2. Materials and methods

2.1. Animals

Normal cycling female *Wistar* rats, weighing 200 g and that were at least 2 months old, were housed, five animals in one polypropylene cage (41 × 34 × 17 cm) with wood shavings on the floor, for 7 days until ovariectomy. After stereotaxic surgery, the animals were housed in pairs. The rats were maintained on a 12 h light-dark cycle (7:00–19:00, 50 lux) in a temperature-controlled room (21 ± 2 °C) and given free access to food and water throughout the experiment, except during testing. The animals were handled three times a week to clean the cages.

Procedures were approved by the research ethics committee of São Paulo State University (Process 553/2009; CEP 015/2009) and were conducted in conformity with the Brazilian Society of Neuroscience and Behavior Guidelines for Care and Use of Laboratory Animals, which are in compliance with international laws and policies. All efforts were made to minimize animal suffering.

2.2. Drugs

Estradiol benzoate (β -estradiol 3-benzoate; Sigma, USA) was dissolved in sesame oil (Sigma, USA) (1200 ng). WAY100.635 (N-[2-(4-[2-Methoxyphenyl]-1-piperazinyl)ethyl]-N-2-

pyridinylcyclohexanecarboxamide) Sigma, USA, a selective 5-HT_{1A} receptor antagonist, was dissolved in saline (100 ng). Control animals received the same volume of the respective vehicle (sesame oil or saline).

2.3. Surgeries

All females were bilaterally ovariectomized under tribromoethanol anesthesia (250 mg/kg, IP; Aldrich, USA). Fourteen days later, a 15 mm guide cannula was implanted into the brain of the animals at a 20° angle, with its tip remaining 1.5 mm above the injection site. This procedure was performed on rats fastened to a stereotaxic instrument (David-Kopf, USA) under the same anesthetic described above, with the addition of a local anesthetic of 2% xylocaine. The following coordinates from the atlas of Paxinos and Watson (2007) were used: anteroposterior = -7.8 mm; lateral = +2.9 mm; depth = -7.5 mm, taking the bregma as reference. The cannula was fixed to the skull with acrylic resin and a stainless steel screw.

At the end of the surgery, all animals were injected (IM) with 0.2 ml of antibiotic preparation (benzylpenicillin and streptomycin;

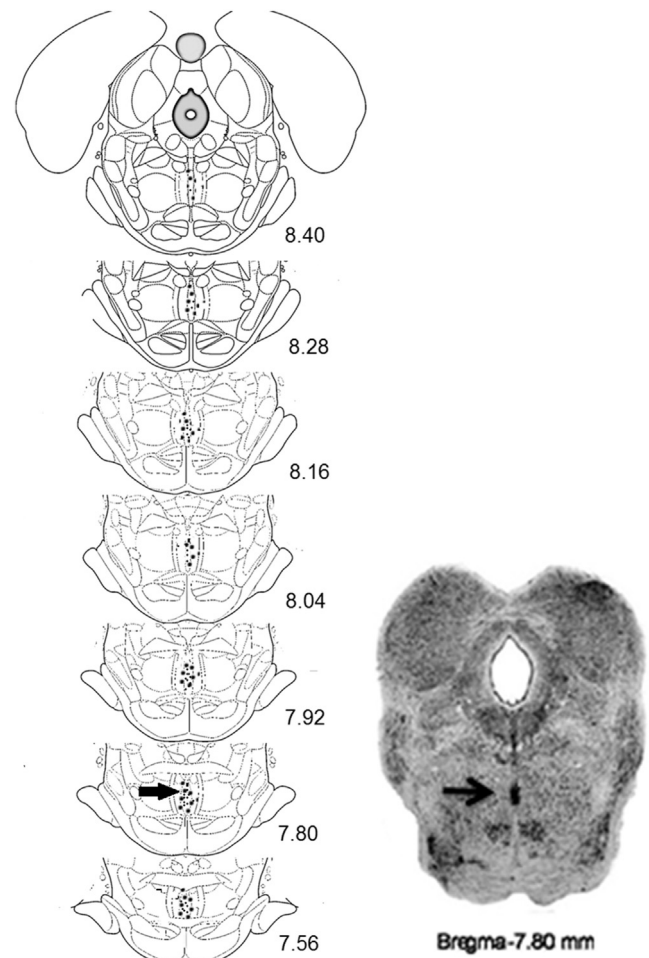


Fig. 1. Diagrammatic representation of coronal sections through the rat brain (mm posterior to bregma) showing the location of injection sites into MRN (Paxinos and Watson, 2007). The number of points in the figures is less than the total number of rats included in the analysis (n = 41) because of several overlaps (on the left side). Photomicrograph of typical injection site (indicated by arrow) in the MRN (on the right side).

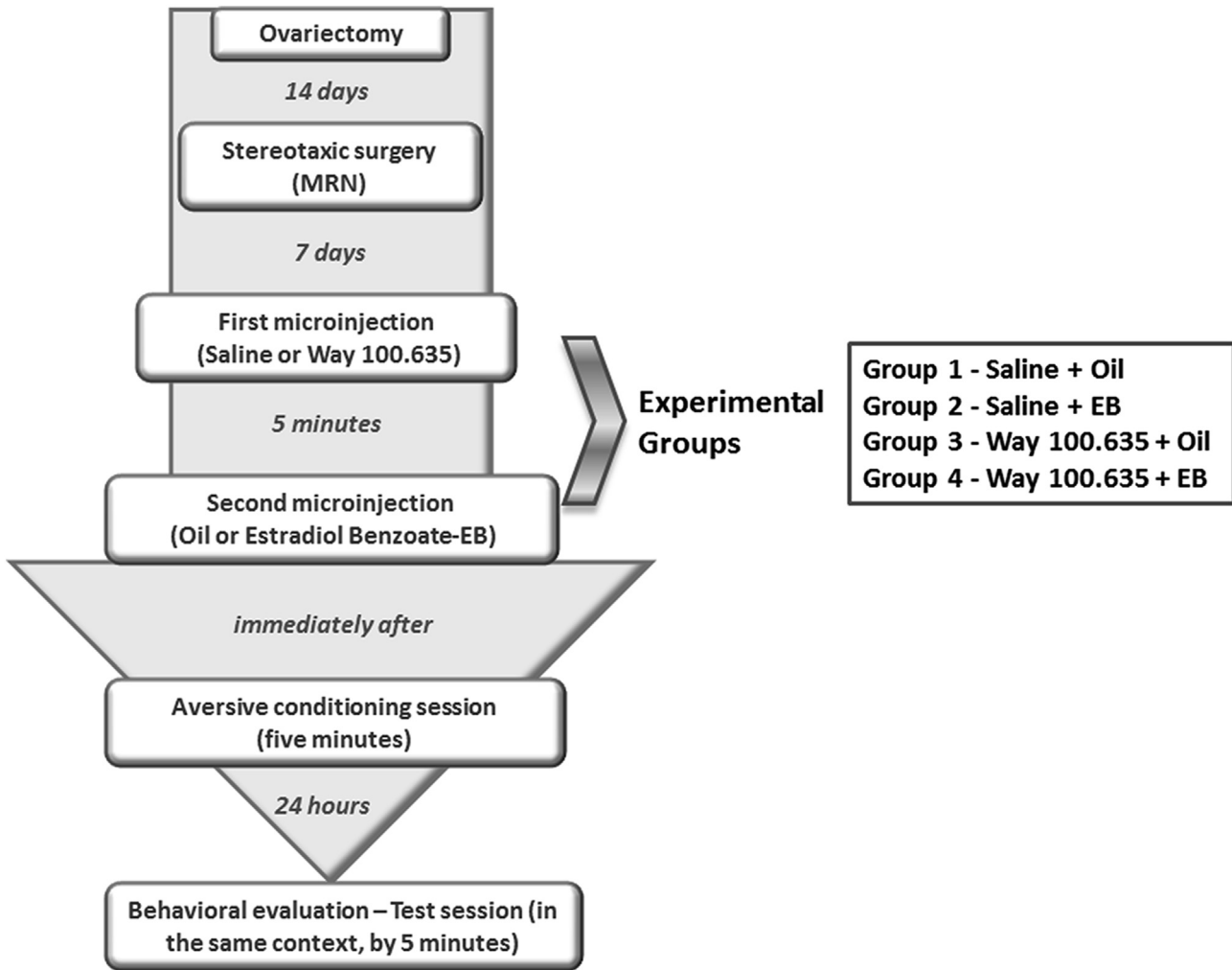


Fig. 2. Experimental design – summary description of the procedures and experimental groups.

Pentabiotico Veterinário Pequeno Porte, Fort Dodge, Brazil) to prevent possible infections.

2.4. Microinjection of drugs into the median raphe nucleus

Microinjection was performed using a micro-syringe (Hamilton, USA) connected to the needle using a polyethylene tube. The micro-syringe was driven by a motor pump (Fisher Scientific Company - USA). A 16.5 mm stainless steel needle (0.3 mm external diameter) was introduced through the guide cannula into the MRN and a volume of 0.2 μ l of each solution was injected during a 1 min period. The needle was held in place for another minute to avoid reflux. The two microinjections were conducted at a 5 min interval. A representative position of the needle tip in the MRN can be seen in Fig. 1.

The dose of EB used was chosen on the basis of previously reported results (Andrade et al., 2005, 2009). The animals were given either saline or WAY100.635 in the first microinjection. Five minutes later, the animals received a second microinjection of sesame oil or EB. The rats were divided into four groups: Group 1 – Saline + Oil (n = 10); Group 2 – Saline + EB (n = 11); Group 3 – WAY 100.635 + Oil (n = 10); Group 4 – WAY100.635 + EB (n = 10). The rats were tested for contextual conditioning immediately after the second microinjection (Fig. 2).

2.5. Contextual conditioning test

Seven days after stereotaxic surgery, immediately after micro-injections, the animals were submitted to aversive conditioning in a Skinner box (30 × 20 × 25 cm). The ceiling, side and back walls of the box were made of stainless steel and the front door of transparent Plexiglas. The grid floor of the chamber consisted of stainless steel rods 1.2 cm. Ten inescapable foot shocks (0.7 mA, 1 s, intertrial interval varied randomly between 20 and 50 s) were delivered through the cage floor by a constant current generator (Insight, Brazil). Contextual conditioning (the test session) was evaluated in the same box for 5 min, 24 h after the aversive conditioning session.

Several behavioral measures were recorded to assess the level of contextual conditioning: the frequency and duration of freezing, rearing and grooming behaviors; the frequency of locomotion; and the number of fecal boluses and bouts of micturition. Freezing was operationally defined as the total absence of movement of the body and vibrissae for a minimal period of 6 s, accompanied by at least two of the following responses: arched back, retraction of the ears, piloerection or exophthalmos. Rearing and grooming were defined respectively as standing with raised forelegs placed either in the air or against the walls of the cage and as licking the fur, beginning by the muzzle, going to the ears and down to the rest of the body (Avanzi and Brandão, 2001; Avanzi et al., 1998).

Both the training and test sessions were conducted between 14:00–17:00 h. The brightness level in the conditioning box was 50 lux. Before positioning the next rat, the apparatus was cleaned with 20% ethanol. The experimenter remained outside the room, and the behavior of the rat was recorded on videotape, which was later analyzed using the standardized software Etholog 2.25, by an investigator blinded to the treatment group of the animal (Othoni, 2000).

2.6. Identification of microinjection sites

Immediately after behavioral assessment, the animals were anesthetized with a lethal dose of sodium pentobarbital (IP). The brain was perfused intracardially with 0.9% saline solution followed by 10% formalin solution before being removed and fixed in a 10% formalin solution at a temperature of 0–4 °C for 3 days, for subsequent evaluation of microinjection sites by histological analysis. Brain slices of 50 μ m were cut by means of a microtome and stained with cresyl violet acetate (Sigma) (Nissl staining). The injection sites were determined using diagrams of the atlas by Paxinos and Watson (2007). Ten animals were excluded from data analysis due to placement of the cannula outside the MRN. Forty-one rats with microinjection sites precisely located within the MRN were included in the study.

2.7. Data analysis

Results were analyzed by repeated measures analysis of variance (ANOVA two way): the behaviors presented in the aversive

conditioning and test sessions (repeated measures) were compared, in addition to the treatments applied (pretreatment: first microinjection with saline or WAY100.635; and treatment - second microinjection with oil or EB). *Post-hoc* comparisons were made using LSD test (the Fisher Least Significant Difference Test) comparing four experimental groups: group 1 – saline + oil; group 2 – saline + EB; group 3 – WAY100.635 + oil; group 4 – WAY100.635 + EB; in each session (conditioning or test). Significant values were established at $p < 0.05$.

3. Results

The frequency of freezing behavior did not differ between the conditioning and test sessions (Fig. 3 – on top) [$F(1,37) = 0.00$; $p = 0.953$]. However, it was affected by pretreatment (saline or WAY100.635) [$F(1,37) = 11.73$; $p = 0.001$], but not by treatment (oil or EB) [$F(1,37) = 3.62$; $p = 0.065$]. There was an interaction between pretreatment and treatment [$F(1,37) = 5.81$; $p = 0.021$]. In the test session, females of the saline + EB group froze less often compared to the saline + oil group ($p = 0.002$), WAY100.635 + oil group ($p = 0.001$) and WAY00.635 + EB group ($p = 0.004$).

In contrast, the duration of freezing behavior (Fig. 3 - bottom) differed between the conditioning and test sessions [$F(1,37) = 6.96$; $p = 0.012$]. The duration of freezing was higher in the test session in saline + oil and WAY100.635 + oil groups

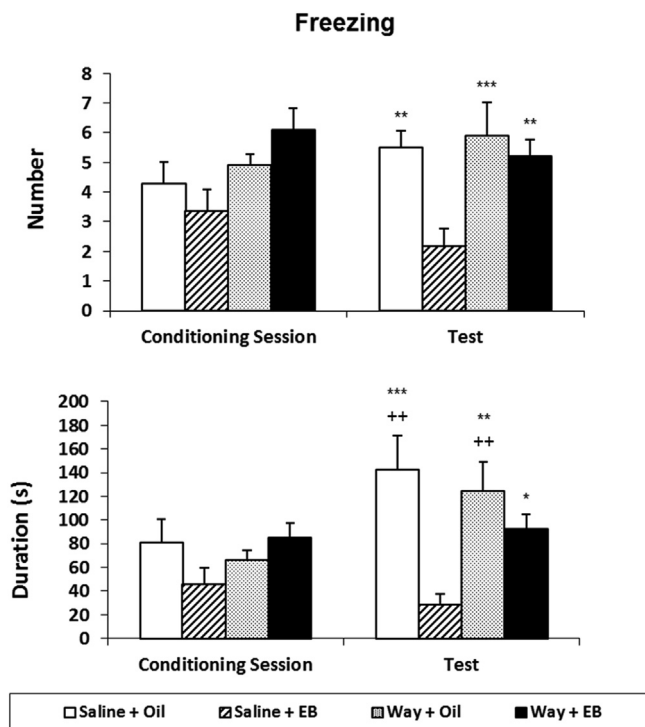


Fig. 3. Frequency and duration (seconds) of freezing (Mean + S.E.M.) during conditioning and test sessions, by ovariectomized rats submitted to microinjections into the MRN prior to aversive conditioning. $N = 10$ –11. Columns represent means and bars represent S.E.M. ANOVA followed by LSD *post-hoc* test: $^{++}p < 0.01$, represent differences between conditioning and test sessions within groups; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ represent differences in relation to the Saline + EB group in the test session.

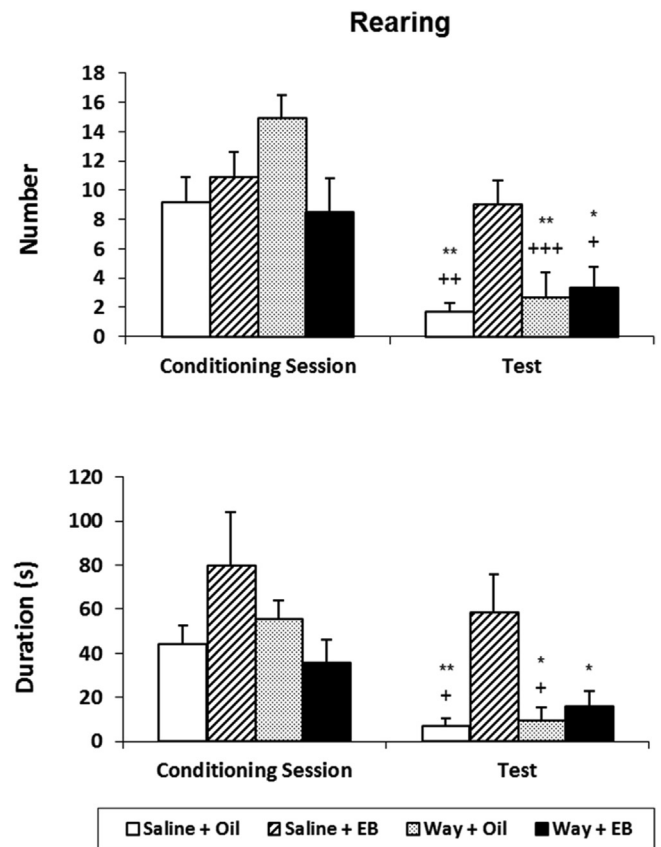


Fig. 4. Frequency and duration (seconds) of rearing (Mean + S.E.M.) during conditioning and test sessions, by ovariectomized rats submitted to microinjections into the MRN prior to aversive conditioning. $N = 10$ –11. Columns represent means and bars represent S.E.M. ANOVA followed by LSD *post-hoc* test: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$ represent differences between conditioning and test sessions within groups; $^{*}p < 0.05$; $^{**}p < 0.01$ represent differences in relation to the Saline + EB group in the test session.

Table 1
Number of locomotion and autonomic responses (Faecal boluses and micturition) shown by ovariectomized rats in the conditioning and test session, submitted to microinjections into the MRN of the Saline + Oil, Saline + EB, WAY 100.635 + Oil or WAY 100.635 + EB previously to aversive conditioning session.

Contextual conditioning test	Groups	Locomotion	Faecal boluses	Micturition
Conditioning Session	Saline + Oil	17.80 ± 2.23	6.70 ± 1.27	1.40 ± 0.75
	Saline + EB	18.73 ± 1.47	3.45 ± 0.92	1.27 ± 0.60
	Way + Oil	23.70 ± 1.52	6.90 ± 0.85	2.10 ± 0.75
	Way + EB	20.30 ± 2.03	6.80 ± 0.81	1.60 ± 0.62
Test Session	Saline + Oil	10.50 ± 1.56	3.10 ± 0.96	1.10 ± 0.67
		+++	++	
	Saline + EB	15.73 ± 1.54	2.73 ± 0.91	0.64 ± 0.24
	Way + Oil	12.70 ± 1.61	3.60 ± 0.86	0.50 ± 0.22 +
		+++	++	
	Way + EB	12.20 ± 1.40	2.70 ± 0.76	0.60 ± 0.22
		+++	++	

Mean ± S.E.M. N = 10–11. LSD test after ANOVA: + < 0.05; ++ *p* < 0.01; +++ *p* < 0.001, represent differences between conditioning and test session in the same group.

(*p* < 0.01). Moreover, pretreatment (saline or WAY100.635) [*F*(1,37) = 1.61; *p* = 0.213] did not affect freezing duration, whereas treatment did (oil or EB) [*F*(1,37) = 8.69; *p* = 0.005], and there was an interaction between pretreatment and treatment effect [*F*(1,37) = 6.06; *p* = 0.019]. In the test session, rats that received saline + EB exhibited shorter duration of freezing than rats that received saline + oil (*p* < 0.001), WAY100.635 + oil (*p* < 0.001) or WAY100.635 + EB (*p* = 0.025).

The number of rearing behaviors – vertical exploratory locomotor activity – differed between the conditioning and test sessions (Fig. 4 – on top) [*F*(1,37) = 32.73; *p* < 0.001]. However, neither pretreatment (saline or WAY100.635) [*F*(1,37) = 0.09; *p* = 0.765] nor treatment (oil or EB) [*F*(1,37) = 0.47; *p* = 0.496] affected the number of rearing behaviors. There was an interaction between the pretreatment and treatment effects [*F*(1,37) = 10.05; *p* = 0.003]: during the test session, females that received saline + EB reared more often than those that received saline + oil (*p* = 0.003), WAY100.635 + oil (*p* = 0.010) or WAY100.635 + EB (*p* = 0.018).

Similarly, the duration of rearing behavior (Fig. 4 – bottom) differed between conditioning and test sessions [*F*(1,37) = 11.42; *p* = 0.002]. There was no effect of pretreatment (saline or WAY100.635) [*F*(1,37) = 4.01; *p* = 0.052], or treatment (oil or EB) [*F*(1,37) = 4.04; *p* = 0.052], but there was an interaction between pretreatment and treatment effects [*F*(1,37) = 7.71; *p* = 0.009]. During the test session, rats that received saline + EB reared for longer time than rats that received saline + oil (*p* = 0.007), WAY100.635 + oil (*p* = 0.010) or WAY100.635 + EB (*p* = 0.022).

The number of locomotion in the Skinner box (horizontal exploratory locomotor activity; Table 1) differed between the conditioning and test sessions [*F*(1,37) = 58.31; *p* < 0.001]. However, there was no effect of pretreatment (saline or WAY100.635) [*F*(1,37) = 1.23; *p* = 0.275], treatment (oil or EB) [*F*(1,37) = 0.16; *p* = 0.687], or interaction between pretreatment and treatment [*F*(1,37) = 3.29; *p* = 0.078].

Similarly, the number of grooming events differed between sessions (Fig. 5 - on top) [*F*(1,37) = 29.08; *p* < 0.001]. However, there was no effect of pretreatment (saline or WAY100.635) [*F*(1,37) = 0.52; *p* = 0.476], treatment (oil or EB) [*F*(1,37) = 0.03; *p* = 0.854], or interaction between pretreatment and treatment [*F*(1,37) = 0.89; *p* = 0.350]. Analysis of the duration of grooming showed similar results: there was a significant effect of session [*F*(1,37) = 37.36; *p* < 0.001;], but there was no effect of pretreatment (saline or WAY100.635) [*F*(1,37) = 0.26; *p* = 0.615], treatment (oil or EB) [*F*(1,37) = 0.30; *p* = 0.583], or interaction between pretreatment and treatment [*F*(1,37) = 0.03; *p* = 0.873] (Fig. 5 - bottom).

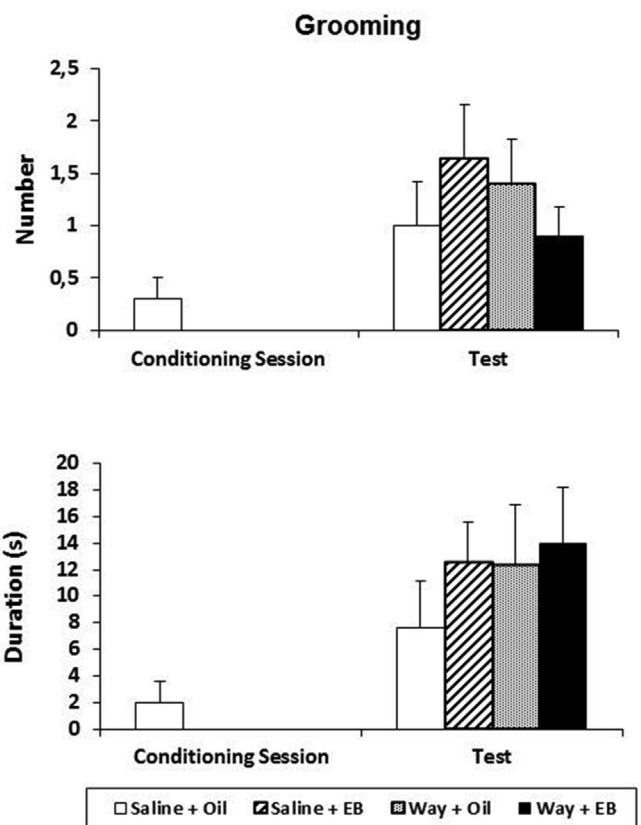


Fig. 5. Frequency and duration (seconds) of grooming (Mean + S.E.M.) during conditioning and test sessions, by ovariectomized rats submitted to microinjections into the MRN prior to aversive conditioning. N = 10–11. Columns represent means and bars represent S.E.M. ANOVA followed by LSD *post-hoc* test: ++ *p* < 0.01, +++ *p* < 0.001 represent differences between conditioning and test sessions within groups.

The autonomic responses measured in this study – the number of fecal boluses and bouts of micturition – both differed between sessions [fecal boluses: *F*(1,37) = 28.70; *p* < 0.001]; bouts of micturition: *F*(1,37) = 7.63; *p* < 0.009]. Even so, these autonomic variables were not affected by pretreatment or treatment (*p* > 0.05) (Table 1).

4. Discussion

In this work we investigated the hypothesis that estradiol in the MRN could contribute for the decrease of the aversive conditioning

process, minimizing anxiety-like behavior in ovariectomized rats by modulating the function of the serotonergic neurons in MRN. For this, we used the Contextual Conditioning. This test is a form of Pavlovian conditioning where an unconditioned stimulus (foot shock – acute aversive stimulus) becomes associated with the context in which the stimulus was given. This form of conditioning has been used to assess anxiety in animals that are subsequently placed in the same context in which they received the acute stressor stimulus. When returned to the context, animals perceived potential danger, which characterizes the state of anxiety. In this condition, animals commonly freeze, characterized as becoming immobile, which constitutes the main behavioral parameter associated with aversive context conditioning (Fanselow, 2000). It has been reported that anxiolytics and antidepressants (acute treatment) can minimize the manifestation of this behavior in this test (Borsini et al., 2002; Miyamoto et al., 2000).

In the present study, ovariectomized females treated with saline + oil into MRN (control animals) showed an increase in the expression of freezing and a decrease in locomotion and rearing, as well as decrease in the fecal boluses elimination in the test sessions, of the same context in which they were exposed to aversive conditioning with electric shocks. Ovariectomized females that received microinjection of the Way100635 + oil into MRN presented with the same behaviors. These results were expected, because the dose used was below threshold for inducing behavioral effects on its own, and this group served as a control group. These findings confirm similar results found in contextual conditioning test in male rats (Avanzi and Brandão, 2001; Avanzi et al., 1998, 2003; Cruz et al., 1993; Fanselow, 1991; Melik et al., 2000; Rudy et al., 2004; Silva et al., 2002, 2004; Vianna et al., 2001) and in ovariectomized rats (Andrade et al., 2009). Previous studies also showed that the removal of contextual signals associated with shock, i.e. when rats were placed in a box in which foot shocks were not given, in a different location, the freezing was absent (Avanzi and Brandão, 2001; Avanzi et al., 1998, 2003; Fanselow, 2000).

A wealth of investigations has demonstrated involvement of the MRN in the manifestation of freezing during contextual conditioning (Avanzi and Brandão, 2001; Avanzi et al., 1998, 2003; Borelli et al., 2005; Melik et al., 2000; Silva et al., 2002, 2004). It is already known that the MRN projects heavily to the septomedial and hippocampal regions, in which a large amount of serotonergic neurons participate (McKenna and Vertes, 2001). In Gray's behavioral inhibition process (Gray and McNaughton, 2000), the septo-hippocampal system has a pivotal role. In particular, the dorsal hippocampus, the main MRN projection, is involved in processing cognitive and spatial information. Therefore, this pathway would be recruited in aversive situations associated with a context, leading to behavioral inhibition. According to hypotheses proposed by Deakin and Graeff (1991), this activation would occur as a result of conditioned and unconditioned chronic stimuli, promoting resilience to stress by strengthening "coping" (Paul and Lowry, 2013).

As the MRN receives afferents from regions related to perception or processing of aversive stimuli, such as the *locus coeruleus* and the hypothalamus (medial and lateral pre-optic areas, anterior and lateral hypothalamus, hypothalamic dorso-medial, and arcuate nuclei) (Harsing, 2006; Hensler, 2006; Marcinkiewicz et al., 1984), serotonergic neurons in the MRN would be activated by stimulation of nor-adrenaline and/or CRH (Corticotropin Releasing Hormone) receptors in this region (Funk et al., 2003; Ohmura et al., 2010). That is, the acute aversive stimuli, the exposure to electric shocks, would activate the MRN-dorsal hippocampus pathway, promoting the storage of spatial information, since the dorsal hippocampus exerts this function.

In addition, there are intra-raphé modulatory mechanisms

dependent on the local GABAergic circuitry, which is characterized by interneurons that exert inhibition of ascending 5-HT transmission (Li et al., 2006; Varga et al., 2002) or of indolaminergic negative feedback (Hensler, 2006). There is a mutual local control through the bilateral interconnection of serotonergic neurons between the dorsal raphe nucleus (DRN) and the MRN (Hensler, 2006). Endogenous serotonin activates 5-HT_{1A} receptors located in the dendrites or the cell body of neurons, also called somatodendritic receptors, reducing neuronal firing and, consequently, the release of 5-HT in terminal area projections (Harsing, 2006; Kusljic and van den Buuse, 2006; Ögren et al., 2008). There is a higher density of 5-HT_{1A} receptors in MRN serotonergic neurons, unlike the DRN, where these receptors are also identified in non-serotonergic neurons (Beck et al., 2004; Kirby et al., 2003).

It is known that inactivation of MRN neurons by 5-HT_{1A} receptor agonists causes anxiolysis in different tests of anxiety, similar to anxiolysis that occurs as a result of lesions of this structure (Andrade et al., 2013). Microinjection of 8-OH-DPAT, a 5-HT_{1A} receptor agonist, in the MRN impairs the acquisition of contextual fear in male rats (Avanzi and Brandão, 2001; Avanzi et al., 1998, 2003; Borelli et al., 2005; Melik et al., 2000; Silva et al., 2002, 2004). On the other hand, prior injections of a silent dose of WAY100.635, a 5-HT_{1A} receptor antagonist, into the MRN reversed the anxiolytic effect of 8-OH-DPAT in the elevated T-maze (Dos Santos et al., 2005, 2008; Vicente et al., 2008) and in a dark-light transition test (Vicente et al., 2008).

These aspects strengthen the hypothesis that MRN serotonergic neurons are involved in anxiety in both innate situations as well as in conditioning experiments. However, the main focus of the present study was to understand female behavior, especially in females with loss of ovarian function (transitional or surgical post-menopause), in situations related to aversive conditioning. It is well established that ovariectomy increases the manifestation of freezing during contextual conditioning tests (Gupta et al., 2001) and that treatment with estradiol decreases it (Barha et al., 2010). Similar effects are observed during proestrus, a period when the levels of sexual steroids (estradiol and progesterone) are elevated (Freeman, 1994), in some anxiety tests (Frye et al., 2000; Markus and Zecevic, 1997). It should be emphasized that not all studies show variation with the estrous cycle in these various tests of anxiety (Dooner and Lowry, 2013 for review). These apparent discrepancies could be related to other factors, for example, the interaction between sexual hormones and glucocorticoids (Solomon and Herman, 2009).

In the present investigation the estradiol was microinjected into MRN of the ovariectomized rats before the conditioning session and the behavioral results (freezing and rearing) were reversed in the test session. This estradiol effect was antagonized by Way 100635. The other parameters evaluated, such as locomotion, grooming and faecal boluses were not affected by treatment with EB. The urinary elimination was not affected either by aversive conditioning or by EB treatment.

The behavioral effects of estradiol in the MRN may be due to a genomic action, a non-genomic action, or to both (for review see Solomon and Herman, 2009). We hypothesize that immediate, non-genomic mechanisms of EB affect the activity of serotonergic neurons: the rapid infusion of EB can hyperpolarize neurons in seconds, through the opening of K⁺ channels that inhibit neuronal activity. These immediate effects of EB on neuronal firing are likely due to the activation of non-genomic mechanisms modulating several neurotransmitters, including 5-HT (Kelly et al., 1999; Kelly and Ronnekleiv, 2002). The functions of estrogen in serotonergic activity are well-described (Deecher and Andree, 2008): it increases the activity of tryptophan hydroxylase (an enzyme involved

in the synthesis of 5-HT), resulting in an increase in overall 5-HT availability; it increases 5-HT by decreasing expression of monoamine oxidases (the enzymes responsible for degradation of 5-HT); it regulates serotonergic tone by modulating expression of the serotonin reuptake transporter (SERT); it modulates serotonin neuronal firing by affecting the distribution and state of 5-HT receptors, specifically presynaptic 5-HT_{1A} autoreceptors and 5-HT_{2A} receptors (Amin et al., 2005; Genazzani et al., 2007). In this same direction, Le Saux and Di Paolo (2005) showed that ovariectomy increased the binding of a 5-HT_{1A} agonist ([3H]8-OH-DPAT) in rat DRN neurons. Treatment with estradiol reversed this effect and restored the binding levels to those of intact animals.

In this investigation, it is possible that ovariectomy caused an increase in the expression of 5-HT_{1A} receptors in the MRN. As estradiol potentiates local 5-HT activity on 5-HT_{1A} receptors, the acute effect of EB in the MRN in ovariectomized rats may also have potentiated the inhibitory role of 5-HT_{1A} receptors on the activity of MRN serotonergic neurons. Therefore, if estradiol chronically desensitizes 5-HT_{1A} receptors (Betha et al., 2002), the loss of estrogen, mainly in menopause (transitional and surgical), could cause an increase in 5-HT_{1A} receptors in the MRN (Amin et al., 2005; Biegon and McEwen, 1982). However, in females at this life stage, there would likely be no endogenous estrogen to potentiate the activity of serotonin on 5-HT_{1A} somatodendritic receptors. Consequently, angiogenesis could be promoted with acute aversive stimuli.

Our previous studies, based on the identification of estrogen receptors in cell bodies of neurons located in the MRN (Alves et al., 2000; Leranth et al., 1999), demonstrated that, in ovariectomized rats, the microinjection of EB into this structure, immediately before the behavioral evaluation, increased open arms exploration in the elevated plus-maze. The same procedure diminished the freezing manifestation (duration in seconds) in contextual conditioning in the test sessions. In addition, these anxiolytic-like effects were reversed by prior injection of WAY100.635 into the MRN (Andrade et al., 2005, 2009).

The results of the present research confirm previous results. However, in this study, the microinjection of EB into the MRN was performed before animals were exposed to the aversive event. This experimental procedure did not affect the behaviors of ovariectomized rats in the conditioning session, but prevented the freezing manifestation in the test session and increased the rearing presentation, 24 h after the aversive conditioning. A rapid non-genomic effect of EB on somatodendritic 5-HT_{1A} receptors in the MRN may have occurred. This action may have damaged the normal functioning of the MRN-dorsal hippocampus pathway, impeding the storage of aversive information (foot shocks) and association of this experience to context in other time.

Grooming, an innate behavior that can generally be increased in two situations, high or low levels of stress (Kalueff and Tuohimaa, 2004), was only increased during the test session, similar to the response of male rats to restraint (Andrade and Graeff, 2001), but was not modified by any treatment in the present study. The electrolytic lesion in the MRN diminished the presentation of grooming in an elevated plus-maze, but specific lesions with 5,7-DHT did not affect this behavior (Andrade and Graeff, 2001). Taken together, these results suggest that grooming could be affected by the function of non-serotonergic neurons originating in the MRN.

5. Conclusion

In conclusion, the microinjection of EB into the MRN, prior to the exposure of the animals to aversive stimulus (foot shocks), did not alter the behaviors of animals in the conditioning session (freezing

and rearing), but neutralized the association of the aversive experience with the context. It is possible that the steroid EB damaged the acquisition of the aforementioned association, and therefore, affected behavioral responses, due to serotonergic activity blockage in the MRN, since this effect was reversed by previous microinjection of WAY100.635. Hence, EB may exert a function on serotonergic neurons originating in the MRN, minimizing the impact of acute aversive experience on behavior by impairing the storage of aversive information. Periods of low estrogen concentration could contribute to increased perception of aversive stimuli, promoting an increase in anxiety-like behavior in female rats. Making an analogy to human experience in a translational approach, anxiety in women is augmented in moments when there is a low concentration of estrogen, and potentiated in environments where aversive experiences happen, or when remembering the place where undesirable or aversive stimuli occurred (Rocca et al., 2008; Seeman, 1997). The results may explain estradiol's mechanism of action in preventing these effects by acting as an inhibitor of the serotonergic pathway MRN–Dorsal Hippocampus and so attenuating the association of the aversive experience to the context.

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