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Effects of sympathectomy on ovarian follicular development and steroid secretion

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Abstract

Recently, the influence of adrenergic activity over ovarian function, and thus fertility, has begun to gain importance. Previous studies have shown that adrenergic activity through norepinephrine (NE) participates in the control of follicular development and steroidal secretion from the ovary, among other functions. To examine this phenomenon, the denervation of the gonad has been widely used to observe changes in the ovary's performance. Nevertheless, the effect of the absence of adrenergic nerves in the ovary has only been studied in short times periods. In the present work, we used guanethidine (a drug that produces an irreversible sympathectomy) during the infantile period of rats, and we observed its effects in the adult rat (6 months old). Our results indicate that ovarian NE content is recovered at 6 months old, alongside with an increase of the adrenal content of NE and a dysfunctional celiac ganglion. Together, these results suggest that the recovery of ovarian NE does not come from a neural origin. In addition, ovarian performance was impaired because the changes in follicular development and steroidal secretion are not recovered despite the recovery of ovarian NE content. In conclusion, these results suggest that the nerve–ovarian connections, which are established during infantile development, are necessary for the accurate response of the ovary to sympathetic stimulation.

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Introduction

Different studies have described that in mammalians, including humans, the ovary is not only controlled by the hormonal axis (hypothalamus, pituitary and ovary) but is also regulated by the nervous system (Burden *et al*. 1985, Gerendai *et al*. 2002, Lara *et al*. 2002, Greiner *et al*. 2005, 2008, Kaaja & Poyhonen-Alho 2006, Cruz *et al*. 2017). Several studies have implicated the sympathetic nervous system (SNS) in ovarian function, which has an important role in folliculogenesis and steroidogenesis (Aguado & Ojeda 1984*b*, Lara *et al*. 1990*a*, Rosa *et al*. 2003, Doganay *et al*. 2010, Zhang *et al*. 2010, Cruz *et al*. 2017). The SNS plays a key role in follicular development, contributing to the growth of the recruited follicles, as well as the synthesis and secretion of progesterone, androgens and oestradiol (Lara *et al*. 2002, Romero *et al*. 2002, Leung 2004). Rodent studies have shown that follicular cells (theca and granulosa cells) have β-adrenergic receptors that respond to catecholamines and β-adrenergic agonists, leading to a physiological follicular development, ovulation and the synthesis and secretion of steroid hormones (Aguado & Ojeda 1984*a*, Barria *et al*. 1993). The cellular neural bodies that project from the celiac ganglion through the superior ovarian nerve (SON) directly innervate the ovary, making this the principal sympathetic innervation that reaches and controls follicular development in the gonad (Lawrence & Burden 1980, Gerendai *et al*. 2002). When SON denervation occurs, a delay in follicular development and a reduction in steroid hormone secretion is observed (Lara *et al*. 1993, Rosa *et al*. 2003, Zhang *et al*. 2010), demonstrating that the technique is useful in understanding the mechanisms by which the SNS influences the ovary. Because of these studies, a surgical cut of the SON has been widely used in avoiding or reducing the stimulation of the ovary by the SNS. However, despite this being a very effective method, the ovary is a plastic organ that, through different molecules such as NGF, rapidly facilitates the growth of new nerve terminals until it fully recovers the SNS innervation and its action (Lara *et al*. 1990*b*, 1991). The complete re-innervation of the ovary is reached 28 days after surgery (Lara *et al*. 1991). This implies that the surgical denervation technique can only be used for short or acute

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time periods. Thus, a method that allows a sympathetic denervation for prolonged times is guanethidine (GD) administration. GD is a drug that causes an irreversible chemical denervation through an immunoreaction that physically destroys the nerve terminal. This avoids nerve stimulation and re-growth over the tissues (Evans *et al*. 1979, Manning *et al*. 1983, Lara *et al*. 1990*a*, Villanueva *et al*. 2002). When prepubertal denervation with GD is produced in rodents (rats and guinea pigs), several effects occur. For example, females display a delay in puberty and alterations in normal follicular populations such as an increase in small antral follicles, a decrease in large antral follicle (Lara *et al*. 1990*a*, Albuquerque-Araujo *et al*. 1995, Riboni 2002*a*,*b*, Trujillo & Riboni 2002, Chavez-Genaro *et al*. 2007), a decline in the steroid secretory response and oestrous cycle alteration (Lara *et al*. 1990*a*,*b*, Albuquerque-Araujo *et al*. 1995, Riboni 2002*b*). However, neonatal GD administration and its effect on ovarian performance have not been examined in later reproductive periods. Thus, the aim of the present work is to study the effect of permanent denervation induced during childhood on ovarian functions during later adulthood. For this, we used a rat model in which the animals were subjected to GD denervation on the seventh day of life, and we observed ovarian performance when the animals are 6 months old.

Methods

Animals and guanethidine treatment

A total of 43 Sprague–Dawley female rats were used in the experiments, 22 for control group (Veh) and 21 for guanethidine group (GD). The animals were divided into: 4 months old ($n=8$; 4 Veh and 4 GD), 5 months old ($n=9$; 4 Veh and 5 GD) and 6 months old ($n=17$; 8 Veh and 9 GD). Half of the 6-month-old rats were used for ovarian morphological analysis and catecholamine measurement while the other half was used to ovarian steroid secretion, ovarian and celiac ganglia RNA extraction and adrenal catecholamine measurements. Finally, 8-month-old rats ($n=9$; 4 Veh and 5GD) were used to performance the beta-adrenergic receptor-binding assays, western blot to tyrosine hydroxylase (TH) and ovarian or adrenal catecholamines measurements. At postnatal day 7, all GD rats were treated with guanethidine monosulfate (Sigma Aldrich). The protocol consisted of an intraperitoneal administration (i.p.) of GD, 50 mg/kg/day, for 5 consecutive days, followed by 2 days without treatment. This was repeated for 3 weeks (Lara *et al*. 1990*a*, Albuquerque-Araujo *et al*. 1995). All control rats receive a saline (vehicle) solution. After treatment, GD and Veh rats were allowed to grow until 4, 5, 6 and 8 months old. The rats were maintained under conditions of free water and food access and 50% light–darkness cycles (12 h of light and 12 h of darkness each day) during all the experiment. Animals were killed by decapitation. The ovaries, adrenal glands, celiac ganglion and trunk blood were collected. The bioethics protocol for laboratory animals was approved by the Bioethics Committee of the Faculty of Chemical and Pharmaceutical Sciences at the University of Chile and the Institutional Animal Experimentation Bioethics Board and the Science Council (FONDECYT) of Chile.

Study of the oestrous cycle

The oestrous cycle of the rat was determined by microscopic observation of the cell type present in the vaginal smear as previously described (Marcondes *et al*. 2002). A full successful cycle was considered when a proestrus was followed by an oestrous and then a diestrous (we considered diestrous to be both metaestrous and diestrous II). The cycles were recorded during the last month of life of the animal (31 days).

In vitro ovarian steroid secretion assay

An *in vitro* steroid secretion from the rat ovaries was performed as previously described (Paredes *et al*. 2011). Briefly, the ovary was halved and preincubated for 20 min in 2 mL of Krebs bicarbonate albumin solution pH 7.4 (118.6 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 25 mM glucose, 0.6 mM ascorbic acid, 0.15 mM EDTA and albumin 0.1 g/L). Then, half of each ovary was incubated for 3 h with Krebs bicarbonate albumin solution pH 7.4 with 0.01 mM isoproterenol hydrochloride (Sigma Aldrich) or 2.5 IU/mL human chorionic gonadotropin (Sigma Aldrich), which was previously described by Barria *et al*. (1993). The other half was incubated in Krebs solution to establish the basal secretion in each case. After the procedure, the incubation medium was stored at −20°C until the determination of steroidal hormones.

Steroid determination

The amount of progesterone and oestradiol were determined in the incubation medium by an enzyme immunoassay according to the manufacturer's instructions. Oestradiol has a catalog number 11 ESTHU-E01 and sensitivity 10 pg/mL and from ALPCO Diagnostic, Windham, NH, USA and progesterone has a catalog number 11-PROHU-E01 and sensitivity of 100 pg/mL and from ALPCO Diagnostic).

Morphological analysis of the ovary

Immediately after killing, the right ovaries of the rats were set in Bouin's fixative solution and embedded in paraffin. Sections 6 µm thick were made and stained with hematoxylin–eosin. All slices were analyzed and preantral, antral follicles, corpora lutea and cystic structures (sum of follicular cyst, precyst and type II follicles) were counted and measured. The classification criteria of the follicular structures were performed according to previous studies (Lara *et al*. 2000, Cruz *et al*. 2012, Fernandois *et al*. 2012). This classification is based on the presence, characteristics and layout of granulosa and theca cells.

Quantification of tyrosine hydroxylase, beta1 and beta2-adrenergic receptor mRNA levels

Total RNA extraction was performed using the Chomczynski–Sacchi method (Chomczynski & Sacchi 1987). After extraction,

total RNA was quantified by spectrophotometrical analysis (260/280 nm). The RNA was stored at −80°C until further use. Total RNA was subjected to reverse transcription to obtain cDNA. The reverse transcriptase reaction was performed with 5 µg of total RNA, 1.6 mM dNTPs, 10 mM DTT, 176 nM of random hexamers, 25 U RNaseOUT (Invitrogen) and 125 U Superscript II reverse transcriptase (Invitrogen). Gene amplification for the mRNAs of the enzyme TH and 18S in the celiac ganglion was performed using real-time PCR (IQ5, Bio-Rad) with specific primers for each gene. The 18S and TH programs consisted in 40 cycles with an alignment temperature of 60°C. The SYBR Green Master mix (Promega) and 0.125 µM of each primer, in a final volume of 20 µL was used for the qPCR mix. Specificity of the product was tested with a melting curve. Each of the amplified PCR products had a single melting point. The amount of mRNA for each gene was interpolated from a standard curve obtained by serial dilutions of a mixed sample. The primer sequences used for 18S were sense: 5′-GGACATCTAAGGGCATCACA-3′ and antisense: 5′ TCAAGAACGAAAGTCGGAGG-3′ (GenBank number: X01117). For TH, sense: 5′-GGTCTACTGTCCGCCCGTGATT-3′ and antisense: 3′-GAGCTTGTCCTTGGCGTCATTG-5′ (GenBank number: NM_012740). For beta1-adrenergic receptor were sense: 5′-AGACGCTCACCAACCTCTTCATCA-3′ and antisense: 5′-ACATAGCACGTCTACCGAAGTCCA-3′ (GenBank number: NM_012701.1). For beta2-adrenergic receptor: sense 5′-CAG GC TATGCTATC GCTTCCTCTAT-3′ and antisense: 5′-GGCTGAGGTTTTGGGCATGAAATC-3′ (GenBank number: NM_012492.2).

Quantification of ovarian norepinephrine

The left ovary was triturated in a glass/glass homogenizer with four volumes of Dulbecco's phosphate-buffered saline (DPBS). Three-quarters of the full homogenate volumes were mixed with four volumes of 0.2 M PCA. NE quantification was performed using high-performance liquid chromatography (HPLC) coupled to an electrochemical detector EICOM ECD-700S. Previously, the samples were filtered in disposable PVDF filters (Millex, Merck Millipore) with a 0.22 µm pore. The calibration curve was constructed with a standard of NE Bitartrate salt (Sigma Aldrich, Catalog A0937). Twenty microliters of the filtrate was injected into the Jasco PU-2089s HPLC system (Jasco, Newtown, PA, USA) coupled to the Jasco LC-NetII/ADC plus digitizer card. To generate the integration of the chromatograms, the computer program ChromPass Chromatography Data System JASCO v1.7.403.1 was used. The mobile phase consisted in 0.1 M NaH_2PO_4 buffer, 75 mg/L octylsulfate, 0.02% EDTA and 1.5% acetonitrile, pH 2.6. The flow rate used in the NE determination was 1 mL/min. The amperometric potential of the detector was set at 650 mV, and the NE retention time under these conditions was 3.2 min.

Quantification of adrenal NE

Both adrenal glands were homogenized in 500 µL of 0.2 N PCA with a glass–glass homogenizer. After centrifugation, the supernatants were diluted 1/1000 for NE quantification with HPLC under the same conditions described earlier.

Statistics

The data were processed using the program GraphPad Prism, v6.0. An unpaired *t*-test was used to detect significances between the control and GD groups. In the *in vitro* secretion assay, significance was obtained using a one-way ANOVA followed by Fisher LSD test, as a multiple comparisons test. A P value <0.05 was considered to be significant. All graphics were made by plotting the mean ± S.E.M.

Results

Systemic effects of early administration of guanethidine in adult rats

Previous studies have shown that sympathectomy by GD delays the onset of puberty (Lara *et al*. 1990*a*, Albuquerque-Araujo *et al*. 1995). Thus, to monitor the effectiveness of the drug, the day of the vaginal opening was determined. As shown in Table 1, GD-treated animals present delays in the onset of puberty. In addition, we observed the oestrous cycle during the last month of life of the animals (the last 31 days of life). Table 1 also shows that between 5 and 6 months of age, GD decreases the number of oestrous cycles by increasing the permanence in the estrus stage. Finally, GD treatment did not alter the weight of the animals nor the ovaries at 6 months old.

Changes of ovarian and adrenal NE content

As shown in Fig. 1, GD administration decreased the concentration of intraovarian NE at 4 and 5 months old, but at 6 months old, no difference in NE concentration was observed (Fig. 1A). Interestingly, the same plot shows a gradual increase in the ovarian NE from 4 months until 6 months old in the GD-treated rats. Because ovarian NE content is recovered at 6 months old, we examined the expression of TH, which is a key enzyme in NE synthesis, in the celiac ganglia, which is where the soma of the SON are located. As is shown in Fig. 1C, the mRNA of *Th* decreases significantly in the celiac ganglia. Regarding the beta-adrenergic receptors, we measure by qRT-PCR the expression of the beta1 and b2-adrenergic receptors subtype. As shown in Fig. 1B, both types of receptors are highly increased in the GD group. In addition, the

Table 1 General parameters of animals at 6 months old.

	Veh	GD	*P* value
Vaginal opening (day)	32.6 ± 0.5099	34.43 ± 0.4286	0.0328*
Number of cycles	3.667 ± 0.6667	1.750 ± 0.2500	0.0144*
% of estrus stage	29.025 ± 1.327	43.550 ± 7.263	0.0484*
Body weight (g)	294.5 ± 6.910	318.5 ± 9.772	0.1383 (ns)
Ovary weight (mg)	33.9 ± 1.9	40.1 ± 3.6	0.2520 (ns)

Data is presented as mean ± S.E.M. Significance was obtained by a Student *t*-test. $P < 0.05$ = *for vehicles vs GD (guanethidine) in each condition. Percentage of estrus stage was calculated considering 31 days as 100%.

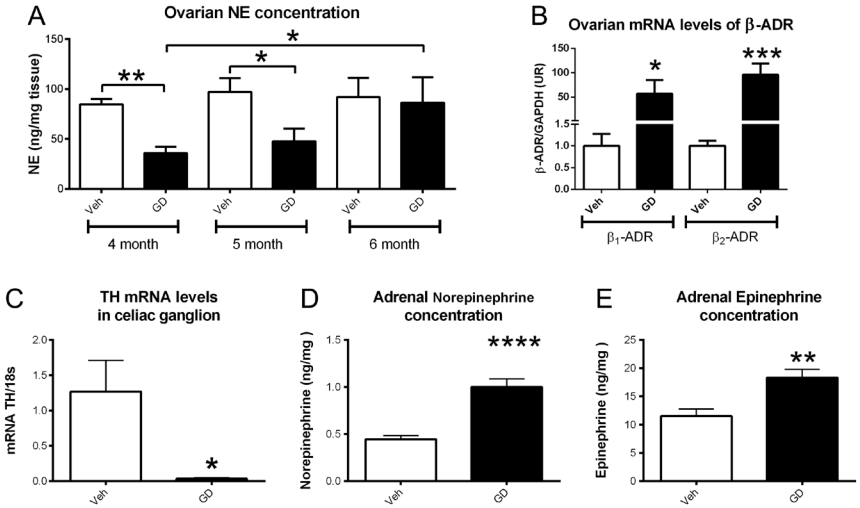

Figure 1 Effects of GD treatment on NE content through reproductive life. (A) Ovarian norepinephrine content in different ages. (B) Beta1 and beta2-adrenergic receptor mRNA in ovary. (C) Tyrosine hydroxylase mRNA in celiac ganglia by qPCR. *Th* qPCR data were normalized by 18S levels and adrenergic receptors by GAPDH. (D) Adrenal gland norepinephrine content and (E) Adrenal gland epinephrine content. For figures A, B and C $N=5$ for vehicle group and $N=4$ for GD group. For D and E, $N=8$ for vehicle group and $N=8$ for GD group. The results are expressed as the mean ± S.E.M. Asterisks represent differences compared to the control with ovaries. $*P<0.05$, $**P<0.01$. The significance was obtained using Student's *t*-test between the vehicle and GD. Veh, vehicle group; GD, guanethidine-treated group.

concentration of NE in the adrenal gland increases in the rats treated with GD. The increase in the adrenal content of NE was 0.445 ± 0.039–1.043 ± 0.084 ng/mg in GD-treated rats; representing a 234% increase in NE (Fig. 1D). For epinephrine content, the increase was 11.54 ± 1.265–18.35 ± 1.473 in GD-treated rats (Fig. 1E). Later, to observe if these effects are maintained for a longer period, we observe the same parameters in ovary, adrenal medulla and celiac ganglia at 8 months old. The results show that at this age, ovarian NE content is similar between GD and Veh groups (Supplementary Fig. 1A, see section on supplementary data given at the end of this article) but NE and epinephrine are increased in the adrenal medulla (Supplementary Fig. 1E and F). More importantly, ovarian beta-adrenergic expression (Supplementary Fig. 1B and C) and ovarian TH protein levels (Supplementary Fig. 1D and H) are increased in GD-treated rats. Finally, *Th* mRNA levels in the celiac ganglia remain decreased in 8-month-old rats (Supplementary Fig. 1G).

Changes in follicular level by administration of guanethidine

Given that the NE content is recovered in 6-month-old rat ovaries, we wanted to assess if the ovary recovers the follicular impairments produced by the drop of NE, as previously reported in the GD model (Lara *et al*. 1990*a*, Albuquerque-Araujo *et al*. 1995). Our results show that GD administration did not cause changes in the number of primordial follicles (Fig. 2A). However, GD caused changes in primary, secondary and healthy antral follicles. GD administration increased the number of primary follicles (Fig. 2B) and the number of total healthy antral follicles (Fig. 2E). In this case, when the size distribution of antral follicles was graphed, a clear Gaussian distribution is observed, and interestingly, we observed a displacement of the curve to the left. This suggests that healthy antral follicles are much smaller in the ovaries treated with GD. No changes in the total number of atretic follicles or their size were observed (Fig. 2G and H).

Because these results could suggest an accumulation of a slowed growth of the follicles, we counted the secondary follicles and measured the serum levels of anti-Müllerian hormone (AMH). As shown in Fig. 2K, the levels of AMH decreased with GD treatment despite the lack of changes in the number of secondary follicles (Fig. 2C). However, when the size distribution of secondary follicles was analyzed, a decrease in the size of secondary follicles was found (Fig. 2D), an effect that is highly similar to that observed with the healthy antral follicles (Fig. 2E and F). On the other hand, rats treated with GD show a decrease in the number of cystic structures (sum of type III follicles, cysts and precyst, Fig. 2I). All of these modifications occurred without affecting the number of corpora lutea in the ovaries (Fig. 2J).

Effects of infant administration of guanethidine on steroid hormone secretion during adulthood

Given that follicular alterations were observed in the ovaries despite the recovered NE content at 6 months old, we tested their response in secreting steroidal hormones. To observe this, we utilized *in vitro* secretion assays using isoproterenol (ISO), which is a beta-adrenergic agonist, to evaluate the response of the beta-adrenergic receptors and human chorionic gonadotropin (hCG), an analogue of luteinizing hormone, to test the response to hormonal stimulation. As shown in Fig. 3A, the control rats subjected to the hCG and ISO stimulation responded by releasing a large amount of progesterone (361.2 ± 152.9 ng/mL/h for hCG and 639.8 ± 473.2 for ISO). In contrast, when we compared the basal-unstimulated release, we observed

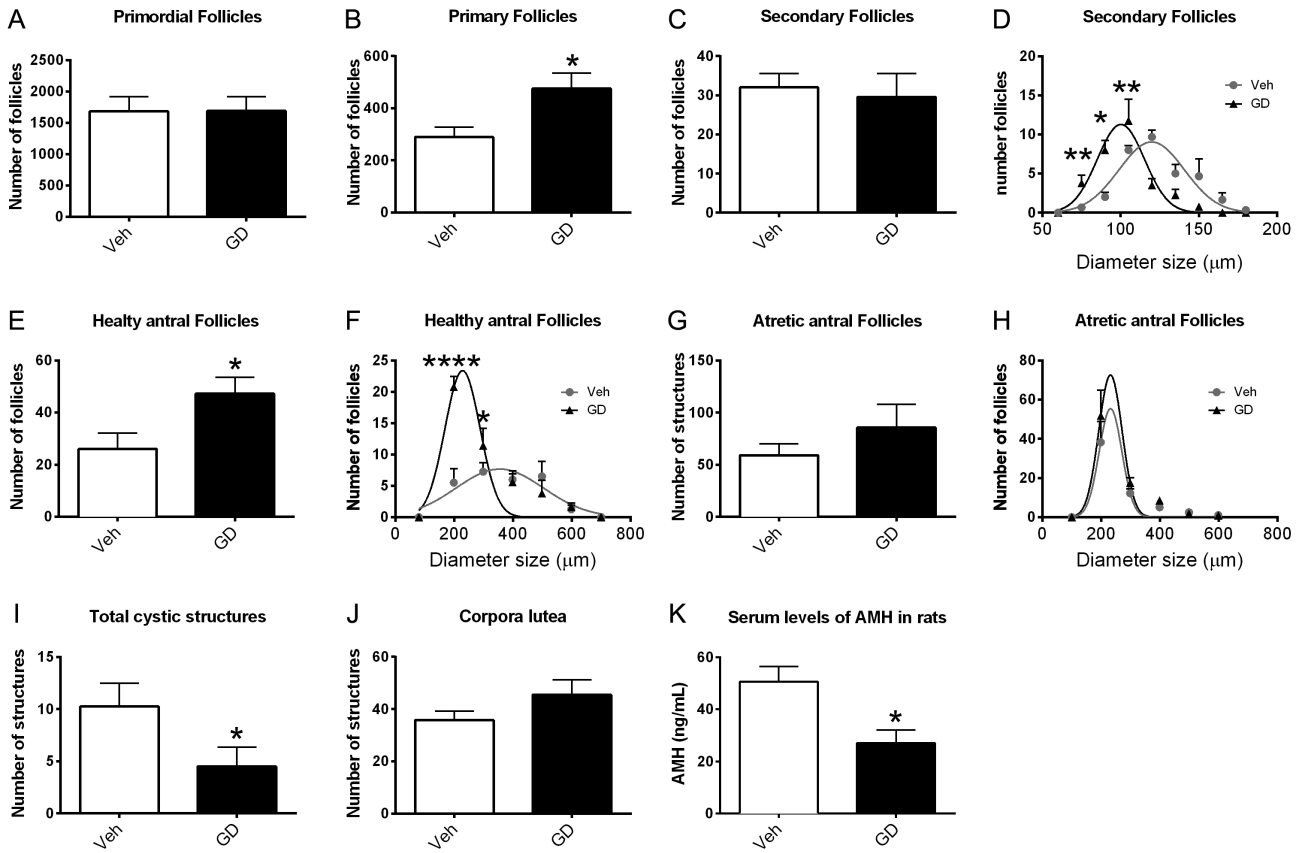

Figure 2 The effects of GD treatment on ovarian follicular development at 6 months old. The quantification of follicles was made by microscopic observation. The graphs show the total number of follicles per ovary. (A) Total number of primordial follicles per ovary. (B) Total number of primary follicles per ovary. (C) Total number of secondary follicles per ovary. (D) Size distribution of secondary follicles plotted as a Gaussian representation of the population of the follicles. Displacement of the curve indicates changes in the follicular growth distribution. (E) Total number of healthy antral follicles. (F) Size distribution of healthy antral follicles plotted as a Gaussian representation of the population of follicles. Displacement and shape of the curve indicate changes in the follicular growth distribution. (G) Total number of atretic antral follicles. (H) Size distribution of atretic antral follicles plotted as a Gaussian representation of the population of follicles. (I) Total number of cystic structures, the number represents the sum of type III follicles, precyst and cyst per ovary. (J) Total number of corpora lutea per ovary and (K) seric levels of anti-Müllerian hormone measured by EIA. The results are expressed as the mean ± S.E.M. of $n = 5$ ovaries. Significance was obtained using Student's *t*-test between the vehicle and GD-treated groups. *$P < 0.05$, **$P < 0.01$ and ****$P < 0.0001$. Asterisks represent differences compared with vehicle ovaries. Veh, vehicle group; GD, guanethidine-treated group.

a decrease in progesterone release between the control and GD-treated rats (46.4 ± 18.3 ng/mL/h in control vs 4.3 ± 1.1 ng/mL/h GD-treated rats). Figure 3A also shows that under hCG stimulation, GD-treated ovaries respond by secreting progesterone (312.2 ± 145.4 ng/mL/h) to the same extent as the control. In contrast, GD-treated ovaries stimulated with ISO released 20.3 ± 5.5 ng/mL/h, which is less than that observed in control ovaries. Because of this secretion pattern, we observed the ratio of change over the basal secretion. Figure 3B shows that progesterone released in GD-treated ovaries is proportionally increased under hCG stimulation but not under ISO stimulation.

For oestradiol, Fig. 3C shows that the basal ovarian secretion was also lower in GD-treated rats (57.4 ± 4.1 pg/mL/h) compared with control rats (158.6 ± 29.3 pg/mL/h). Control animals were able to respond more than 6 times higher than the basal and equally for the hormonal (1069.0 ± 269.1 pg/mL/h) or ISO stimulation (995.2 ± 575.1 pg/mL/h) as seen with the progesterone secretion (Fig. 3A). However, when GD-treated ovaries were assessed, we found that they respond to hCG (258.9 ± 51.9 pg/mL/h) by increasing oestradiol secretion approximately 4.5 times more than the control (Fig. 3D). Instead, the ISO stimulation only produced an increase of 109.6 ± 30.4 pg/mL/h, which is less than half of the levels reached by the hCG stimulation (Fig. 3D).

Discussion

Adrenergic system function

This research was conducted to evaluate if ovarian sympathetic innervation during early life is necessary to

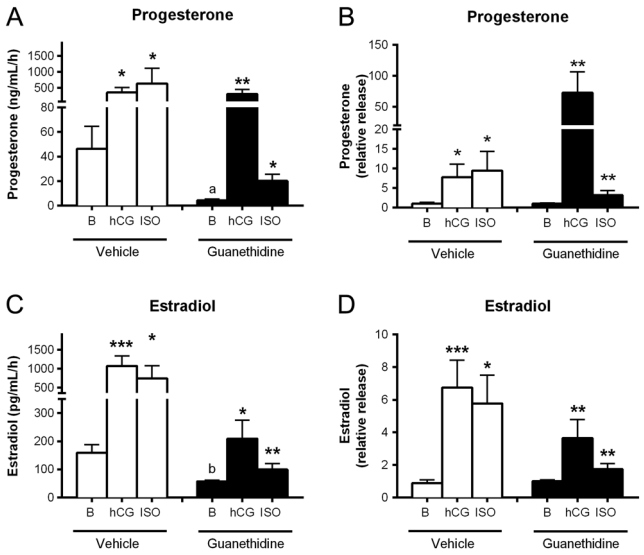

Figure 3 The effects of GD treatment on steroidal secretion at 6 months of age. (A) Progesterone release in the incubation medium. (B) The ratio of the increase in progesterone release vs the basal levels. (C) Oestradiol release in the incubation medium. (D) The ratio of the increase in oestradiol release vs the basal levels. The ratios of increase were calculated by dividing the stimulated progesterone or oestradiol release vs the corresponding basal levels of the group. The results are expressed as the mean ± S.E.M. B, basal; hCG, human chorionic gonadotropin; ISO, isoproterenol. $^{*}P<0.05$ and $^{**}P<0.01$. Asterisks represent differences against basal secretion in each group. $^{a}P<0.05$ and $^{b}P<0.01$. Letters represent differences between basal of vehicle group and basal guanethidine-treated rats. $N=4$ rats for each group. Significance was obtained using a one-way ANOVA followed by a multiple comparisons test.

establish the correct development of follicles and steroid secretion in the adult rat. We administered GD (50 mg/kg i.p.) from postnatal day 7 according to a previously reported protocol by Lara *et al*. (1990*a*). This previous study observed a degeneration of the SON evidenced by a nearly absence of ovarian TH and neuropeptide Y (NPY) immunoreactivity at 30 days old. The absence of the neurons projecting from the celiac ganglion to the ovary leads to a significant decrease in the ovarian levels of NE (Lara *et al*. 1990*a*, Albuquerque-Araujo *et al*. 1995). In the present study, we found a recovery of ovarian NE content at 6 months old, as a result of a progressive increase on the NE content since denervation (Fig. 1A). Our results suggest that re-innervation process does not produce this recovery. As previous works demonstrated, the noradrenergic innervation begins to be recovered only 12 months after GD administration and few cellular bodies are detected in the ganglia (Evans *et al*. 1979), which are not enough to increase ovarian norepinephrine content. Consistent with this idea, we observed that *Th* mRNA levels in celiac ganglia (where somata of the ovarian superior nerve reside) remained significantly decreased in rats treated with GD. Even more, ovarian *Th* levels 8 months after GD treatment are insufficient to explain the NE recovery. This apparent inconsistency of normal ovarian NE levels, combined with a probable absence of nerves, led us to think that a paracrine or even endocrine source of NE could be the cause of the NE content recovery. In this sense, GD does not affect the chromaffin cells of the adrenal medulla (Villanueva *et al*. 2002). Even more, as we found here, and previous publications have described, GD denervation produces an adrenal hypertrophy with an increased secretion of epinephrine and predominantly of NE (Ostman-Smith 1976, Qiu *et al*. 1999, Riboni 2002*a*). GD-mediated denervation impede the sympathetic control on arterial blood pressure and heart rate (Blythe *et al*. 1976, Glaubiger *et al*. 1978, Bennett & Gardiner 1986), producing an increase in plasmatic angiotensin (De Moura *et al*. 1970) and angiotensin receptors (AT1 and AT2) in the adrenal gland (Qiu *et al*. 1999, Pawlikowski *et al*. 2001, 2008). Since AT2 evokes catecholamine release (Belloni *et al*. 1998, Harada *et al*. 2010) and both ATs induce cell proliferation (McEwan *et al*. 1996*a*,*b*, Pawlikowski *et al*. 2001), the angiotensin increase is producing the hypertrophy of the adrenal gland.

On the other hand, granulosa cells of the ovary express functional NE and dopamine transporters, which are able to incorporate NE from the outside media (Greiner *et al*. 2008). Therefore, a non-neuronal NE accumulation in the granulosa cells conceivably contributes to the amount of this neurotransmitter that we measured in the ovary. Despite the function of this NE stored in granulosa cells from the outer media is unknown, it seems to have a different function than the neuron, which is related to follicular development and steroidal secretion.

Ovarian morphological changes

The sympathetic system controls ovarian function and is a key factor in ovarian pathophysiology (Aguado & Ojeda 1984*b*, Lara *et al*. 1993, 2000, 2002, Luna *et al*. 2012). The sympathetic denervation with GD delays the follicular development, leading to an increase in small-sized healthy antral follicles (Lara *et al*. 1990*a*, Riboni 2002*a*, Trujillo & Riboni 2002, Chavez-Genaro *et al*. 2007). Here, the ovarian morphological analysis showed the same results; however, we have to consider that ovarian NE content was similar between groups. NE influences mainly primary and secondary follicle growth because they lack control by gonadotropins (Mayerhofer *et al*. 1997). In our results, an accumulation of the primary follicles and a delay in the growth of secondary follicles (Fig. 2B and D) is consistent with the observation that NE from a neuronal origin is almost absent in our experiment. In addition, the serum marker AMH can illustrate the decrease in overall follicular development. Plasmatic AMH levels are proportional to secondary and small antral follicles (Baarends *et al*. 1995, Sadeu *et al*. 2008); thus, the decrease in serum AMH observed

in GD-treated rats (Fig. 2K) is a true reflection of the low follicular growth observed in this group.

On the other hand, NE is also implicated in the formation of cystic structures in the ovary (Dorfman *et al*. 2003, Acuna *et al*. 2009, Fernandois *et al*. 2012, Luna *et al*. 2012). As expected, the absence of neuronal NE found in this work is accompanied by a lower number of follicular cysts in GD-treated animals. In other words, it seems that the NE from adrenal origin is not functional enough to fully recover follicular development in GD rats.

Changes in the ovarian steroid secretion

Studies have observed that NE influences the secretion of steroid hormones by the ovary (Aguado & Ojeda 1984*a*, Barria *et al*. 1993). In the present work, we observed a decrease in basal-unstimulated secretion (Fig. 3A and C) of both oestradiol and progesterone, which is coherent with the poor development of follicles and thus a low number of granulosa cells in the ovary. However, when analyzing the responsiveness of the ovary to hCG, a decrease in oestradiol, but not in progesterone, is observed. Because of a low number of follicular cells is found, this could suggest that each ovarian steroidogenic cell is producing more progesterone and less oestradiol, a fact that is better observed when the ratio hCG stimulated/basal secretion is analyzed (Fig. 3B and D). An explanation for this profile of secretion is a decrease in CYP19 aromatase expression in each granulosa cell. In this context, it is known that beta-adrenergic stimulation induces FSHR expression (Mayerhofer *et al*. 1997) and in consequence aromatase expression (Adashi & Hsueh 1982, Fitzpatrick *et al*. 1997). Interestingly, despite that the beta-adrenergic receptors expression and its plasma membrane localization in GD-treated ovaries are increased, a stimulation of incubated ovaries with ISO was unable to induce oestradiol and progesterone secretion, as occurs with hCG in control rats. Our results show that the decreased ovarian secretion of oestradiol and progesterone after ISO stimulation is corresponding with a reduced sympathetic sensitivity or uncoupled beta-adrenergic response in ovarian cells. In summary, the chronic decrease of NE from neuronal origin in the ovary (from infantile age until 8 months old) and the ovarian sympathetic hyporesponsiveness could be lowering the aromatase levels in granulosa cells and, in consequence, decreasing oestradiol synthesis and increasing progesterone accumulation in the follicles. Taken together, we speculate that well-established neural-follicle connections are necessary for an adequate coupling/response of beta-adrenergic receptors to adrenergic stimuli. In the rat, ovarian nerves become fully functional close to puberty onset (Ricu *et al*. 2008). Despite this, they are present in the ovary before birth (Malamed *et al*. 1992) and continue growing postnatally until they are functional (Kannisto *et al*. 1986, Owman *et al*. 1986, Schultea *et al*. 1992). Given this, the loss of interaction between the ovary and nerve fibers in GD-mediated sympathectomy during the infantile period could be deeply affecting the development of a functional responsiveness in the beta-adrenergic receptors of the gonad to NE. Even though the nature of these molecules in the ovary are still obscure, future studies would have significant importance in understanding the genesis of the functional neuronal ovarian/follicular cell connections.

In conclusion, our work indicates that after denervation during the infantile period in the rat, the ovarian NE content can be recovered. Despite this, the long-term absence of NE from a neural origin affects the sensitivity and responsiveness of beta-adrenergic receptors in the ovary, which is reflected by a decreased follicular development and altered steroid hormone secretion. Finally, we suggest that the neural–ovarian connection established during postnatal development is necessary for a correct ovarian response to adrenergic stimulation during the reproductive period.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-17-0318.

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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