REPRODUCTION
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Maternal sympathetic stress impairs follicular development and puberty of the offspring

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Abstract

Chronic cold stress applied to adult rats activates ovarian sympathetic innervation and develops polycystic ovary (PCO) phenotype. The PCO syndrome in humans originates during early development and is expressed before or during puberty, which suggests that the condition derived from *in utero* exposure to neural- or metabolic-derived insults. We studied the effects of maternal sympathetic stress on the ovarian follicular development and on the onset of puberty of female offspring. Timed pregnant rats were exposed to chronic cold stress (4 °C, 3 h/daily from 1000 to 1300 h) during the entire pregnancy. Neonatal rats exposed to sympathetic stress during gestation had a lower number of primary, primordial, and secondary follicles in the ovary and a lower recruitment of primary and secondary follicles derived from the primordial follicular pool. The expression of the FSH receptor and response of the neonatal ovary to FSH were reduced. A decrease in nerve growth factor (*NGF*) mRNA was found without change in the low-affinity *NGF* receptor. The FSH-induced development of secondary follicles was decreased. At puberty, estradiol plasma levels decreased without changes in LH plasma levels. Puberty onset (as shown by the vaginal opening) was delayed. Ovarian norepinephrine (NE) was reduced; there was no change in its metabolite, 3-methoxy-4-hydroxyphenylglycol, in stressed rats and no change in NE turnover. The changes in ovarian NE in prepubertal rats stressed during gestation could represent a lower development of sympathetic nerves as a compensatory response to the chronically increased NE levels during gestation and hence participate in delaying reproductive performance in the rat.

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Introduction

Stress as an adaptive response of the body produces a wide range of biochemical and behavioral manifestations to respond to a threat (Chrousos 1998, Szabo 1998, Tache & Brunnhuber 2008). This apparently beneficial response of the body can become a problem when the stress becomes chronic. (Goldstein & Kopin 2008). Cold stress increases the noradrenergic tone in the rat, without producing changes in serum epinephrine or adrenocorticotropic hormone levels (Pacak *et al.* 1998*a*,*b*). We previously used this stress paradigm and demonstrated that when applied to adult rats it increased ovarian norepinephrine (NE) levels and induced ovarian function alterations, which led to polycystic ovarian morphology in rats (Paredes *et al.* 1998, Dorfman *et al.* 2003, Greiner *et al.* 2005). Polycystic ovary is the most common ovarian disease in women during their reproductive life (Fauser *et al.* 2012), is expressed early in development, thus proposing the likelihood of fetal origin.

The preferential sympathetic component of the chronic cold stress paradigm permits one to study the neural noradrenergic component of the physiological response (Goldstein & Kopin 2008) and the factors regulating the adequate establishment of the sympathetic innervation, such as nerve growth factor (*NGF*), by the respective innervated organ, including the ovary (Levi-Montalcini 1987).

The two important emerging questions are what are the implications of stress during gestation life and whether and how stress may affect the predisposition for, or programing of adult disease (Glover *et al.* 2010, Nugent *et al.* 2012, Rinaudo & Wang 2012), Alteration of reproductive function by early exposure to stress would compromise the survival of the species. Furthermore, the impact of prenatal programing of adult disease can be transmitted to future generations. In this regard, pre-eclampsia is characterized by high sympathetic activity that affects blood pressure in gestating mothers and induces changes that affect the progeny (Schobel *et al.* 1996, Takiuti *et al.* 2003), presumably through

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changes in the availability of NE to the fetal environment. Thus, newborn babies have changes in weight and metabolic parameters.

To understand the risk of chronically enhanced sympathetic nerve activity to the *in utero* development, we studied whether chronic cold stress (as a direct sympathetic stimulus) applied to rats during the entire gestation period may induce alterations in ovarian function during postnatal development of the progeny and whether it may affect reproductive performance during adult life.

Materials and methods

Animals

Sprague–Dawley rats weighing 250–300 g were maintained at 20 °C with a 12 h light:12 h darkness cycle. Water and food were provided *ad libitum*. Estrual cycling activity of rats was monitored by vaginal smears. In the afternoon of proestrus, rats were mated and the next morning pregnancy was confirmed by checking a vaginal sperm plug. We randomized 23 pregnant rats into two groups of ten control rats and 13 stressed rats. The control rats were maintained at room temperature during the entire pregnancy and the stressed rats were moved to a cold room at 4 °C for 3 h/each day during the entire pregnancy. All experimental procedures were approved by the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences at the Universidad de Chile and complied with national guidelines (CONICYT Guide for the Care and Use of Laboratory Animals). All efforts were made to minimize the number of animals used and their suffering.

Experimental design

After birth, the male rats were given to a foster mother and the female pups stayed with their mothers up to postnatal day 4 (PND4), at which time they were mixed and used for the experiments. The neonates used for prepubertal studies were maintained with their mothers up to 30 days old.

Study in neonatal rats

Four neonates from the control group and six neonates derived from cold-stressed dams were killed at PND4; the right ovary was fixed in Bouin's fixative and the left ovary was frozen at −80 °C. The ovaries of a second group of 4-day-old neonates ($n=8$ control and ten stressed rats) were immediately frozen and stored at −80 °C. A third group of neonates ($n=16$ control and 22 stressed rats) was used to study the '*in vitro*' effects of follicle-stimulating hormone (FSH) on the ovary.

Study in 30-day-old prepubertal rats

A group of nine control rats and 14 stressed prepubertal rats were killed at 30 days old; the right ovary was fixed in Bouin's fixative and the left ovary was frozen at −80 °C. Plasma samples were collected and stored at −20 °C for steroid analysis.

Study of vaginal opening as index of puberty

A group of six control rats and nine stressed rats born from control and stressed dams, respectively, was followed to study the age of vaginal opening as an index of puberty. We analyzed the capacity of these rats to develop a cyclic estrual activity.

Incubation assay

Pairs of ovaries from stressed and control rats of 4 days old were incubated for 24 h at 37 °C with 95% oxygen and 5% CO_2, as described previously (Mayerhofer *et al.* 1997). Four experimental conditions were tested: control + incubation media, stress + incubation media, control + FSH, and stress + FSH. The incubation media utilized was DMEM/F12 Gibco (Invitrogen Corporation), supplemented with 100 U of antibiotics (penicillin + streptomycin) and 0.5 mM 3-iso-4-butyl, methylxantine to inhibit phosphodiesterase. We used human FSH (F4021, 7000 IU/mg, Sigma Chemical Co.) at a final concentration of 10 IU/ml incubation media.

After 24 h of incubation, the ovaries were fixed to perform morphometric analysis of follicles; the incubation media were frozen at −80 °C for determination of cAMP.

Morphometry

Ovaries previously fixed were embedded in paraffin, cut into 6-μm sections, and stained with hematoxylin and eosin. Morphometric analyses of whole ovaries were done as described previously (Cruz *et al.* 2012).

Measurements of mRNA levels by real-time RT-PCR

Total RNA from the ovaries was prepared using the Chomczynski & Sacchi (1987) method of RNA extraction. The total RNA extracted was submitted to RT reaction to obtain cDNA. Then, cDNA obtained was amplified in real-time PCR equipment (IQ5 thermocycler, Bio-Rad Laboratories, Inc.) using specific primers for *NGF*, low-affinity *NGF* receptor ($p75^{NTR}$), and receptor for FSH (*FSHR*) as described previously (Dissen *et al.* 2000, Romero *et al.* 2002). All of the samples were analyzed in triplicate.

Determination of ovarian levels of NE, 3-methoxy-4-hydroxyphenylglycol by HPLC

Ovaries previously stored at −80 °C were weighed and then homogenized in perchloric acid, 0.25 M, and centrifuged at 12 000 ***g*** for 10 min at 4 °C. The resulting supernatant was filtered and used for the measurement of NA and 3-methoxy-4-hydroxyphenylglycol (MHPG) using a HPLC system (Sotomayor-Zarate *et al.* 2008, 2011) as previously described.

Determination of cAMP concentrations

All cAMP concentrations were determined by EIA, using a cAMP EIA Kit (Cayman Chemical Co., Ann Arbor, MI, USA) in accordance with the manufacturer's instructions. The coefficient of variation was <10% (Pradelles *et al.* 1989).

Determination of serum levels of estradiol

Estradiol (E_2) levels were determined using an enzyme immunoassay according to the manufacturer's instructions (Alpco Diagnostic, Windham, NH, USA). Intra- and inter-assay variations were $<5\%$ for E_2. The minimal detectable value was 10 pg/ml.

Statistical analyses

The data are expressed as the mean$\pm$S.E.M. values. To determine significant differences among multiple groups, we used one-way ANOVA followed by the Newman–Keuls *post hoc* test. To determine the differences between two groups, we used Student's *t*-test.

Results

Effects of stress during gestation in early follicular development

Early follicular development was assessed by morphometric analysis of all follicles of neonatal ovaries at PND4. Representative microphotographs of the ovaries are shown in Fig. 1. There were several differences between the ovaries from the control (Fig. 1A) and stressed (Fig. 1B) groups. First, the ovary of the control rats had a higher number of developing follicles than the ovary of stressed rats. Second, in the stressed rats (Fig. 1B), there was an increase in naked primordial follicles (arrows head), which are found primarily at the periphery of the ovary. Main differences were found in the number of healthy primordial follicles (Fig. 1A, black arrows), which have a healthy nuclei with a spherical shape compared with apoptotic follicles (Fig. 1B, arrow head), which present pycnotic nuclei with an irregular shape and some granulosa cells. The total number of follicles (including apoptotic follicles which are not more than 60 per ovary of stressed rats) was decreased in the offspring of rats stressed during gestation compared with the control rats (Fig. 1C). The more numerous follicles at this age are primordial follicles, also named 'follicular reserve pool', which were diminished in the stress group compared with the control group (Fig. 1D). This decrease is accompanied by an increase in apoptotic follicles undergoing the process of attrition (Fig. 1E) and thus total primordial pool (healthy+apoptotic) was unchanged. The primary and secondary follicles were also decreased in the stress group compared with the control group (Fig. 1F and G).

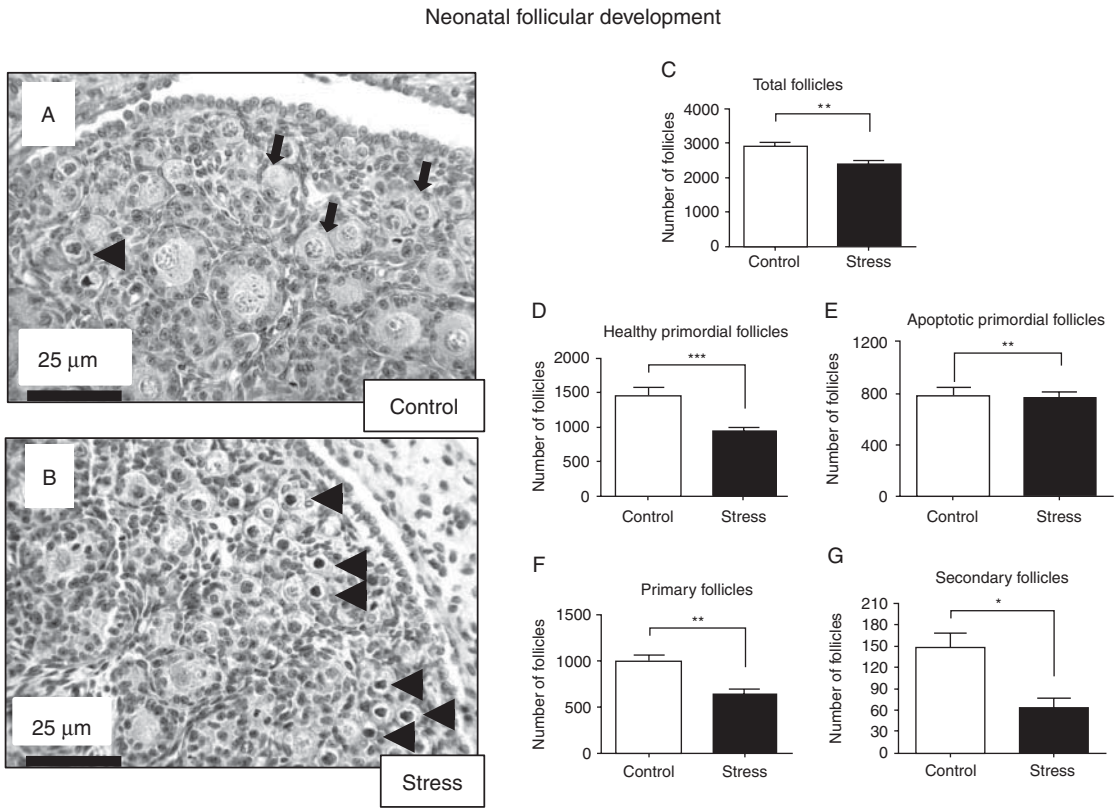

Figure 1 Representative pictures of neonatal ovaries from offspring rats from control (A) and stressed mothers (B). Black bar at the bottom of each picture corresponds to magnification of the picture. Arrows head represent apoptotic follicles. (C–G) Neonatal ovarian morphometry. (C) The total number of follicles in the control and stress groups; (D) healthy primordial follicles in the control and stress groups; (E) the number of atretic follicles in the control and stress groups; (F) the primary follicles in the control and stress groups; and (G) the secondary follicles in the control and stress groups. Results represent the mean$\pm$S.E.M. values of three rats for control and six rats for stress group. *$P<0.05$ vs control; **$P<0.01$ vs control; and ***$P<0.001$ vs control.

In addition, the ratio between developing follicles (primary + secondary) and total healthy follicles (healthy primordial + primary + secondary) was decreased in the stress group compared with the control group (control group 39.30 ± 2.91 vs stress group 28.75 ± 2.189, $P<0.05$ vs control, $n=5$ in each condition), which suggests that in addition to a lower number of follicles there is a decreased passage from the primordial stage to additional stages of development.

The effect of stress during gestation on the ovarian mRNA expression of NGF, $p75^{NTR}$, *and* FSHR *of the female progeny*

Early works (Dissen *et al.* 2001, 2009) describe the role of *NGF*, $p75^{NTR}$, and *NTRK1* (*TRKA*) in early follicular development and in the expression of FSHR. Thus to correlate the early changes in follicular development found in this study that are induced by a neurogenic stimulus such as sympathetic stress, we measured the expression of *NGF* and its receptors in addition to its functional expression as the FSHR. The levels of mRNA for *NGF*, $p75^{NTR}$, and *FSHR* obtained by qRT-PCR are shown in Fig. 2. The stress group presented a significant decrease in the levels of *FSHR* and *NGF* in the ovary (Fig. 2A and B). No differences were found in $p75^{NTR}$ levels between the control and stress groups (Fig. 2C).

The effect of stress during gestation on the neonatal ovarian responsiveness to FSH of the female progeny

To understand the ovarian response to FSH, we incubated PND4 ovaries for 24 h with FSH or with the incubation media. The number of secondary follicles after incubation with FSH was higher in the control rats than in the stress group (Fig. 3A). In addition, the secondary follicles reached a higher diameter in the control ovaries under FSH stimulation compared with the secondary follicles from stressed rats (Fig. 3B). In the stressed group, we observed a lower capacity of ovaries to produce cAMP under the incubation with FSH, which strongly suggests that there is a decreased responsiveness of the ovary to FSH and thus to develop secondary follicles under a gonadotropic stimulation (Fig. 3C).

The effect of stress during gestation on the age of vaginal opening and follicular development near to puberty in the female offspring

To analyze follicular development during the prepubertal stage of development, we studied the ovaries of control and stressed rats at the age of 30 days to quantify antral follicular development by morphometry and to relate it with plasma E_2 levels. There was a decrease in the number of larger-size antral follicles preovulatory at 30 days old in the stress group (Fig. 4A). The stressed rats presented decreased plasma E_2 levels (Fig. 4B).

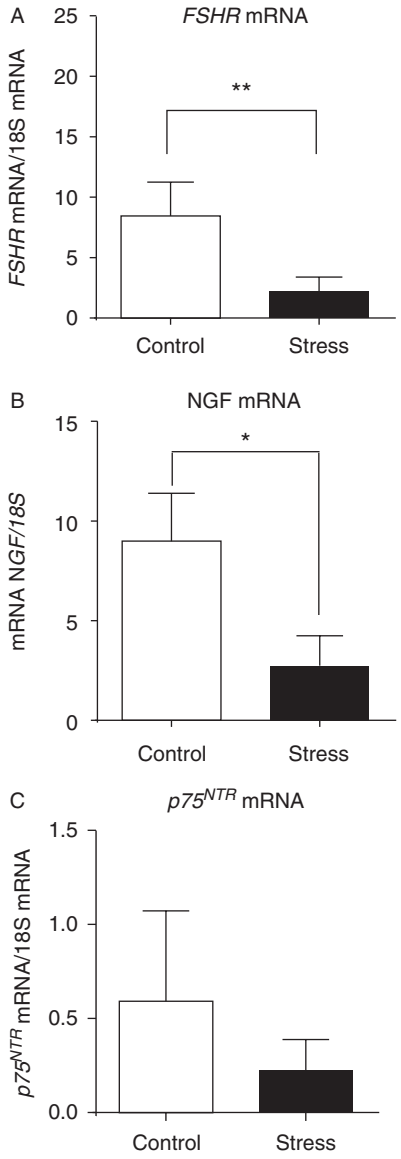

Figure 2 Quantification of the levels of mRNA to *FSHR* (A), *NGF* (B), and $p75^{NTR}$ (C) by qRT-PCR. Results are expressed as the mean ± S.E.M. values of four experiments in each condition. $^*P \leq 0.05$; $^{**}P<0.01$ vs control.

Another group of rats was monitored to determine at which stage they reached vaginal opening as an index of puberty. Rats exposed to stress during gestation had delayed puberty. Figure 4C demonstrates that 50% of rats reached vaginal opening by 32 days of age in the control group, whereas in the stress group, 50% of rats reached vaginal opening at ~35 days of age.

The effect of stress during gestation on ovarian NE and turnover of the female offspring

Previous evidence showed that extrinsic noradrenergic innervation develops just before puberty in the rat, as demonstrated by an increase in the concentration of NE

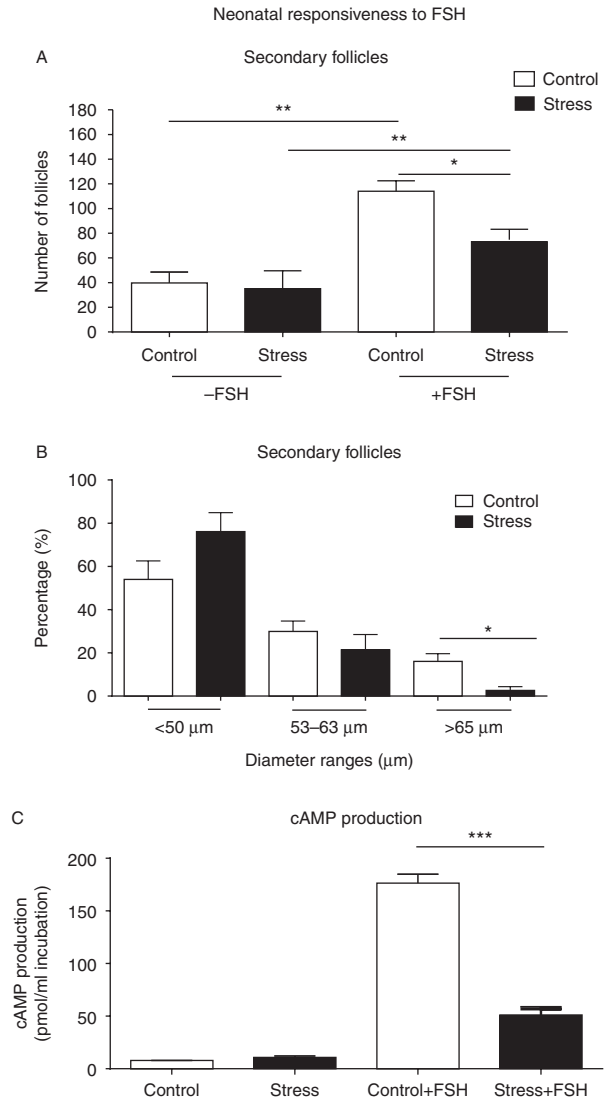

Figure 3 Responsiveness of the neonatal (4 days old) ovary to *in vitro* incubation with FSH (A) shows the presence of secondary follicles after 24 h; (B) a morphometric analysis of the secondary follicles; and (C) the concentration of cAMP determined in the incubation media. Results are expressed as the mean ± S.E.M. values of four experiments in each condition. *$P \leq 0.05$; **$P < 0.01$; and ***$P < 0.001$ vs control.

and in the induced release of NE from the tissue (Ricu *et al.* 2008). At PND30, there was a decrease in the concentration of NE in the progeny of rats stressed during gestation compared with the progeny of the control group (Fig. 4D). This decrease in NE was followed by a similar decrease in its metabolite MHPG (Fig. 4E); thus, the turnover rate was similar (Fig. 4F).

The effect of stress during gestation in the acquisition of regular cycling activity in the offspring

Vaginal cytology analysis is commonly used to determine the presence of ovulatory or anovulatory cycles. Approximately, 80% of the control rats reached normal cycling activity after vaginal opening (Fig. 5A) compared with fewer than 40% of the offspring of the mothers stressed during gestation reached normal cycling activity at puberty (Fig. 5B). The total number of cycles after the first month of observation was significantly decreased in the stressed rats (Fig. 5C); this effect was preferentially found during the first two weeks of observation (Fig. 5D). No differences were found during the last two weeks of observation (Fig. 5E).

Discussion

There is ample evidence linking an exposure to stress during gestation with harmful effects observed in the offspring. Different types of gestational stressors have been identified to program adult disease (Glover *et al.* 2010, Rinaudo & Wang 2012). In this study, we found that chronic prenatal sympathetic stress during gestation decreases the follicular reserve pool and impairs early follicular development during neonatal development in the offspring and the acquisition of female reproductive capacity and most likely the end of reproductive life.

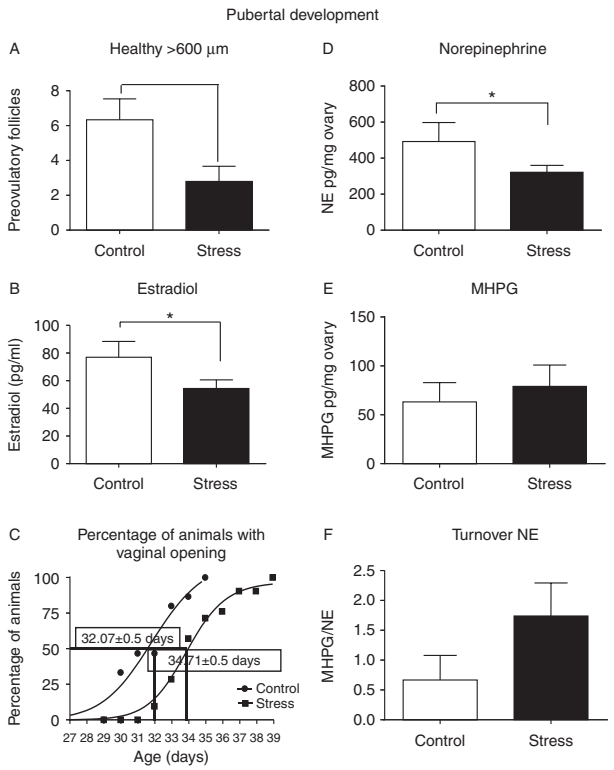

Figure 4 Effect of gestational stress in the number of antral preovulatory follicles (A), in the plasma concentration of estradiol (E_2) (B), and in the age of vaginal opening (C). Data for follicles and E_2 correspond to the mean ± S.E.M. values of four and six individual animals, respectively, at the age of 30 days. *$P < 0.05$ vs control. In D–F are shown the norepinephrine (NE) concentration and turnover. (D) ovarian NE, (E) 3-methoxy-4-hydroxyphenylglycol (MHPG) concentration, and (F) the turnover ratio. Results correspond to the mean ± S.E.M. values of four rats for control and seven for gestational stressed rats. *$P < 0.05$ vs control.

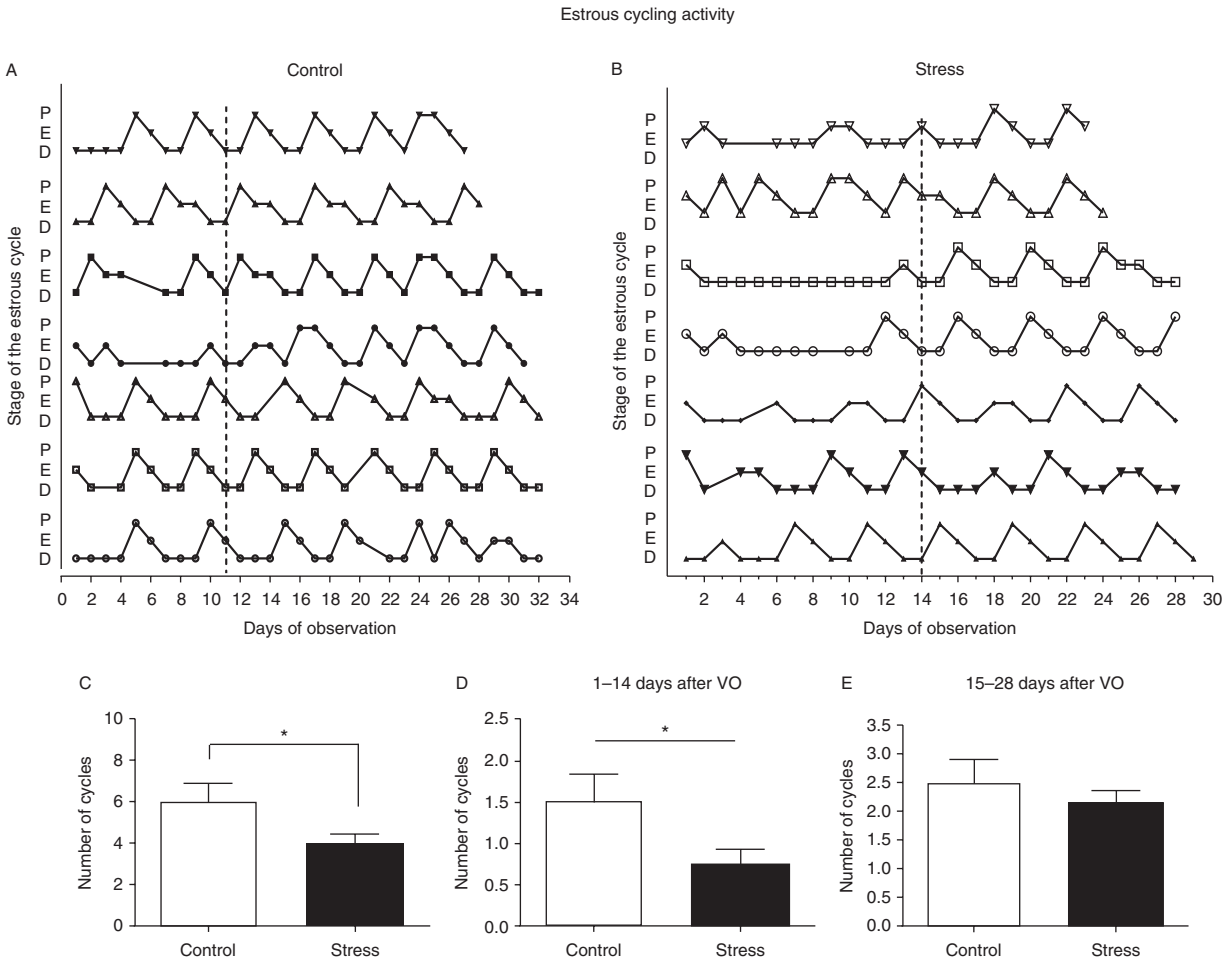

Figure 5 Gestational stress and its consequence on the estrual cycling activity after vaginal opening. Results in (A and B) represent data for representative animals for each group and day 0 corresponds to the first day after rats presented vaginal opening. Rats were observed for 4 weeks. The period of observation was divided in two groups. (C) The total number of cycles during the 4-week period. (D and E) Correspond to the estrual cycling activity during the first 2 weeks (D) and during the following 2 weeks (E). Results correspond to 12 control and 12 rats with gestational stress and represent the mean ± S.E.M. values. *$P<0.05$ vs control.

Stress during gestation decreases the follicular reserve pool

To the best of our knowledge, there is no previous evidence regarding prenatal stress exposure and its effect on early ovarian function. The similar number of total primordial follicles (healthy + apoptotic) in the stress group compared with the control group indicates that both groups of rats likely presented with similar amounts of oocytes before follicular assembly occurred. The decreased number of healthy primordial follicles and the increased number of oocytes undergoing apoptosis (apoptotic follicles) at PND4 indicate a likely failure during the follicular assembly process in animals under gestational stress. The bulk of primordial follicle assembly occurs within days 1–4 after birth and the process is commanded by paracrine regulators, such as NGF and others (Dissen *et al.* 2001, Pepling 2012). The increased number of apoptotic follicles at PND4 could be the result of the decreased expression of *NGF*. This neurotrophin supports oocyte survival and favors proliferation and differentiation of granulosa cells in follicles at different stages of development (Dissen *et al.* 1995, 2000, 2009). Interestingly, the *NGF* knockout mice have a similar follicular distribution of primordial follicles as the one observed in this study, i.e. a decrease in healthy follicles and an increase in nonassembled follicles that strongly support our finding (Dissen *et al.* 2001).

Stress during gestation impairs the initial recruitment of follicles and decreases the progression from primary to secondary follicles

While the follicular assembly is occurring, some already assembled follicles begin to differentiate in primary follicles and subsequently in secondary follicles (Pepling 2012). At PND4, the primordial, primary, and secondary follicles develop in the ovary (Pepling 2012). The decreased number of primary follicles found in the stress

group compared with the control group could be the product of a decreased number of healthy primordial follicles that result from a poor follicular assembly. However, the decreased ratio observed between developing follicles and total follicles (including primordial) indicates that independent of the number of primordial follicles, there is an alteration of the recruitment of follicles or an alteration in its control. *NGF* has been postulated to participate in the recruitment of follicles because the peptide is necessary for the differentiation of pregranulosa cells into granulosa cells (Dissen *et al.* 2009, Pepling 2012). In addition to this role, the incubation of ovaries with *NGF* enhances the development of primary follicles by increasing the expression of *FSHR* (Romero *et al.* 2002). The decrease in *Ngfb* mRNA found in this study could be paralleled with a similar decrease in the NGF peptide, as we previously demonstrated to occur in the adult rat neonatally treated with estradiol valerate, (EV) (Sotomayor-Zarate *et al.* 2008). The small size of the ovary made it impossible to get enough tissue for the EIA to measure NGF.

Hence, the decrease in *NGF* mRNA levels could be the cause of the decreased mRNA levels of *FSHR* that we observed in this study. The decreased *FSHR* levels can result from the slow trafficking from primary to secondary follicles, which finally leads to a poor amount of secondary follicles in the stressed group of rats.

A loss of sensitivity to FSH is the cause of low development of secondary follicles in the offspring of rats stressed during gestation

The expression of *NGF* is functionally coupled to the expression of *FSHR* (Romero *et al.* 2002); if there is a decreased expression of *FSHR* in early developing follicles, we should expect a decreased capacity of FSH to promote the transition from primary to secondary follicles and the growth of this type of follicles. To examine this decreased expression of *FSHR*, we incubated 4-day-old ovaries from the stress and control groups with FSH for 24 h. The development of preantral follicles depends on several paracrine factors and on the extrinsic regulation performed by gonadotropins and innervations (Mayerhofer *et al.* 1997, Dissen *et al.* 2009, Sirotkin 2011). After 24 h of incubation with only the media, the number of secondary follicles was similar between control and stress groups, which suggest that an extrinsic factor is involved in the decreased development of secondary follicles observed *in vivo* in the stress group. Interestingly, the incubation with FSH for 24 h increases the development of secondary follicles in both groups; however, the number of secondary follicles in the stress group was lower compared with that in the control group. In addition, as FSH promotes the proliferation and differentiation of granulose cells (DeManno *et al.* 1999) leading secondary follicles to grow, the decrease in larger-size secondary follicles observed in the stress group is due to a lower sensitivity to FSH. The low levels of cAMP reached after FSH stimulation in the stress group compared with the control group indicate that the lower sensitivity to FSH is due to a lower response of follicles because of low *FSHR* expression. Altogether, these results demonstrate that the lower response to FSH leads to a lower trafficking from primary to secondary follicles and to a lower development of secondary follicles in the offspring of rats submitted to sympathetic gestational stress.

Slow trafficking of follicles may cause delayed puberty in the offspring of rats stressed during gestation

The decreased number of follicles at all stages of development and the decreased sensitivity of preantral follicles to FSH in the stressed progeny suggest a condition persisting through pubertal development. The decreased number of larger-size antral follicles seen at 30 days old (>600 μm), which corresponds to the first cohort of follicles that will ovulate, was a likely consequence of a lesser amount of follicles capable of responding to FSH during early development in the stress group. This type of follicles is the primary source for the increasing levels of E_2 before puberty, which produces the positive feedback on luteinizing hormone (LH). Coherently, we observed that prepubertal rats aged 30 day had lower levels of E_2.

Programing effect of sympathetic stress

We previously described that stress-induced sympathetic activation using a 4-week chronic cold stress to adult rats (Dorfman *et al.* 2003) increased NE and NGF concentrations in the ovary in a manner that is causally related to a centrally originated increase in sympathetic activity (Fiedler *et al.* 2006, Jara *et al.* 2010). The decreased ovarian NE concentration found in the ovary of prepubertal rats could be the result of a compensatory response to the chronic hyper-noradrenergic condition during gestation. The fact that there is a similar change in the metabolite MHPG explains the lack of change observed in the turnover rate of the sympathetic neurons, which could indicate that the decrease in NE results from poorly developed noradrenergic nerves. The immunoblockade of NGF during neonatal development in rats decreased NGF and blocked sympathetic nerve development (Lara *et al.* 1990*a*,*b*), thus the decreased *NGF* expression found at early stages of development could be the responsible of the low NE activity and probably nerve development. We also observed decreased follicular development, delayed puberty, and low E_2 plasma levels (Lara *et al.* 1990*b*). Similarly, the lower number and poor development of the larger-size follicles observed in this study were correlated with decreased serum levels of E_2 measured in stressed rats.

Preovulatory follicles are the primary source of plasma levels of E_2. This first wave of follicles is necessary to reach the increasing levels of E_2 and to produce a feed-forward on release of LHRH and subsequently increasing release of LH that induces ovulation (Ebling 2005). This increase in E_2 levels produces changes in the vaginal epithelium leading to vaginal opening when the first ovulation occurs. The lower level of E_2 can also be responsible for the delayed vaginal opening compared with the control group. Thus, the late development of follicles delays the time at which this critical mass of follicles is reached. This suggestion is also reinforced by the delayed capacity of stressed rats to maintain a regular estrus cycling activity after vaginal opening. We cannot discard, however, that reproductive hypothalamus development *in utero* could be affected by the gestational stress.

Conclusion

We demonstrate for the first time that gestational stress produces early changes in follicular development and long-term impairments in ovarian function that finally leads to delayed puberty. Prenatal stress also reduces the pool of primordial follicles, which is closely related to the onset of reproductive senescence (Adhikari & Liu 2009). Although we studied several possible mechanisms that explain the alteration of physiological processes in the ovary by the time they occur, the mechanism that explains how stress can induce these changes in ovarian function has not been established. A compensatory programing effect on sympathetic nerve activity that decreases the development of ovarian sympathetic nerves by a neurotrophic-dependent mechanism could be one of the causes that merit additional exploration.

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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