A single dose of allopregnanolone affects rat ovarian morphology and steroidogenesis

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Abstract

Allopregnanolone, a progesterone metabolite, is one of the best characterized neurosteroids. In a dose that mimics serum levels during stress, allopregnanolone inhibits sexual receptivity and ovulation and induces a decrease in luteinizing hormone levels. The aim of this work was to examine the effect of an intracerebroventricular administration of allopregnanolone on ovarian morphophysiology; serum and tissue levels of progesterone and estrogen; and enzymatic activity of 3 β -hydroxysteroid dehydrogenase, 20 α -hydroxysteroid dehydrogenase and 3 α -hydroxysteroid oxido-reductase in the ovary and in the medial basal hypothalamus on the morning of estrus. Ovarian morphology was analyzed under light microscopy. The hormone assays were performed by radioimmunoassay. The enzymatic activities were measured by spectrophotometric analysis. The morphometric analysis revealed that, in allopregnanolone-treated animals, the number of secondary and Graafian follicles was decreased, whereas that of atretic follicles and cysts was significantly increased. Some cysts showed luteinized unruptured follicles. There were no differences in the number of tertiary follicles or corpora lutea in comparison with the corresponding control groups. In allopregnanolone-treated animals, progesterone serum levels were increased, whereas ovarian progesterone levels were decreased. Moreover, 3 β -HSD and 3 α -HSOR enzymatic activities were increased in the medial basal hypothalamus, whereas ovarian levels were decreased. The enzyme 20 α -hydroxysteroid dehydrogenase showed the opposite profile. The results of this study showed that allopregnanolone interferes on ovarian steroidogenesis and ovarian morphophysiology in rats, providing a clear evidence for the role of this neurosteroid in the control of reproductive function under stress situations.

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Introduction

Steroids are synthesized by the brain *de novo* from cholesterol or from an *in situ* metabolism of peripheral hormone precursors. As a whole, these steroids are known as 'neurosteroids' (Robel & Baulieu 1994, Baulieu 1997, Melcangi & Panzica 2006, Melcangi *et al.* 2011). Neurosteroids are synthesized, stored and released in the central nervous system (CNS) and in the peripheral nervous system independently of classical steroidogenic glands, such as gonads and adrenals (Robel & Baulieu 1985, Corpéchet *et al.* 1993, Baulieu 1997, Melcangi & Panzica 2006). These neurosteroids include pregnenolone, progesterone (Pg) and allopregnanolone (ALLO). In particular, ALLO, also called 3 α -hydroxy-5 α -pregnan-20-one or 3 α -5 α -tetrahydroprogesterone, is a metabolite of Pg

(Majewska *et al.* 1986) and is synthesized in astrocytes (Micevych *et al.* 2003) and oligodendrocytes (Mensah-Nyagan *et al.* 1999). ALLO synthesis implies the conversion of Pg to pregnenolone by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and the reduction of this steroid by 3 α -hydroxysteroid oxido-reductase (3 α -HSOR) (Patte-Mensah *et al.* 2010). Pg can also be metabolized by 20 α -hydroxysteroid dehydrogenase (20 α -HSD) (Clementi *et al.* 2004). These enzymes are present in different regions, including the hypothalamus (Guenoun *et al.* 1995, Vidal *et al.* 2000) and the ovary (Vega Orozco *et al.* 2012). Micevych and Sinchak (2008) mentioned that neurosteroids are not isolated from peripheral steroid sources. Mutual interactions modulate their levels in the brain and periphery. Moreover, to provide a reservoir of steroids, circulating hormonal steroids modulate the site-specific synthesis

of neurosteroids and their cognate receptors. This dual regulation of neurosteroidogenesis and post-synaptic receptor expression has profound implications for neurosteroid function. Interactions of peripheral steroids with neurosteroid synthesis are involved in regulating reproduction in the hypothalamus. The contribution from the brain to the pool of ALLO measured in serum is minimal (in the order of nM), whereas the peripheral contribution of the sum of adrenal and ovarian production is in the order of μM (Purdy *et al.* 1990, 1991, Micevych & Sinchak 2008).

ALLO is a positive allosteric modulator of the GABA_A receptor and its effects are similar to those of benzodiazepines and include sedative and anticonvulsant activities (Kokate *et al.* 1999, Laconi *et al.* 2001). Its action on the GABA_A receptor is related to its neuro-protective, neuro-modulatory and antigonadotropic properties (Purdy *et al.* 1991, Concas *et al.* 1996). The potency of ALLO in increasing GABA-activated Cl⁻ currents is comparable to high-potency benzodiazepine. As in Cl⁻ flux studies (Morrow *et al.* 1987), low nanomolar concentrations of ALLO increase GABA-activated Cl⁻ currents, whereas higher concentrations, in the low micromolar range, directly activate a bicuculline-sensitive Cl⁻ current.

The affinity of ALLO for GABA_A receptors is comparable to that of benzodiazepines, and ALLO is one of the most potent GABA_A receptor ligands. ALLO actions are mediated by synaptic and extra-synaptic receptors. ALLO interacts with synaptic GABA_A receptors to produce phasic inhibition via specific bindings (Paul & Purdy 1992).

Pg and ALLO have been studied in clinical trials of psychiatric disorders such as depression, anxiety, premenstrual irritability and menopausal syndrome as well as in neurodegenerative diseases such as Parkinson or Alzheimer and post-traumatic neuronal repair (Bicíková & Hampl 2007).

Previously, we reported that ALLO increases GnRH release through the glutamatergic system and NMDA receptors (Giuliani *et al.* 2011). Other authors reported that ALLO, in different strains of GT1 neurons, might either stimulate or have no effect on the release of GnRH (Sleiter *et al.* 2009). Moreover, we have reported that i.c.v. administration of ALLO induces an increase in endogenous dopamine concentration with a decrease in the dopamine/dopac turnover rate in the medial basal hypothalamus (MBH), indicating an increase in dopamine metabolism. This action is mediated by the GABA_A receptor (Laconi & Cabrera 2002). In addition, ALLO is able to reduce LH serum levels and anxiety levels and to inhibit lordosis in female rats (Laconi *et al.* 2001, Laconi & Cabrera 2002, Pelegrina *et al.* 2015). Moreover, Sleiter and coworkers (2009) found that Pg inhibits GnRH release through an action on membrane Pg receptors, but more evidence is needed to clarify the role of ALLO in GnRH release (Giuliani *et al.* 2011).

Circulating levels of ALLO are usually according with Pg levels but stress or pathological situations could alter both ALLO and Pg levels (Purdy *et al.* 1991, Genazzani *et al.* 1995). Stress is one of the main factors that alter ALLO circulating levels (Purdy *et al.* 1991), which could alter the reproductive axis. Bäckström and coworkers (2011) have shown that neurosteroid concentrations are variable, especially those acting on the GABA_A receptor and can induce mood changes in women. These changes become more apparent during the premenstrual phase, when the levels of Pg and ALLO are the highest. Studies in our laboratory have shown that the anxiolytic effect of ALLO in female rats is associated with their hormonal status (Laconi *et al.* 2001, 2007, Laconi & Cabrera 2002). Recently, we studied the effect of central administration of ALLO doses that mimic the circulating levels during stress and found that ALLO inhibits LH and the ovulation rate and increases prolactin serum levels. In addition, ALLO inhibits corpus luteum apoptosis (Laconi *et al.* 2012) and the loss of ovulation may be due to its effect over the hypothalamic–pituitary axis.

Pg stimulates luteal cells to secrete more Pg in a paracrine manner, protecting corpora lutea from cell death (Stocco *et al.* 2007). The functional and structural luteal development of luteal cells is controlled by the action of several luteotropic hormones secreted by the pituitary gland, the endometrium and the placenta, in the case of pregnancy. Among the best-known luteotropic hormones are PRL and LH (Niswender *et al.* 2000). ALLO could also be a candidate to control the previously mentioned process (Laconi *et al.* 2012).

Ovarian cysts are an important cause of subfertility in mammals, as well as of the polycystic ovarian syndrome and the luteinized unruptured follicle (LUF) syndrome in women (Summaria *et al.* 1998, Ali 2015). Cysts can be subdivided into follicular and luteal cysts, which could be different forms of the same disorder. Follicular cysts are dynamic structures that develop when one or more follicles fail to ovulate (Vanholder *et al.* 2006). Some kinds of cysts do not interfere with the estrous or menstrual cycle (Douthwaite & Dobson 2000, Noble *et al.* 2000) and can appear in the absence of clear clinical signs, such as LUFs, which are formed from Graafian follicles in the absence of oocyte expulsion in women with normal menstrual cycles and animal models (Killick & Elstein 1987, Van de Lagemaat *et al.* 2011). During the follicular phase, granulosa cells acquire luteinization potential, which is suppressed until ovulation (Williams & Erickson 2012). In the LUFs, the process of ovulation is dysregulated. Failure of ovulation due to the luteinization of follicles under the action of LH is one of the main causes of infertility in women (Summaria *et al.* 1998, Qublan *et al.* 2006).

Considering our previous findings, the aim of this work was to determine the effect of a dose of ALLO (6 μM i.c.v.) on the ovarian morphophysiology, Pg and

17 β -estradiol serum and ovarian levels, and 3 β -HSD, 3 α -HSD and 20 α -HSD enzymatic activities in the ovary and MBH.

Materials and methods

Animals

Adult female Sprague–Dawley rats (60–90 days old; body weight 200–250 g) bred in our laboratory were used. Animals were housed at room temperature (22 $\pm$ 2°C) with a 12 h light:12 h darkness photoperiod in an air-conditioned environment. Food and water were available *ad libitum* (standard rat chow Cargil, Córdoba, Argentina). Only animals with two consecutive 4–5-day cycles were used for the experiment. The stages of the estrous cycle were determined daily by vaginal cytology.

Experimental design

In the morning of proestrous, rats were injected i.c.v. with ALLO (6 μ M, 1 μ L injection volume, for 60 s). Control animals were injected with KREBS solution (as vehicle) containing propylene glycol at concentrations equivalent to those used in the experimental groups. The chosen dose of ALLO mimics the serum levels during stress in rats (Purdy *et al.* 1991) and is the same dose used in our previous reports (Laconi *et al.* 2001, 2002, 2012, Giuliani *et al.* 2013, Pelegrina *et al.* 2015). Six rats per group were used in each experiment, which was performed only once. In the morning of estrous (09:00 h), vaginal smears were analyzed. Then, the rats were killed by decapitation. The brains were rapidly removed and cooled on ice and the MBH explants dissected out. The anterior border of each block of tissue was made by a coronal cut just anterior to the entry point of the optic chiasm and the posterior border by a coronal cut just behind the pituitary stalk. The lateral limits were the hypothalamic fissures and the in-depth limit was the sub-thalamic sulcus. The MBH of each animal was labeled for subsequent measurement of enzymatic activity.

Serum samples were collected after blood centrifugation and stored at –30°C until used for radioimmunoassay (RIA). The ovaries were removed and cleaned free of fat, and oocytes were collected by the puncture of the ampulla and counted under a light microscope. The right ovary was frozen to measure Pg and enzymatic activity, whereas the left ovary was fixed in Bouin solution (Biopur Diagnostics) for subsequent microscopic analysis. All protocols were previously approved by the Experimental Animal Committee of the Universidad Nacional de Cuyo, Argentina (CICUAL N° 141021) and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academies, U.S.A., 8th Edition, 2011).

Drugs

Allopregnanolone (ALLO) (3 α -hydroxy-5 α -pregnan-20-one) (Sigma Chemical Co.), Penicillin G Benzathine (Richet,

Argentina), Ketamine HCL (Holliday-Scott S.A, Buenos Aires, Argentina) and Xylazine (Koning Laboratories, Buenos Aires, Argentina) were used for experimental and surgical procedures. Dihydroprogesterone (5 α -Pregnan-3,20-dione) (Sigma Aldrich), Pregnenolone (3 β -hydroxy-5-pregnen-20-one) (Sigma Aldrich), NAD⁺ (β -Nicotinamide adenine dinucleotide hydrate) and NADPH (β -Nicotinamide adenine dinucleotide phosphate) (all from Sigma Aldrich) were used for enzymatic activity determination. ALLO was prepared as described in our previous papers (Laconi *et al.* 2001, 2012). Stocks of ALLO were initially dissolved in propylene glycol to a concentration of 0.6 mM. The dose of ALLO used in the experiment (6 μ M) was obtained by dilutions in Krebs Ringer Bicarbonate glucose (KRBG) buffer at pH 7.4, to make negligible the final amount of propylene glycol. Control animals were injected with KRBG buffer at pH 7.4 as vehicle. KRBG preparation contained propylene glycol in a concentration equivalent to that used in the experimental groups.

Determination of the estrous cycle and the ovulation rate

The estrous cycle stage was determined daily (07:00–09:00 h) using vaginal smears observed with a light microscope. The ovulation rate was determined on the morning of estrous after the rats were killed to confirm our previous results (Laconi *et al.* 2012). After killing, the ovary was placed on a petri dish, moistened slightly and the ampulla was gently punctured. Then, oocytes were removed and counted under a light microscope. ADVANCE \d 1

Surgical procedures

A stainless-steel cannula was stereotaxically inserted into the right lateral ventricle in rats anesthetized with an intraperitoneal injection of Ketamine HCL (80 mg/kg) and xylazine (4 mg/kg). A stainless-steel needle was placed into the guide cannula and connected by a silicone catheter to a Hamilton microliter syringe. After inoculation, the injection cannula was maintained for an additional minute to avoid reflux. The following coordinates from bregma were used, in accordance with Paxinos and Watson's Atlas (2009), AP: 0.4 mm, L: –1.5 mm and DV: –4 mm. At the end of the surgery, the cannula was sealed with a stainless-steel wire to protect it from obstruction. To prevent infections, each animal received a subcutaneous injection of 0.2 mL of Penicillin G Benzathine (1,200,000 IU; 1 IU = 0.6 μ g; 72 mg/rat). After surgery, animals were housed singly in Plexiglas cages and maintained undisturbed for a week for recovery. At the end of the experiments, the location of the guide cannula into the lateral ventricle was confirmed by the injection of blue ink. Only animals with confirmed microinjection into the right lateral ventricle were included in the study.

On the morning of proestrous (09:00 h), the experimental group ($n=6$ rats) received a single i.c.v. injection of ALLO (6 μ M) and the vehicle group received an i.c.v. injection of KREBS solution. The total volume of ALLO or vehicle injected was 1 μ L for 60 s.

Ovarian morphology

The left ovaries from both experimental groups were removed and immediately fixed in Bouin solution (Biopur) for 12 h, dehydrated in ethanol series, cleared in xylene and embedded in paraffin. Histological sections were made for staining with hematoxylin and eosin (Merck). Ovaries were cut in serial sections at 5 μ m on a rotary microtome, mounted on slides at 50- μ m intervals to prevent counting the same structure twice and examined under a light microscope (Zeiss). From each ovary, the number of secondary (SF), tertiary (TF), Graafian (GF) and atretic (AtF) follicles as well as corpora lutea (CL) and cysts (C), including LUFs, were examined under a light microscope (Zeiss). The follicles were classified in accordance with Williams and Erickson (2012): SFs have multiple layers of granulosa cells around the oocyte and a theca layer; TFs contain a small cavity or 'antrum' filled with follicular fluid; in GF, the cavity occupies most of the total follicular volume and the cumulus appears; AtFs were those with more than 10 pycnotic nuclei per follicle, which also had a degenerate oocyte and precocious antrum formation or both (Banka & Erickson 1985, Sadrkhanloo et al. 1987). The CLs of each individual were counted and classified in new and old (previous cycle) according to Westwood (2008), as follows: New CL: easily found during estrous. They are generally small, but defined, with basophilic cell cytoplasm, central fluid-filled cavity and no fibrous tissue (Fig. 1C); Old CL: might be found throughout the whole cycle. They can present more cytoplasmic vacuoles indicative of active steroidogenesis and fibrous tissue proliferation in the central cavity (Fig. 1D).

Follicular cysts were defined as follicles with or without oocytes that contain a large antral cavity and a thin granulosa layer. LUFs were defined as structures with an oocyte surrounded by luteal and granulosa cells, with neo-vascularization (Wang et al. 2008, Fernandois et al. 2012). The number of these different ovarian structures was determined in six ovarian sections from each ovary ($n=6$ ovaries/group) and

expressed as mean $\pm$ S.E.M. The mean diameter of TF, GF and CL was recorded using ImageJ software.

Radioimmunoassay for progesterone and estradiol determination in serum and ovarian tissue

Trunk blood was collected and centrifuged at 402 g for 15 min (Beckman TJ-6RS). The serum obtained was kept frozen (-30°C) until hormone assays were run. RIA was performed using a commercially obtained kit (New England Nuclear Products, Boston, MA, USA) and used to measure progesterone concentrations in serum and ovaries. In both cases, Pg was extracted according to Sánchez-Criado and coworkers (1992). The sensitivity of the assay was 0.02 ng/mL, and inter- and intra-assay coefficients of variation were 5% and 6% respectively, for serum measures. 17β -Estradiol (E2) concentration in serum was determined by RIA using a commercial kit (Radim, Pomezia, Italy) based on competition between antigens labeled with iodine 125 (radioactive conjugate) and non-labeled antigens (calibrator sample) for specific binding sites in antiserum-coated tubes. After incubation, all unbound material was removed and radioactivity was measured. Uncoated tubes were prepared for measurements of total activity (T) and non-specific binding (NSB). Tubes coated with rabbit antibody against E2 were prepared for measurements in the zero calibrator (Bo), calibrators 1–6, control serum and samples as follows. First, 100 μ L of Bo was added to the NSB tube and 100 μ L of each additional calibrator as well as the control serum and samples was pipetted into the corresponding tube. Next, 500 μ L of the radioactive conjugate was pipetted into all the tubes, whose contents were then mixed by vortex. After incubation for 3 h at 37°C , the contents were carefully aspirated by pump from all tubes except the uncoated T tube. The radioactivity in the tubes was measured with a γ -counter. The sensitivity of the assay was 2 pg. The intra-assay coefficient of variation (CV) was 3%. In the case of the ovarian Pg measures, the concentration was expressed as ng/mg ovary/mL, and assay

Figure 1 Top panel: Light micrographs of whole ovaries from rats after treatment with vehicle (A) or ALLO (B). Inset: Luteinized unruptured follicle (LUF) containing an oocyte (O). A secondary follicle (SF), a tertiary follicle (TF), corpora lutea (CL), an atretic follicle (AtF) and a cyst (C) are also shown. Bottom panel: Representative micrographs of a new corpus luteum (C) with basophilic cells (BC) and a fluid-filled cavity (FFC); and an old corpus luteum (D) with central fibrous tissue formation (FT) and luteinized cells (LC). Scale bars in A and B represent 200 μ m, in C and D 100 μ m.

Table 1 Morphometric features of ovarian follicles in ovaries after treatment with ALLO or vehicle in female rats.

Structures	Control (n=6)	ALLO (n=6)	P value
Secondary follicles (n)	3.94±0.56	2.77±0.33	<0.05
Tertiary follicles (n)	7.5±0.96	6.2±0.95	ns
Graafian follicles (n)	5.1±0.45	3±0.66	<0.01
Atretic follicles (n)	2.75±1.1	4.77±0.9	<0.001
Corpora lutea (n)	7.65±2.20	6.35±1.1	ns
New CL (n)	3.55±1.16	1.60±0.85	<0.01
Old CL (n)	3.25±2	5.76±2.26	<0.001
Cysts and LUFs (n)	2±0.55	6±1.33	<0.001
Diameter of tertiary follicles (µm)	428.87±102.49	432.53±104.28	ns
Diameter of Graafian follicles (µm)	650.85±97.62	603.62±65.12	ns
Diameter of corpora lutea (µm)	758±65.01	836.3±82.3	ns

Values expressed as mean±S.E.M., ns, not significant. CL, corpora lutea; LUF, luteinized unruptured follicles.

sensitivity was less than 5 ng Pg/mL. The inter- and intra-assay CVs were less than 10.0%. For the sake of comparison, some previously published data regarding Pg serum levels are shown together with Pg ovarian levels.

Spectrophotometric analysis of enzymatic activity (ovary and MBH)

The right ovary from each animal was used both for Pg determination by RIA (see previous paragraph) and for enzymatic activity determination. First, the ovaries were homogenized in buffer Tris-HCl, and then an aliquot was taken for determination of enzyme activities. The remaining aliquot was used for RIA determination. The activities of 3β-HSD, 3α-HSOR and 20α-HSD were measured as described by Kawano and coworkers (1988) and Giuliani and coworkers (2013), with slight modifications (Tellería & Deis 1994). The method of Lowry and coworkers (1951) was used for protein determination using bovine serum albumin (BSA) as standard. The ovaries and MBH were homogenized in 0.7 mL of 0.1 M Tris-HCl and 1 mM EDTA buffer (pH 8) at 0°C with a glass homogenizer. The homogenates were centrifuged at 30,000 rpm for 60 min, using a Beckman L T40.2 ultracentrifuge. The supernatants were used for determining 20α-HSD activity. The precipitates were re-homogenized with 0.25 M sucrose and then centrifuged at 3000 rpm for 5 min. The supernatants obtained were used as the enzyme solution to determine 3β-HSD activity. Then, to start the assays, the substrate for the reaction of 3α-HSOR, pregnenolone, was added to the reaction mix. The latter contained glycine-NaOH (pH=9.4), BSA, NAD⁺ and a fraction of the enzyme solution. The enzymatic activities were assayed spectrophotometrically using a Zeltec spectrophotometer. The assay of each enzyme measured the reduction of NAD⁺ or the oxidation rate of NADPH at 340 nm respectively (Kawano et al. 1988, Takahashi et al. 1995, Escudero et al. 2012) as an increase in absorbance in 1 min at 37°C. A fraction of the enzymatic solution was reserved for protein quantification. The values of enzymatic activity were expressed as U/mg protein/min.

Data analysis

Data were expressed as the mean±S.E.M. Statistical analysis was performed using the unpaired Student's *t* test. Values of

P<0.05 were considered significant. Data were statistically analyzed using Prism v 5.0.

Results

Estrous cycle and ovulation

ALLO administration at proestrous caused a significant decrease in the ovulation rate. The percentage of inhibition was of 75%, whereas the administration of vehicle had no effect (data not shown). Interestingly, the estrous cycle was not modified in any of the experimental groups.

Ovarian morphology

The mean numbers of follicles and CL in the ALLO-treated and control groups are shown in Table 1. In the ALLO-treated group, the number of SF and GF was lower than that in the control group (*P*<0.05 and *P*<0.01 respectively). In contrast, the number of

Figure 2 Radioimmunoassay of progesterone (top panel) and estrogen (bottom panel); serum levels (ng/mL) (A and C) and ovarian tissue levels (ng/mg) (B and D). Allopregnanolone (ALLO). Bars represent the mean±S.E.M. (n=6; ****P*<0.001).

follicular cysts and LUFs was increased in the ALLO-treated group ($P < 0.001$). No significant differences were found in the number of TF and CL between both groups. ALLO increased the number of old CL and decreased the number of new CL, compared to the untreated group. There were no significant differences between the diameters of TF, GF and CL between the ALLO-treated and control animals (Table 1). Figure 1 (upper panel) shows representative photomicrographs of a whole control ovary (A) and a whole ALLO-treated ovary (B), which display a LUF with retained oocyte, a large antrum and intense vascularization. Figure 1 (lower panel) shows photomicrographs of new (Fig. 1C) and old corpora lutea (Fig. 1D).

Progesterone and estrogen levels

ALLO administration induced a significant increase in Pg serum levels with respect to the control group

($P < 0.001$, Fig. 2A). However, the opposite results were found in the ovaries, where Pg levels were lower than those of the control group ($P < 0.001$, Fig. 2B). The administration of ALLO did not alter serum or ovarian estradiol levels when compared with the control group (Fig. 2C and D).

3 β -HSD, 3 α -HSOR and 20 α -HSD enzymatic activity

The 3 β -HSD activity in MBH of ALLO-treated animals was significantly higher than that of the control group ($P < 0.05$, Fig. 3A). In the ovaries, the opposite results ($P < 0.05$, Fig. 3B) were found: 3 β -HSD was lower in the ALLO-treated groups than that in the control group. The same changes in the enzymatic activity of 3 α -HSOR were observed in both MBH and ovarian samples ($P < 0.05$, Fig. 3C and D). Finally, ALLO administration induced a decrease in the activity of 20 α -HSD in the MBH and an increase in the activity of 20 α -HSD in the ovary ($P < 0.05$, Fig. 3E and F).

Discussion

Ovulation is one of the main female reproductive events. It is a consequence of sequential steps that begin early in life with the formation of primordial follicles and then, during the fertile period with cyclic follicular development. This process is controlled by the hypothalamic–pituitary–ovarian axis, which is accompanied by an increased sympathetic tone. ALLO plays a determinant role in the regulation of the reproductive function in female rats. We have previously shown that ALLO modifies the ovulation pattern, acting at the level of the dopaminergic, GABAergic and glutamatergic systems (Laconi *et al.* 2001, 2012, Laconi & Cabrera 2002, Giuliani *et al.* 2013). Based on these previous findings, this study was designed to analyze the putative effect of a single dose of ALLO i.c.v. over morphometric parameters and ovarian and hypothalamic steroidogenesis.

We confirmed that the i.c.v. administration of ALLO inhibited ovulation, a mechanism controlled primarily by the pituitary LH. ALLO administration did not alter the estrous cycle. These results are in agreement with those of Genazzani and coworkers (1995), who had already found that after the administration of anti-ALLO serum, the anovulatory effect of the neurosteroid was reversed.

In the present study, we observed that ALLO affects the process of follicle maturation. In the ALLO-treated group, the number of SF and GF was significantly lower than that in the control group, whereas their diameter was not affected. On the other hand, ALLO increased the number of AtF. As shown in one of our previous works (Laconi *et al.* 2012), ALLO interferes with gonadotropin release. At a concentration in the order of μM , ALLO

Figure 3 Spectrophotometric analysis of ALLO effect over 3 β -HSD (A and B), 3 α -HSOR (C and D) and 20 α -HSD (E and F) enzymatic activities in the medial basal hypothalamus (MBH left panel) and in the ovary (right panel) of estrous rats. Bars represent the mean $\pm$ S.E.M. ($n = 6$; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

is able to decrease LH serum levels, whereas at a concentration in the order of nM, it is able to increase GnRH levels (Giuliani *et al.* 2011). These findings support the idea that ALLO alters gonadal steroidogenesis and thus disrupts follicular development. ALLO may also alter the balance between survival and death factors in follicular cells, leading to the atresia of developing follicles.

The ovulatory process, which involves the breakdown of the theca layers, which in turn allows the release of the oocyte, is dependent on Pg and the regulation of proteolytic activity. In addition, this process is dependent on the pre-ovulatory LH surge that induces the secretion of follicular Pg (Gaytán *et al.* 2002). Our findings suggest that ALLO inhibits ovulation by decreasing the ovarian levels of Pg, an effect that seems to be mediated by the inhibition of 3β -HSD activity and by an increase in 20α -HSD activity.

Previously, we found that ALLO affects luteal regression through the inhibition of apoptosis (Laconi *et al.* 2012). In this study, we observed that in animals injected with ALLO, the mean number of CL and their diameters had no differences with respect to the control group. Although the difference of the total number of CL remained statistically not significant, there was a difference in the number of new and old CL between the ALLO-treated group and the control group. The decrease in the number of new CL could be a consequence of the inhibition of ovulation or of the increase in the number of atretic and cystic follicles. The increase in the number of old CL could be associated with a decrease in the apoptotic process in the CL (Laconi *et al.* 2012).

The increase in 20α -HSD activity and the decrease in 3β -HSD activity observed in the present study, together with the decline in the number of new CL, would be the cause for the decrease in ovarian Pg levels. Luteal regression initiates with a decline in the biosynthesis of Pg (Clementi *et al.* 2004), which is then followed by the activation of the catabolism of Pg by 20α -HSD, an established marker for luteal regression.

Moreover, as is well known, follicle maturation is a process regulated by gonadotropins, hormones and local growth factors (Fortune *et al.* 2004). Follicular growth and oocyte maturation are dependent primarily on FSH and LH (Canipari *et al.* 2012). Mattheij and Swarts (1995) linked a deficiency in the secretion of LH in the period before ovulation with the formation of LUFs. Therefore, the central effect of ALLO over the reproductive function may be due to decreased LH levels, which affect folliculogenesis and thus inhibit ovulation. This process would be involved in the formation of ovarian cysts and, in particular, LUFs. This is in agreement with that found by Vanholder and coworkers (2006) who mentioned that low LH levels lead to the formation of cystic structures, which do not interfere with the normal ovarian cycle in cows. Women with LUFs have a normal menstrual cycle without ovulation (Summaria *et al.* 1998). The same

situation occurred in our experimental model, where the rats presented a normal four- to five-day cycle, with regular vaginal smears, with a significant reduction of the ovulation, even though follicular cysts and LUF were increased.

In our model, ALLO probably affects the selection-recruitment of dominant follicles to ovulate, preventing them to reach the GF state, thus leading to the formation of cystic structures. This idea reinforces our hypothesis about the importance of this neurosteroid in the reproductive function, in particular in the functionality of ovarian structures.

On the other hand, we found that ALLO increased serum Pg levels and decreased ovarian Pg levels, but did not affect serum or ovarian estrogen levels. All these results suggest that ALLO acts both at central (CNS) and peripheral levels (adrenal and ovarian levels) (Micevych & Sinchak 2011).

In this study, we measured the ovarian and MBH enzymatic activities of 3β -HSD, 3α -HSD and 20α -HSD, which mediate the synthesis and metabolism of Pg and ALLO respectively. We found that the activities of both 3β -HSD and 3α -HSD had the same profile. They were increased at MBH and decreased in ovarian tissue, suggesting a relationship between the central and ovarian effect of ALLO. However, 20α -HSD activity followed the opposite profile: in the MBH, it might regulate the availability of locally produced Pg from Pg receptors, and thus, control the influence of Pg over neuronal activity; in the ovary, it plays a relevant role in the induction of luteolysis (Pelletier *et al.* 2004, Stocco *et al.* 2007).

De Rensis and Scaramuzzi (2003) have shown the effect of heat stress over female fertility. The decrease in fertility is associated with an increased body temperature that alters ovarian function and oocyte health (Hansen 2007). Wolfenson and coworkers (1997, 2000) have reported that stress can alter follicular development, lead to the formation of suboptimal CL and low Pg concentration and reduce steroid hormone production. Similarly, we reported that a concentration of ALLO that mimics stress levels also has a deleterious effect on GF, leading to the formation of cystic and luteinized structures. ALLO, at stress level concentrations, may generate a cascade of effects from the hypothalamus and pituitary gland to the ovary, impairing the whole equation of female fertility. It alters luteal function and follicular development, reduces ovarian Pg concentration, decreases the enzymatic activities of 3β -HSD and 3α -HSD and increases 20α -HSD activity.

In conclusion, ALLO alters key enzymes of its own synthesis and generates a special microenvironment, causing alterations in steroidogenesis, perhaps responsible for the morphological changes in follicles and the development of cystic structures, reinforcing the idea that ALLO is a potent modulator of female reproductive function.

More studies are needed to ascertain if ALLO actions could affect the ovarian tissue directly. We are currently studying the effect of an ALLO intra-bursal injection and determining ALLO serum levels.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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