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A single dose of allopregnanolone affects the ovarian morphology and steroidogenesis

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A single dose of allopregnanolone affects the rat ovarian morphology and steroidogenesis

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 3β-hydroxysteroid dehydrogenase, 20α-hydroxysteroid dehydrogenase and 3α–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 while 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, while ovarian progesterone levels were decreased. Moreover, 3β-HSD and 3α-HSOR enzymatic activities were increased in the medial basal hypothalamus while ovarian levels were decreased. The enzyme 20α-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.

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" (Baulieu 1997, Robel & Baulieu 1994, Melcangi & Panzica 2006, 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, Corpéchot et al. 1993, Baulieu 1997, Melcangi & Panzica 2006). These neurosteroids include pregnenolone, progesterone (Pg) and allopregnanolone (ALLO). In particular, ALLO, also called 3α-hydroxy-5α-pregnan-20-one or 3α-5α-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 3β-hydroxysteroid dehydrogenase (3β-HSD) and the reduction of this steroid by 3α–hydroxysteroid oxido-reductase (3α-HSOR) (Patte-Mensah et al. 2010). Pg can also be metabolized by 20α-hydroxysteroid dehydrogenase (20α-HSD) (Clementi et al 2004). These enzymes are present in different regions, including the hypothalamus (Guennoun et al. 1995, Vidal et al. 2000) and the ovary (Vega Orozco et al. 2012). Micevych & 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 and 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 anti-gonadotropic properties (Concas et al. 1996; Purdy et al. 1991). 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 et al. (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 1995). Stress is one of the main factors that alter ALLO circulating levels (Purdy et al. 1991), which could alter the reproductive axis. Bäckström et al. (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, Laconi & Cabrera 2002, Laconi et al. 2007). 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 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 (William & 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 (Qublan et al., 2006; Summaria et al., 1998).

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 17β-estradiol serum and ovarian levels, and 3β-HSD, 3α-HSOR and 20α-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±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 μM, 1 μL injection volume, for 60 sec). 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 (09.00h), vaginal smears were analyzed. Then, the rats were sacrificed 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 labelled 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

154 to the National Institutes of Health Guide for the Care and Use of Laboratory Animals of
155 the National Research Council (National Academies, U.S.A., 8th Edition, 2011).
156
157 **Drugs**
158 Allopregnanolone (ALLO)[3α-hydroxy-5α-pregnan-20-one] (Sigma Chemical Co., St.
159 Louis, MO, USA), Penicillin G Benzathine (Richet, Argentina), Ketamine HCL (Holliday -
160 Scott S.A, Buenos Aires Argentina) and Xylazine (Koning Laboratories, Buenos Aires,
161 Argentina) were used for experimental and surgical procedures. Dihydroprogesterone [5α-
162 Pregnan-3, 20-dione] (Sigma Aldrich, Argentina), Pregnenolone [3β-hydroxy-5-pregnen-
163 20-one] (Sigma Aldrich, Argentina), NAD+ [β-Nicotinamide adenine dinucleotide hydrate]
164 and NADPH [β-Nicotinamide adenine dinucleotide phosphate] (all from Sigma Aldrich,
165 Argentina) were used for enzymatic activity determination. ALLO was prepared as
166 described in our previous papers (Laconi et al. 2001, 2012). Stocks of ALLO were initially
167 dissolved in propylene glycol to a concentration of 0.6 mM. The dose of ALLO used in the
168 experiment (6 μM) was obtained by dilutions in Krebs Ringer Bicarbonate glucose
169 (KRBG) buffer at pH 7.4, to make negligible the final amount of propylene glycol. Control
170 animals were injected with KRBG buffer at pH 7.4 as vehicle. KRBG preparation
171 contained propylene glycol in a concentration equivalent to that used in the experimental
172 groups.
173
174 **Determination of the estrous cycle and the ovulation rate**
175 The estrous cycle stage was determined daily (07:00-09:00 am) using vaginal smears
176 observed with a light microscope. The ovulation rate was determined on the morning of
177 estrous after the rats were sacrificed to confirm our previous results (Laconi et al., 2012).
178 After sacrifice, the ovary was placed on a petri dish, moistened slightly and the ampulla
179 gently punctured. Then, oocytes were removed and counted under a light microscope.

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 UI; 1UI = 0.6 µg; 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 µM), and the vehicle group received an i.c.v. injection of KREBS solution. The total volume of ALLO or vehicle injected was 1 µL for 60 seconds.

Ovarian morphology

The left ovaries from both experimental groups were removed and immediately fixed in Bouin solution (Biopur) for 12 hours, dehydrated in ethanol series, cleared in xylene and embedded in paraffin. Histological sections were made for staining with hematoxylin-eosin (Merk). Ovaries were cut in serial sections at 5 µm on a rotary microtome, mounted on slides at 50-µm intervals to prevent counting the same structure twice and examined under

206 a light microscope (Zeiss, Germany). From each ovary, the number of secondary (SF),
207 tertiary (TF), Graafian (GF) and atretic (AtF) follicles as well as corpora lutea (CL), and
208 cysts (C), including LUFs, were examined under a light microscope (Zeiss Germany). The
209 follicles were classified in accordance with Williams & Erickson, 2012: SF have multiple
210 layers of granulosa cells around the oocyte and a theca layer; TF contain a small cavity or
211 "antrum" field with follicular fluid; in GF, the cavity occupies most of the total follicular
212 volume and the cumulus appears; AtF were those with more than 10 pycnotic nuclei per
213 follicle, which also had a degenerate oocyte and precocious antrum formation, or both
214 (Banka & Erickson 1985, Sadrkhanloo et al. 1987). The CL of each individual were
215 counted and classified in new and old (previous cycle) according to Westwood (2008), as
216 follows: New CL: easily found during estrous. They are generally small, but defined, with
217 basophilic cell cytoplasm, central fluid-filled cavity and no fibrous tissue. (Fig 1, C); Old
218 CL: might be found throughout the whole cycle. They can present more cytoplasmic
219 vacuoles indicative of active steroidogenesis, and fibrous tissue proliferation in the central
220 cavity (Fig 1, D).
221 Follicular cysts were defined as follicles with or without oocytes that contain a large antral
222 cavity and a thin granulosa layer. LUFs were defined as structures with an oocyte
223 surrounded by luteal and granulosa cells, with neo-vascularization (Wang et al. 2008,
224 Fernandois et al. 2012). The number of these different ovarian structures was determined
225 in six ovarian sections from each ovary (n=6 ovaries/group) and expressed as Mean ±
226 S.E.M. The mean diameter of TF, GF and CL was recorded using Image J software.
227
228 **Radioimmunoassay for progesterone and estradiol determination in serum and**
229 **ovarian tissue**
230 Trunk blood was collected and centrifuged at 3000 rpm for 15 min (Beckman TJ-6RS).
231 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 Sanchez-Criado et al. (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 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 to 6, control serum, and samples as follows. First, 100 μL of Bo was added to the NSB tube, and 100 μL of each additional calibrator as well as the control serum and samples was pipetted into the corresponding tube. Next, 500 μL 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 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

258 homogenized in buffer TRIS-HCL, and then an aliquot was taken for determination of
259 enzyme activities. The remaining aliquot was used for RIA determination. The activities of
260 3β-HSD, 3α-HSOR and 20α-HSD were measured as described by Kawano et al. (1988)
261 and Giuliani et al. (2013), with slight modifications (Tellería & Deis, 1994). The method of
262 Lowry et al. (1951) was used for protein determination using bovine serum albumin (BSA)
263 as standard. The ovaries and MBH were homogenized in 0.7 mL of 0.1 M Tris-HCl, 1 mM
264 EDTA buffer (pH 8) at 0° C with a glass homogenizer. The homogenates were centrifuged
265 at 30000 rpm for 60 min, using a Beckman L T40.2 ultracentrifuge. The supernatants were
266 used for determine 20α-HSD activity. The precipitates were re-homogenized with 0.25 M
267 sucrose and then centrifuged at 3000 rpm for 5 min. The supernatants obtained were used
268 as the enzyme solution to determine 3β-HSD activity. Then, to start the assays, the
269 substrate for the reaction of 3α-HSOR, pregnenolone, was added to the reaction mix. The
270 latter contained Glycine-NaOH (pH=9.4), BSA, NAD+ and a fraction of the enzyme
271 solution. The enzymatic activities were assayed spectrophotometrically using a Zeltec
272 spectrophometer. The assay of each enzyme measured the reduction of NAD+ or the
273 oxidation rate of NADPH at 340 nm respectively (Kawano et al. 1988; Takahashi et al.
274 1995; Escudero et al. 2012) as an increase in absorbance in 1 min at 37°C. A fraction of the
275 enzymatic solution was reserved for protein quantification. The values of enzymatic
276 activity were expressed as mU/mg protein/min.
277
278 **Data analysis**
279 Data were expressed as the mean ± SEM. Statistical analysis was performed using the
280 unpaired Student`s t test. Values of $p<0.05$ were considered significant. Data were
281 statistically analyzed using Prism v 5.0.
282
283 **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 in the control group ($p<0.05$ and $p<0.01$, respectively). In contrast, the number of 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 1, C) and old corpora lutea (Fig 1, D).

Progesterone and estrogen levels

ALLO administration induced a significant increase in Pg serum levels with respect to the control group ($p<0.001$, Fig 2, A). 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 2, C and D)

310

311 **3β-HSD, 3α-HSOR and 20α-HSD enzymatic activity**

312 The 3β-HSD activity in MBH of ALLO-treated animals was significantly higher than that
313 of the control group ($p<0.05$, Fig. 3A). In the ovaries, the opposite results ($p<0.05$, Fig.
314 3B) were found: 3β-HSD was lower in the ALLO-treated groups than in the control group.
315 The same changes in the enzymatic activity of 3α-HSOR were observed in both MBH and
316 ovarian samples ($p<0.05$, Fig. 3C and D). Finally, ALLO administration induced a
317 decrease in the activity of 20α-HSD in the MBH and an increase in the activity of 20α-
318 HSD in the ovary ($p<0.05$, Fig. 3 E and F).

319

320 **Discussion**

321 Ovulation is one of the main female reproductive events. It is a consequence of sequential
322 steps that begin early in life with the formation of primordial follicles and then, during the
323 fertile period with cyclic follicular development. This process is controlled by the
324 hypothalamic-pituitary-ovarian axis, which is accompanied by an increased sympathetic
325 tone. ALLO plays a determinant role in the regulation of the reproductive function in
326 female rats. We have previously shown that ALLO modifies the ovulation pattern, acting
327 at the level of the dopaminergic, GABAergic and glutamatergic systems (Laconi et al.
328 2001, 2012, Laconi & Cabrera 2002, Giuliani et al. 2013). Based on these previous
329 findings, this study was designed to analyze the putative effect of a single dose of ALLO
330 i.c.v. over morphometric parameters and ovarian and hypothalamic steroidogenesis.
331 We confirmed that the i.c.v administration of ALLO inhibited ovulation, a mechanism
332 controlled primarily by the pituitary LH. ALLO administration did not alter the estrous
333 cycle. These results are in agreement with those of Genazzani et al. (1995), who had
334 already found that after the administration of anti-ALLO serum, the anovulatory effect of
335 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 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 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 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 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 & Swarts (1995) linked a deficiency in the secretion of LH in the period prior to 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 et al. (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-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

386 ALLO acts both at central (CNS) and peripheral levels (adrenal and ovarian levels)
387 (Micevych and Sinchak, 2011).

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

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

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

412 More studies are needed to ascertain if ALLO actions could affect the ovarian tissue
413 directly. We are currently studying the effect of an ALLO intra-bursal injection and
414 determining ALLO serum levels.
415
416 **Declaration of interest**
417 The authors declare that there is no conflict of interest that could be perceived as
418 prejudicing the impartiality of the research reported.
419
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430
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Figure legends

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 μm, in C and D 100 μm.

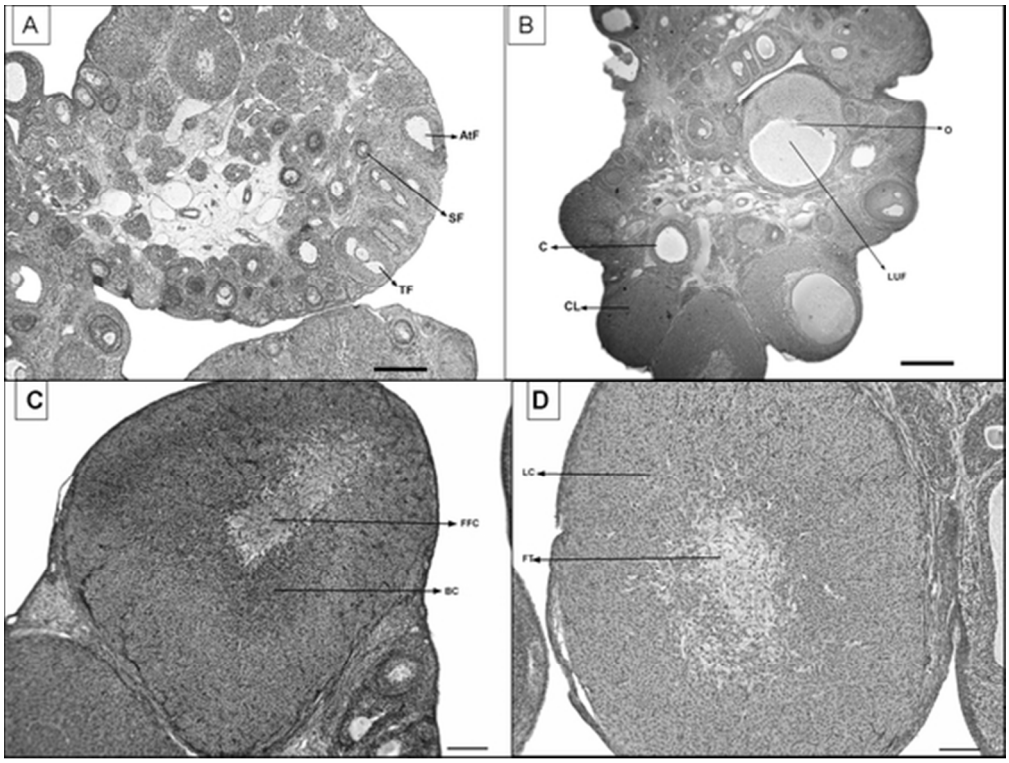
Figure 2: Radioimmunoassay of progesterone (top panel) and estrogen (bottom panel) serum levels (ng/mL) (A-C) and ovarian tissue levels (ng/mg) (B-D). Allopregnanolone (ALLO). Bars represent the mean ± S.E.M. (n= 6; ***p<0.001).

Figure 3: Spectrophotometric analysis of ALLO effect over 3β-HSD (A-B), 3α-HSOR (C-D) and 20 α-HSD (E-F) enzymatic activities in the Medial Basal Hypothalamus (MBH left panel) and in the ovary (right panel) of estrous rats. Bars represent the mean ± S.E.M. (n= 6; *p<0.05, **p<0.01 and ***p<0.001).

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	$p < 0.05$
Tertiary follicles (n)	7.5 ± 0.96	6.2 ± 0.95	ns
Graafian follicles (n)	5.1 ± 0.45	3 ± 0.66	$p < 0.01$
Atretic follicles (n)	2.75 ± 1.1	4.77 ± 0.9	$p < 0.001$
Corpora lutea (n)	7.65 ± 2.20	6.35 ± 1.1	ns
New CL (n)	3.55 ± 1.16	1.60 ± 0.85	$p < 0.01$
Old CL (n)	3.25 ± 2	5.76 ± 2.26	$p < 0.001$
Cysts and LUFs (n)	2 ± 0.55	6 ± 1.33	$p< 0.001$
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; LUF: Luteinized unruptured follicles, CL: corpora lutea.



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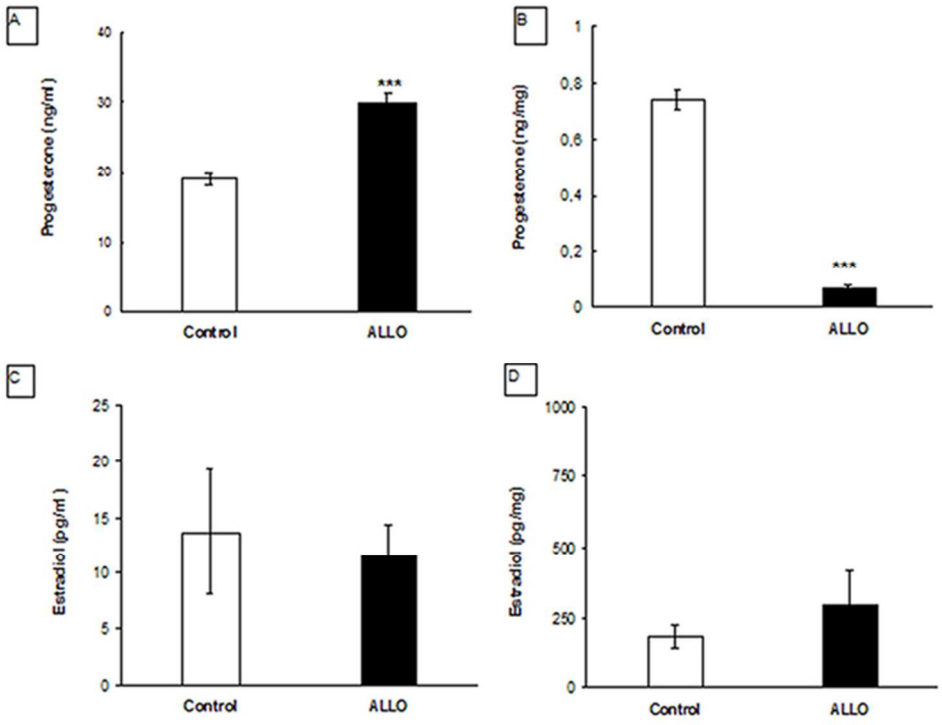

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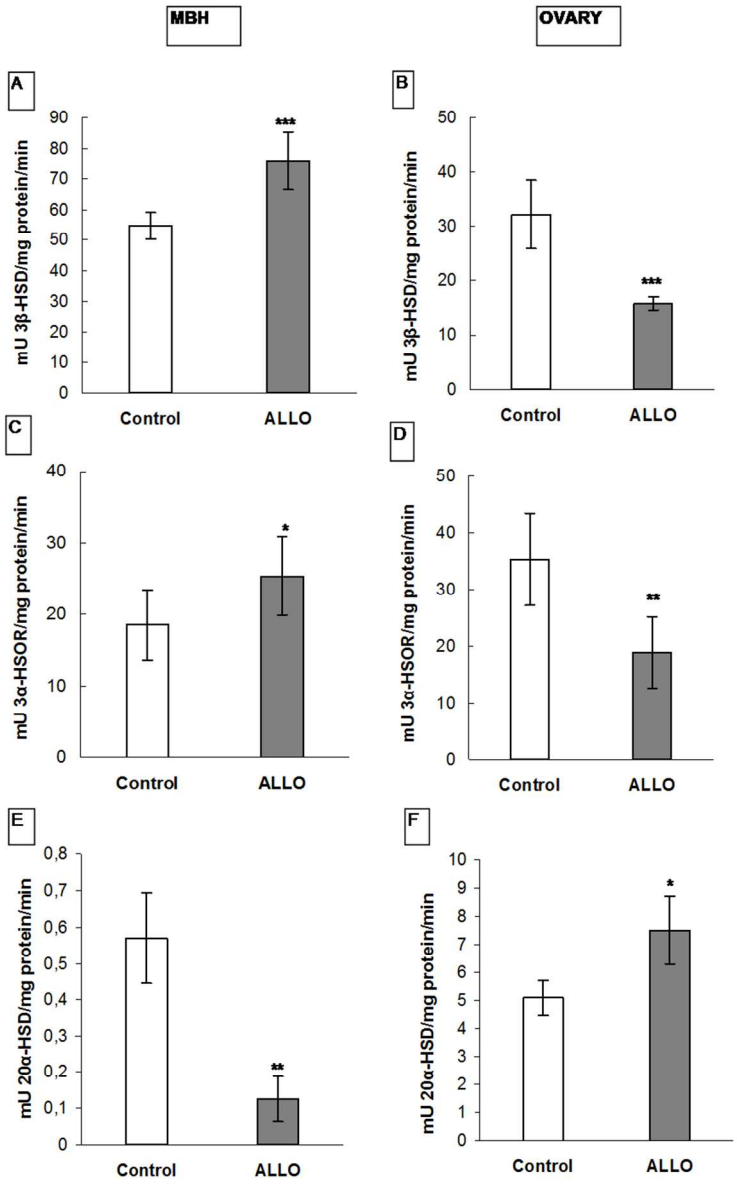

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