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Pharmacological effect of one icv dose of allopregnanolone in the female rat: behavioural profile

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Abstract. We have previously observed that intracerebroventricular allopregnanolone (ALLO) injection produced an anxiolytic effect and inhibited sexual receptivity when the test was performed in a separate manner. Also, ALLO reverts learning deficit in female rats in the hippocampi. To study the behavioural effects of an acute treatment with ALLO in the right lateral ventricle we used two approaches: a- A battery test to analyze the anxiety and mating behavior. And b- The avoidance test and novel object recognition test to evaluate its effect on memory and learning. Ovariectomized rats were injected with estrogen and progesterone. After it ALLO or vehicle were administered into the right lateral ventricle. To reach the objective (a) rats were put in a sequential battery test in the next order: 1-Open field. 2- Plus maze task. 3- Mating behavior. For the aim (b) it was performed a Novel Object Recognition Test and Step-down Inhibitory Avoidance Task. ALLO did not affect locomotors-exploratory behavior. Animals treated with ALLO, spent more time and had more entries into the open arm in a plus maze task and lordosis quotient was lower than in the control group. ALLO increased the latency in step down test and had no effects on discrimination index test in NORT. Here we demonstrated that one pharmacological dose of ALLO in ovariectomized primed rats is enough to generate all changes observed in the battery test. Moreover, the acute treatment with ALLO in lateral ventricle enhanced the memory acquisition in an avoidance task.

Keywords: Allopregnanolone, Neurosteroids, Behavior, Female rat, Memory.

Abbrevation list: ALLO: Allopregnanolone; E: oestrogen; NORT: novel object recognition test; OVX: ovariectomized; P: progesterone; LV: Lateral ventricle; ICV: intracerebroventricular.

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Introduction

Steroids are produced not only in traditional organs, such as the ovaries, adrenals, and placenta but also in the brain and peripheral nerves (McEwen, 1991). Steroids synthesized in the central nervous system are called “neurosteroids”. They are produced and metabolized in several brain areas (Laconi et al., 2012). Moreover, the

term “neuroactive steroids” refers to steroids, independently of their origin, that are able to modify neural activity (Majewska, 1992; Robel and Baulieu, 1994). Changes in neurosteroid levels could contribute to development many physiological and pathological processes such as stress, depression, epilepsy and Alzheimer’s disease (Bicíková and Hampl, 2007).

Allopregnanolone (ALLO, 3α-hydroxy-5α-pregnan-20-one) is one of the most active progesterone derivate and is a neurosteroid synthesized the novo in the brain and in the peripheral nerves. ALLO levels in the nervous system are a product of metabolism from peripheral gland-derived from precursors and its subsequent accumulation in neural tissues (Robel and Baulieu, 1994; Laconi et al., 2001). The quantities of ALLO in the nervous system can be much greater than the circulating ones and even persist after removal of the glands that produce pregnane steroids in the body (Majewska, 1992; Paul and Purdy, 1992; Robel and Baulieu, 1994; Mellon, 1994; Bicíková and Hampl, 2007).

Neurosteroids are substances with very interesting behavioral effects. They are able to alter cognitive functions, particularly in memory processes (Wang, 2013). Both, systemic or intracerebral administration of neurosteroids, like pregnenolone, enhances memory in young and old rats (Nanfaro et al., 2010; Mayo et al., 2001). However, there is controversial evidence about the effects of ALLO in hippocampus. Johansson et al. (2002) have demonstrated that ALLO inhibits learning in the Morris water maze and, on the other hand Modol et al. (2011) has demonstrated that intra-hipocampal administration of ALLO have no effects on retention learning. Previously, in our laboratory, it were investigated the effects of ALLO action in exploration, anxiety, social and reproductive behaviors in separate tests. We have reported that ALLO injection into the lateral ventricle (LV) modified the female rat behavior producing an anxiolytic effect in an ovarian hormonal-dependent manner (Paul and Purdy, 1992). Moreover, we showed that intra-cerebroventricular (ICV) administration of ALLO in ovariectomized (OVX) estrogen/progesterone primed rat inhibited lordosis behavior (Laconi et al., 2002). In agreement with previous sentence, Santuro et al. (2014) have reported a decreased level in brain ALLO concentrations induced by hormonal contraceptives is associated with a reduction in social behavior and sexual motivation in female rats. Recent studies showed that the use of a short battery of tasks exploration, anxiety, social interaction and paced mating, increases ALLO synthesis in the mid-brain of male rats (Frye et al., 2014, 2012). These data support that the mating behavior can be utilized as a measure of ALLO’s action as well as a way to manipulate ALLO levels in the mid-brain.

The purpose of the present work was study the effects of ALLO on behavior using a pharmacological dose of ALLO (previously probed in others neurochemical and histological experiments). ALLO was injected into lateral ventricle (LV) under two different approaches:

a) The effect on the anxiety and mating using a behavioral battery test with female rats. The rats were put in a sequential way battery:

1- Exploratory behavior measured in an open-field device;

2- anxiety behavior by the plus-maze test; and

3- mating behavior measure by lordosis quotient test.

b) The effect on memory and learning using a Novel Object Recognition Test (NORT) and Step Down Inhibitory Avoidance Task.

Materials and methods

Animals

Adult female Sprague-Dawley rats (60-90 days old; 200-220 g) bred in our laboratory were used. They were housed in groups of four per cage until surgery. Animals were maintained at constant temperature (22 °C± 1 °C) and lightning (12/12 light/dark cycle) with food and water available ad libitum. Animals were kept and handled according to the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Research Council, 2011). All efforts were made to minimize animal suffering.

Drugs

Allopregnanolone (Sigma Chemical Co., St. Louis, MO, USA); penicillin G benzathine (Riched, Argentina), and ketamine HCL (Hollliday - Scott S.A, Buenos Aires, Argentina), xylazine (Koning Laboratories, Buenos Aires Argentina) were used. ALLO was prepared as we describe in our previous papers (Laconi et al., 2012). The dose of ALLO used (6 μM) is a pharmacological dose that mimic the blood concentration during stress (Laconi et al., 2001, 2002, 2007, and 2012). Control animals were injected with Krebs-Ringer bicarbonate glucose (KRBG - vehicle) buffer at pH 7.4. KRBG preparation contains propyleneglycol in equivalent concentrations to that used in experimental groups. Drugs were injected into lateral ventricle in a 1 μL injection volume.

Surgical procedures

The stainless-steel cannulae assembly were stereotaxically inserted into the right LV in rats in animals anesthetized with intraperitoneally injected ketamine HCL (80 mg /kg) and xylazine (4 mg/kg). The following coordinates from bregma were used, in accordance to the Paxinos and Watson's Atlas, AP: +0.4 mm, L: − 1.5 mm, and DV: − 4 mm (Paxinos and Watson, 1997). In order to protect cannulae of obstruction, at the end of the surgery, each cannula was sealed with a stainless steel wire. In the same surgery the female rats were ovariectomized bilaterally (OVX). To prevent infections, each animal received an injection of 0.2 mL intramuscular of 1 200 000 UI penicillin G benzathine (1 UI = 0.6 μg; 72 mg/rat). After surgery, animals were housed singly in plexiglas cages and were maintained undisturbed for a week.

Experimental design

Ovariectomized rats were injected subcutaneously (s.c.) with oil oestradiol benzoate (E, 0.1 mg/kg) and progesterone (P, 4 mg/kg), 48 and 4 h before the training session, respectively. For both objectives, the experiments were made in rats treated with ALLO (6 μM; n = 8) or with vehicle (n = 8; group control) these were injected into the right LV (ICV) of freely moving rats in a volume of 1 μL during 60 s. A stainless-steel needle was placed into the guide cannula and connected by a silicone catheter to a Hamilton microliter syringe. The injection cannula was left placed for an additional minute to avoid reflux. Thirty minutes after drug injection, animals were put into the arena to perform the training session. After the behavioral test, animals were decapitated and they were injected with 1 μL of blue ink through the guide cannula to confirm the location of the guide cannula into LV. Only animals with microinjections in LV were included in any experimental protocol.

Battery test

The effect of ALLO was evaluated in the following battery, on locomotor activity, sexual and anxiety behavior. Rats were individually tested in behavioral test in a special room (noise isolated), in a sequential manner, with no breaks between tasks (other than time needed to clean apparatus and move rats from one task to the next). Testing apparatus were brightly-lit from above with three fluorescent bulbs. Behavioral testing was performed by 1 of 3 observers (98% concordance), who were blind to experimental conditions. Rats were tested through the following battery in the order described below:

1- Open field. Behavior displayed in the open field is used as a tool to measure the exploration and locomotion (Frye and Erskine, 1990). A commercial apparatus, a photoelectric device known as Opto-Varimex (OVM; Columbus Instruments, USA), designed to measure photobeam interruptions in individually tracked photocells, was used to assess the locomotor and exploratory activity of the animals. The OVM consisted of a plexiglas transparent cage (30 x 42 x 42 cm) with a homogenous black plastic floor. The walls housed infrared emitters and detectors in order to automatically register several measures: 1) horizontal activity: all movements performed on the horizontal axis; 2) ambulatory activity: all movements detected as displacement; 3) non-ambulatory activity: all movements performed by the animal while remains in the same place; 4) number of movements: number of episodic or consecutive

movements performed by the animal; and 5) vertical activity: number of times the subject rises on their rear feet in the air or against the walls during at least 2 s. Regarding any measure other than discrete ones (for example vertical activity), the measures are referred to as total counts/5 min, scoring a count as an interruption of the photobeam per second (Nanfaro et al., 2010). The numbers of movements were recorded during a 5 min (Laconi et al., 2001, 2002).

2- Elevated plus maze. The evaluation of behavior in the elevated plus-maze is used to measure exploration and anxiety. The total number of arm entries and time spent on the open arm are an index of animal anxiety. The elevated plus maze consisted of four arms, 45 cm long and 7 cm wide, elevated 45 cm off the ground. Two arms are enclosed by walls 30 cm high while the others are exposed. The number of entries and the amount of time spent into the open and closed arms were recorded during a 5 min. test (Laconi et al., 2001; Frye et al., 2000; File, 1990).

3- Paced mating. Paced mating behavior was measured by lordosis quotient during mating. It consists in the quantification of the lordosis postures assumed by female rodents during mounts of the male. It was carried out as was previously reported (Laconi and Cabrera, 2002; Erskine et al., 1985; Frye and Erskine, 1990). Behavioral testing was conducted in a red, dimly illuminated, and sound proof animal room. Mating tests were conducted in a 50 x 50 x 30 cm chamber. Sexually experienced male rats were introduced into the arena at least 10 min before the female. Later, a female rat was gently dropped into the corner most distant from the males. After every mount, which consisted of the male clasping the female, the female was scored as to whether or not the female has displayed lordosis. It was considered positive, after completely dismounting, when the female showing pelvic thrusting during the mount. Each test consisted in 10 mounts, and the number of lordosis responses was expressed as the lordosis quotient, equivalent to the number of lordosis responses per 10 mounts, expressed as a percentage of the total mounts.

Novel object recognition test and Step down inhibitory avoidance task: Effect of ALLO in learning and memory

In order to test learning and memory parameters different groups of animals were used. Behavioral tests were performed by 1 of 3 observers (98% concordance), who were blind to experimental conditions. Previously, to exclude the possible effect of drugs administration on locomotors and exploratory activity, we assessed each experimental group with open field test before NORT and step down inhibitory avoidance task.

Novel object recognition test

The apparatus used in this test was described in detail by our working group's previous work (Nanfaro et al., 2010). Animals were maintained undistured into the experimental room during a period of at least one hour. The day before training, each animal freely explored the apparatus with no objects for a 2 min period. A training session (T1) was followed by a test session (T2) 24 h later. During training session, animals were placed in the arena containing two identical objects (pink truncated pyramids). In the test session a familiar object was changed for an unfamiliar one (grayish opaque candlestick). Duration of both training and test stages were 3 min each one. The position of the objects (familiar and unfamiliar) and the extreme of the box used to place the objects were randomly exchanged for each experimental animal in order to avoid the use of potential confounding spatial clues. Exploration was defined as the orientation of animal's snout towards the object, within a range of 2 cm or less from the object (Ennaceur and Delacour, 1988). Running around the object or sitting on it was not recorded as exploration. The objects and floor were carefully cleaned with ethanol (10%) after each individual trial to equate olfactory cues. The experiments were recorded with a camcorder digital camera JVC Everio GZ-MG330 (Japan) using a black and white recording mode in order to improve the

register. The measures during the object recognition test were as follows: 1) total time spent by the subject exploring both objects during training (T1); 2) total time spent by the subject exploring just the novel object during T2; and 3) discrimination index, the difference between time spent exploring unfamiliar and familiar objects during T2 (Prickaerts et al., 2002).

Step down inhibitory avoidance task

Animals were trained in a one-trial step down inhibitory avoidance task, a learning task in which stepping down from a platform present in a given context is associated with a foot shock, resulting in an increase in step-down latency, and tested for retention 24 h later. The training apparatus consisted of a 50-25 x 25 cm Plexiglas box with a 4 cm high, 8.5 cm wide, and 17 cm long platform on the left end of a series of bronze bars that constitute the floor of the box. During training, animals were gently placed into the platform facing the left rear corner of the training box (Cammarota et al., 2008). When they stepped down and placed their four paws on the grid, they received a 3 s, 0.5 mA scrambled foot shock and were immediately withdrawn from the training box. Memory retention was evaluated in test sessions carried out 24 h after training. At test, trained animals were put back on the training box platform until they eventually stepped down to the grid. The latency to step down was taken as an indicator of memory retention. A 180 s ceiling was imposed on step-down latency during test sessions. Additionally, the step-down latency during training sessions were reported as additional control (Escudero et al., 2012).

Statistical analysis

The discrimination index for object recognition as well as the locomotor and exploratory behaviors were analysed using a one way analysis of variance (ANOVA), followed by a post hoc Newman-Keuls test. Pair comparisons were analysed using the Student-t test. Data were expressed as the mean ± SEM; $p < 0.05$ was considered as minimum criterion for assigning statistical significance. For the statistical analysis we utilized the software StatView for Windows (Abacus Concepts, Berkely, CA, USA).

Results

1- Effect of ALLO on the behavior battery test

a) Exploratory behavior. Open field test. Parameters were not modified by ALLO infusions when compared with the control group in the Open Field. Not differences were observed in ambulatory activity ($p = 0.500$), non-ambulatory activity ($p = 0.247$), number of movements ($p = 0.660$), vertical activity ($p = 0.639$) and horizontal activity ($p= 0.877$) (Table 1).

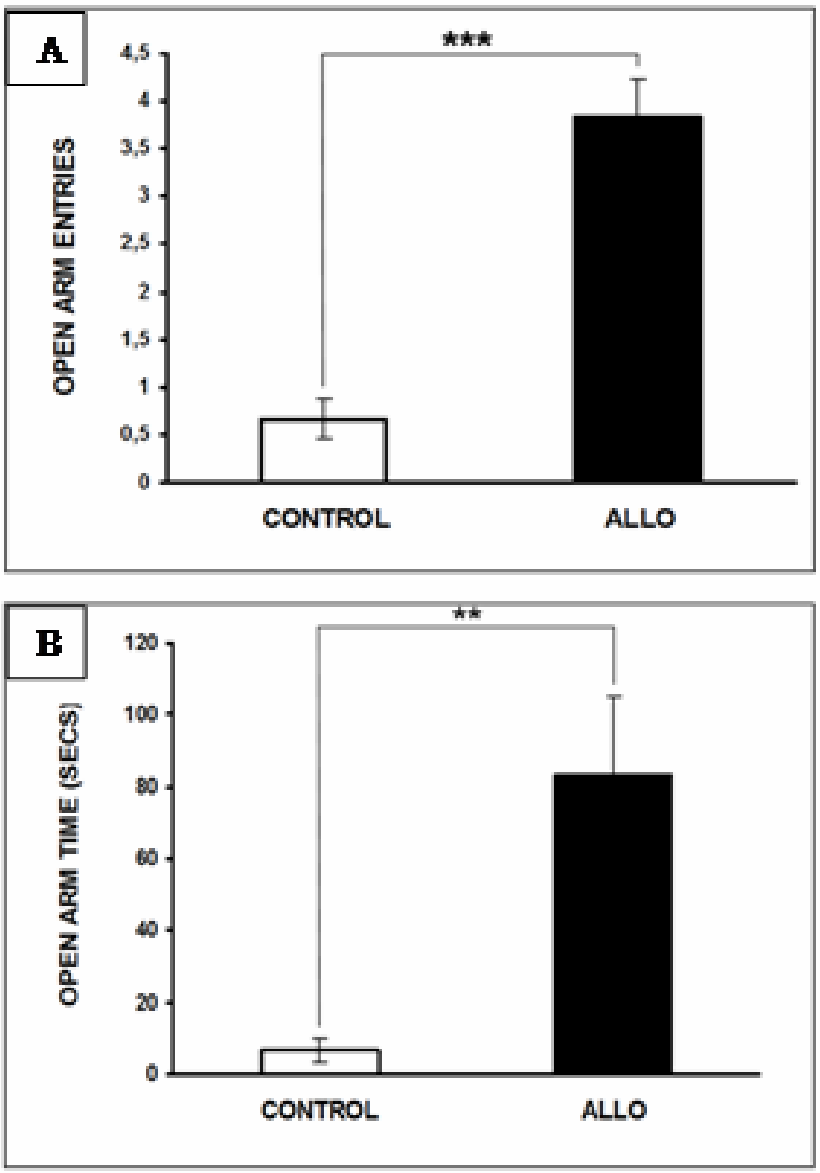
b) Anxiety behavior. Plus-maze test. Animals treated with ALLO spent more time and had more entries into the open arm ($p < 0.01$ and $p < 0.001$, respectively) than control group (Figure 1, A and B).

c) Mating behavior. Lordosis quotient test. In ALLO treated animals, lordosis quotient was significantly lower compared to control group ($p < 0.001$) (Figure 2).

Table 1. Effects of ALLO on locomotor and exploratory activity. Results are expressed as mean ± SEM of recorded movements recorded in open field, from OVX EP-primed rats infused with vehicle (control group, n = 8) or ALLO (Experimental group, n=8) into LV.

Type of movements	Control group	Experimental group	t test
Horizontal	4,765 ± 307	4,838 ± 339.6	p = 0.877
Vertical	35 ± 3.17	36.8 ± 1.88	p = 0.639
Ambulatory	3,795 ± 375	4,148 ± 330	p = 0.500
Non ambulatory	936 ± 75	819.2 ± 54.82	p = 0.247
Numbers of movements	124.5 ± 5.2	128.3 ± 6.91	p = 0.660

Figure 1. Effect of ALLO on anxiety test. Results are expressed as mean ± SEM of open arm time (Panel A) and open arm entries (Panel B) of OVX EP-primed rats infused with vehicle (control group, white) or ALLO (black) into LV (for each group n = 8, ** $p < 0.01$ and *** $p < 0.001$, respectively).



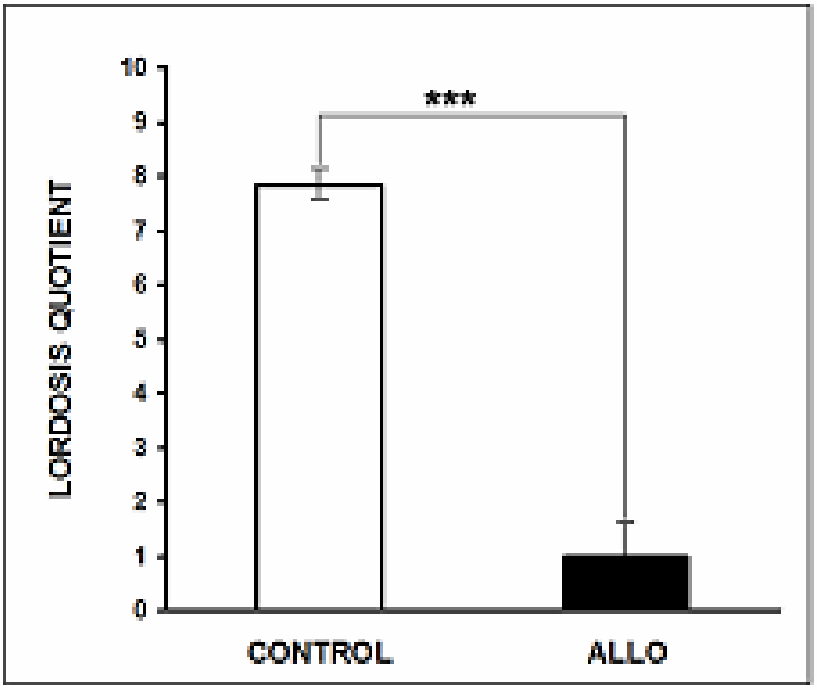
2- Effect of ALLO in learning and memory: NORT and step-down inhibitory avoidance task

Statistical analysis showed that neither of the parameters evaluated were modified by ALLO administration in the open field test (Data not shown).

a) Novel object recognition test. In the discrimination index test there were no significant differences between animals treated with ALLO and control group (Figure 3.B). Additionally, there were no significant differences between control and treated group regarding total exploration time, the time spent by the animals exploring both objects, both during training session (T1) and during the test session (T2) (Figure 3.A).

b) Step down inhibitory avoidance task. In ALLO treated animals, the latency in step down was significantly higher than in the control group ($p < 0.001$, Figure 4). The step-down latency, measured during training sessions was reported for

Figure 2. Effect of ALLO on sexual behavior. Results are expressed as mean ± SEM of lordosis quotients of OVX EP-primed rats infused whit vehicle (control group, white) or ALLO (black) into LV (for each group n = 8, *** $p < 0.001$).



each group as a control and there were no significantly differences (data not shown).

Discussion

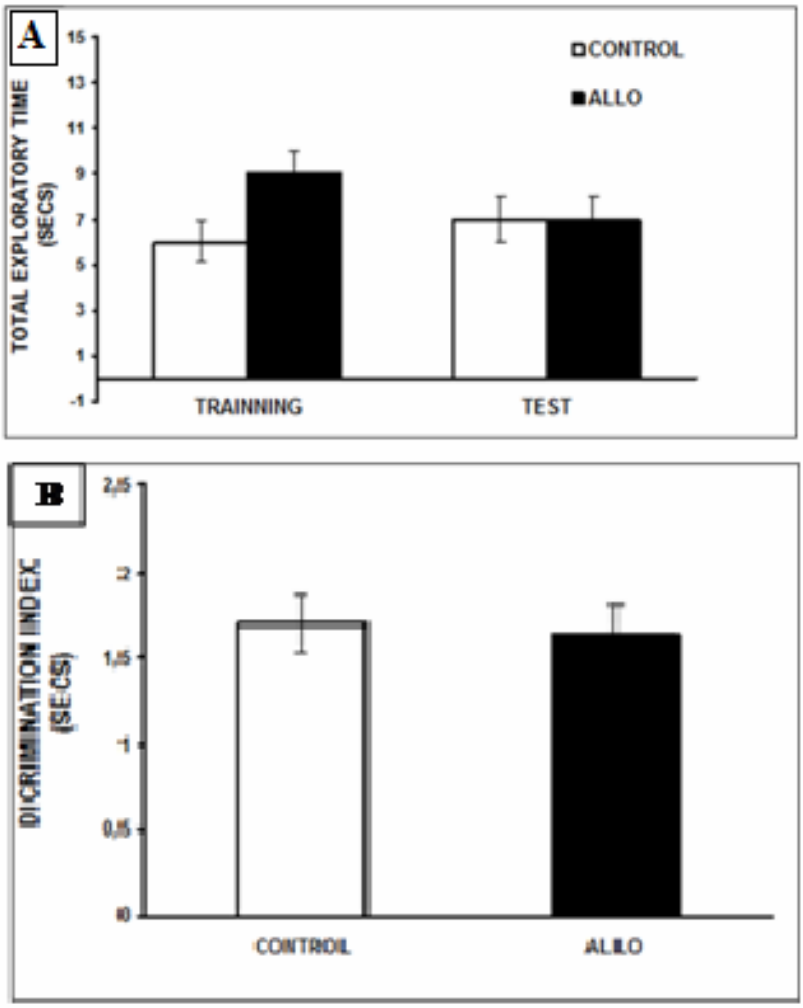
The results of the present study show that one injection of ALLO into the right lateral ventricle is enough to modulate exploratory, anxiety and reproductive behaviors in a behavioral battery task. Both, locomotor as exploratory activity are important to exclude the eventual effect of confounding variables. The ALLO injection didn't modify exploratory/ambulatory behavior (Table 1).

As is well known, ALLO is a positive modulator of $GABA_A$ receptors. And has pharmacological properties as anxiolytic and anticonvulsant activity (Kokate et al., 1999). It has also been shown that ALLO is able to regulate anxiety and depression-like behaviors in a model of chronic social stress by isolation in rodents (Evans et al., 2012). Moreover, in the brain the ALLO level under stress situation is usually augmented (Concas et al., 1996). ALLO has been shown to facilitate GABA action at nanomolar concentrations and to open the chloride channel at micromolar concentrations. This neurosteroid modulates the general inhibitory system in the Central Nervous System by enhancing the effect of GABA (Laconi et al., 2001; Laconi and Cabrera, 2002).

Clinical trials support that ALLO can mediate the effects of anxiety and depressive symptoms in women with premenstrual dysphoric disorder (Freeman et al., 1993; Endicott at et al., 1999; Gracia et al., 2009) as well as self-reported anxiety in men with post-traumatic stress disorder following exposure to trauma reminders (Casada et al., 1998). Furthermore, the reductions of ALLO levels in depressive symptoms of women diagnosed with major depression are correlated with higher brain spinal fluid levels of ALLO (Concas et al., 1996; Frye et al., 2012).

Previously, we reported an anxiolytic effect of ALLO in an ovarian hormonal dependent way in a simple task, suggesting that ALLO needs the traditional nuclear mechanism of action of ovarian steroids to induce anxiolytic effects (Laconi et al., 2001). In this work, ALLO injection

Figure 3. Effect of ALLO on exploratory activity, learning and memory (novel object recognition test). Results are expressed as mean ± SEM of total exploratory time of both object during training and test sessions (Panel A) and discrimination index (Panel B) of OVX EP-primed rats infused whit vehicle (control group, white, n = 8) or ALLO (black, n = 8) into LV.

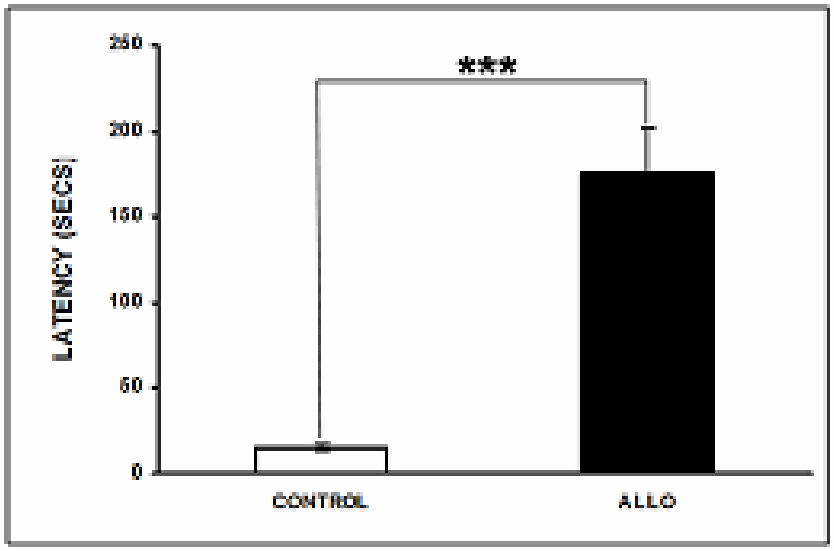


produced a clear anxiolytic effect in OVX-EP rats that was measure by time spend and entries in open arm in the plus maze (Figure 1). These results showed that ALLO produced an anxiolytic change consistent with those observed in other animal models of anxiety (Laconi et al., 2001; Freeman et al., 1993; Gracia et al., 2009).

In this work, we analysed mating by lordosis quotient test. ALLO treated animals showed that lordosis quotient was lower compared to control group (Figure 2). These data suggest that the administration of ALLO intra-brain can reduce the sexual behavior and that are not associated with non-specific effects on ambulatory behavior. In accordance with this, Laconi and Cabrera (2002) reported that ALLO influences the dopaminergic and GABA systems with the consequent alteration in LH levels and sexual receptivity in female rats. In addition, mating can induce ALLO formation in the nervous system. Then, the mating behavior can be utilized as a measure of the effects of ALLO in the midbrain and as a measure of ALLO levels (Frye et al., 2007; 2014; Frye and Bayon, 1999).

Taken together, these findings suggest that one injection of ALLO in OVX-EP female rats is sufficient to generate all changes observed in the battery test. In agreement with this, one previous report comparing males tested in a similar battery of anxiety tasks versus the individual anxiety tasks did not reveal

Figure 4. Effect of ALLO on memory test (step down inhibitory avoidance task). Results are expressed as mean ± SEM of latency in step down of OVX EP-primed rats infused whit vehicle (control group, w, n = 8) or ALLO (black, n = 8) into LV. *** $p < 0.001$.



differences in behavioral and/or endocrine measures (Edinger and Frye, 2005).

The other aim of this work was to study the effect of one injection of ALLO in the same dose (acute treatment) and analyse the effect on memory and learning in two different tests.

Chronic treatment with other compounds acting on the $GABA_A$ receptor, such as benzodiazepines, ethanol and medroxy-P acetate has been associated to cognitive decline and/or increased risk for dementia. ALLO declines with age and neurodegenerative disease. In a series of studies using in two transgenic Alzheimer's disease model, Swe/Arc and Swe/PS1 mice, was demonstrated that chronically elevated levels of ALLO (physiological range), accelerated Alzheimer's disease development. After one month of exposure to chronically elevated levels of ALLO, impaired learning and memory pattern occurred in the Alzheimer's disease mice (Brinton, 2013; Wang, 2013). In opposition, an intermittent ALLO exposure increased liver X receptor and pregnane X receptor expression, reduced amyloid-β and microglial activation, and increased markers of myelin and white matter generation (Brinton, 2013).

We evaluate the effect of one injection of ALLO in LV in learning and memory trough a NORT and a step down task (inhibitory avoidance task). Open field test was performed to all the animals before tasks. Statistical analysis showed that ALLO infusions did not modify exploratory, locomotors activity and it had no sedative effects.

ALLO had no effect on the discrimination index tested in NORT (Figure 3). This test shows the rats' natural exploratory tendencies and it is considered to be less demanding than tasks that incorporate aversive stimuli (Cavigelli and McClintock, 2003). However, in this test we demonstrated that ALLO did not modify significantly discrimination index tested, we only observed a positive tendency.

The step down task involves an associative memory component together with a component of operant conditioning, where the animal learns that a particular location must be avoided, due to is associated with an aversive event. In previous reports, we studied the effects of sub-cutaneous administration of ovarian steroid in OVX rats in combination with an intra-hippocampal infusion of ALLO on learning and memory processes. We used a passive avoidance test. And we observed an important role of ALLO in memory impairment processes through the regulation of the enzyme responsible for its

synthesis (Escudero et al., 2012). One injection of ALLO 6 μM in LV increased latency in comparison with control group in a Step Down inhibitory Avoidance Task (Figure 4). So, now we postulated that the acute treatment of ALLO enhanced the memory acquisition in the OVX-EP in comparison of the control. These finding emphatize the importance of GABAergic stimulation as one mechanism to enhance cognition. In accordance with this, co-administration of E and P can enhance cognitive performance (Sandstrom and Williams, 2001; Sato et al., 2004). Freeman et al. (1993) have demonstrated that P or ALLO administration increased the percentage of time spent exploring the object in a novel location. Other studies have not demonstrated a beneficial effect of progestins for cognitive performance (Zou et al., 2000). Differences in the results could be by dose-dependent effects for behavior. Additionally, since the drugs were administered before training and to exclude the eventual confounding effect, the step-down latency during training sessions was reported for each group as a control and no difference was found. These results confirm that none of the drugs have any effect on locomotor activity and exploratory activity (data not shown).

The neuro-active steroids, could modulate one or more memory systems. According to the place and without changing any basic molecular and cellular mechanism, not to mention the dose utilized, it could be obtained an inhibitory or stimulatory result.

Taken together all previous results, we can think in a possible clinical uses of neurosteroids under different kind of pathological conditions. Further studies are required in order to explain the specific role of ALLO (endogenous or exogenous). In resume, we consider that the results discussed in this paper add to the understanding the mechanisms by which an acute treatment of ALLO in LV could be playing a modulator role in anxiety and reproductive behaviors, also in learning and memory processes, which are useful to increase the knowledge of the different physiological and patho-physiological conditions in the female.

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Conflict of interest statement

Authors declare that they have no conflict of interests.

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