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.


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 1984b, Lara et al. 1990a, 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 1984a, 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. 1990b, 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
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. 1990a, 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. 1990a, Albuquerque-Araujo et al. 1995, Riboni 2002a, b, Trujillo & Riboni 2002, Chavez-Genaro et al. 2007), a decline in the steroid secretory response and oestrous cycle alteration (Lara et al. 1990a, b, Albuquerque-Araujo et al. 1995, Riboni 2002b). 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 5 GD) 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. 1990a, 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 prooestrus was followed by an oestrus and then a diestrus (we considered diestrus to be both metaoestrus and diestrus 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 ovarian steroid secretion from the 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 KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 2.5 mM CaCl2, 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 oestriadiol 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 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, prevacuolated 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,
Ovarian sympathetic denervation 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 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' - TCAAGAAGCAGATCGGAGG-3' (GenBank number: X01117). For TH, sense: 5' - GGTCTACTGTCCGACCGTGATT-3' and antisense: 3' - GAGCTGTCTCTTGGGGCATATTG-5' (GenBank number: NM_012740). For beta1-adrenergic receptor were sense: 5' - AGACGCTCAACACCTCTCTAT-3' and antisense: 5' - ACAAGACAGCTCTACCGAAGTCCA-3' (GenBank number: NM_012701.1). For beta2-adrenergic receptor: sense 5' - CAG GC TATGCTATC GCTTCCTAT-3' and antisense: 5' - GGCTGAGGTTTGGGGCATAAAATC-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 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 A9037). 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 NaH2PO4 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. 1990a, 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.

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

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%.
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. 1990a, 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 ± 174.2 for ISO). In contrast, when we compared the basal-unstimulated release, we observed...
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
The sympathetic system controls ovarian function and is a key factor in ovarian pathophysiology (Aguado & Ojeda 1984b, 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. 1990a, Riboni 2002a, 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 ovaries. 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 steroid secretion.

**Ovarian morphological changes**

The sympathetic system controls ovarian function and is a key factor in ovarian pathophysiology (Aguado & Ojeda 1984b, 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. 1990a, Riboni 2002a, 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 (Dorffman 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 1984a, 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 steroiogenic 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 infanthile age until 8 months old) and the ovarian sympathetic hyposresponsiveness 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.

**Funding**

The authors of this manuscript where supported by FONDECYT-Chile grant number 1090159 to A P.

**Acknowledgments**

The authors thank and appreciate the help of Dr Cruz N for the critical analysis and suggestion on the manuscript revision. They also thank to Michelle Tiszavari for her help in the morphological analysis.

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Received 24 May 2017
First decision 14 June 2017
Revised manuscript received 14 November 2017
Accepted 21 November 2017