Neonatal Exposure to Estradiol Valerate Programs Ovarian Sympathetic Innervation and Follicular Development in the Adult Rat[1]

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ABSTRACT

A single injection of estradiol valerate (EV) to 14-day-old rats (when the ovarian follicle population has been already established) disrupts cyclicity, increases the activity of key enzymes of androgen biosynthesis, and develops polycystic ovary by a causally related increase in ovarian noradrenaline (NA). The current study examined an early window of ovarian development to look for a specific stage of development at which estradiol can induce such changes in sympathetic activity and follicular development. A single dose of EV applied to rats before the first 12 h of life rapidly increases (after 24 h) the ovarian expression of nerve growth factor (*Ngfb*) and p75 low-affinity neurotrophic receptor (*Ngfr*) mRNAs. When adults, rats presented early vaginal opening, disrupted cyclicity, appearance of follicular cyst, absence of corpus luteum, and infertility. Total follicles decreased, mainly due to a reduced number of primordial follicles, suggesting that estradiol acts in the first stages of folliculogenesis, when primordial follicles are organizing. These changes paralleled a 6-fold increase in NA concentration. No changes in NA content were found in the celiac ganglia, suggesting a local, non-centrally mediated effect of estradiol. Surgical section of the superior ovarian nerve (the main source of sympathetic nerves to the ovary) to rats neonatally treated with EV decreased intraovarian NA, delayed vaginal opening, and blocked the development of follicular cyst and that of preovulatory follicles. Therefore, we can conclude that early exposure to estradiol permanently modifies ovarian sympathetic activity and causes profound changes in follicular development, leading to the polycystic ovary condition.

early development, estradiol, infertility, neonatal estrogenization, neurotransmitters, ovary, ovulation, PCOS, sympathetic nerves

INTRODUCTION

The exposure to estrogens or androgens disrupts normal endocrine function and decreases fertility in humans and wildlife [1, 2]. The fact that these effects could be provoked by a single administration of the steroids during specific windows of reproductive cycles resembles the concept firstly elaborated by Lucas [3], who defined the concept of "programming" as referring to the physiological setting by an early stimulus or insult (e.g., maternal food restriction, low body weight, influence of endocrine-disrupting compounds) during a critical hormone-sensitive period, resulting in negative effects in adult life [1, 4]. Animal research has primarily focused on fetal exposures (fetal programming) [1, 5–7], but recent research has expanded the concept of programming to early postnatal exposures (neonatal programming) during crucial phases of development in early life [8–14].

Studies on neonatal treatment with diethylstilbestrol [2, 11] have reported on negative effects over the normal morphology of the reproductive tract. Along this line, we have previously demonstrated [15, 16] that estradiol valerate (EV) administration to adult rats disrupts cyclicity and induces multifollicular cysts in the ovary through a neurotrophic mechanism causally related to the activation of sympathetic ovarian nerve [15]. Likewise, EV administration to 14-day-old female prepubertal rats [17] caused early puberty and disrupted ovarian follicle development, as evidenced by the absence of corpus luteum and the presence of ovaries with multifollicular cysts. In both cases (EV administration to adult or to prepuberal rats), there was a recovery of ovulatory function, such as estrous cyclicity, appearance of corpus luteum, and a reduction in the number of cysts after surgical sectioning of the sympathetic ovarian nerves to the adult rat, giving strong evidence for the involvement of an increased adrenergic tone in the development of the polycystic condition. At present, however, we do not know whether the early effect reported for estradiol on follicular development also is derived from an early induced increase in ovarian sympathetic activity.

Compared with the human, the rat is an immature mammal regarding ovarian development. It reaches the ovarian primary follicle population shortly after birth, unlike humans, in which these steps of development occur during the third trimester of pregnancy [18]. Thus, the neonatal rat appears a good model to investigate whether the effect of estradiol on early follicular development [15, 17] is able to permanently modify ovarian function in a manner dependent on sympathetic nerve activity.

MATERIALS AND METHODS

Animals

Ovaries from neonatal (36, 60, and 84 h old), young (42 days old), and adult (60 days old) Sprague-Dawley rats derived from a stock maintained at the University of Chile were used. We divided the experimental animals in three groups: 1) to study the short-term effect of neonatal EV administration on the expression of neurotrophic components of the ovary; 2) to study the long-term effect of neonatal EV administration on reproductive function; 3) to study the effect of sympathetic denervation on the effect of neonatal EV administration on follicular development.

For the first objective, we did four individual experiments for each time point (24, 48, and 72 h). For each experiment, we used six pups (72 control pups and 72 EV-treated pups). Each of these groups (control and EV-treated rats) were killed at 24, 48, and 72 h after hormone or corn oil administration to analyze the acute effect of EV in nerve growth factor (*Ngfb*) and p75 low-affinity neurotrophic receptor (*Ngfr*) expression. For the second group, we used

[1]Supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) grant 105-0765 to H.E.L. and grant PG/63/2005 to R.S.Z. (Departamento de Postgrado y Postítulo, Universidad de Chile). R.S.Z. is the recipient of a Ph.D. in Pharmacology fellowship UCH-208 from the Mejoramiento de la Calidad y Equidad de la Educación Superior (MECESUP) Chilean Program.

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20 newborn female pups divided in two groups of 10 animals each. The first 10 animals were injected during the first 12 h of life with a single subcutaneous injection of 0.1 mg EV dissolved in 50 µl corn oil per pup; the other group of 10 rats was injected with corn oil and used as controls. The dose of EV used was calculated according to the dose previously used in adults and prepubertal rats [15, 17]. Another consideration was the dose previously published by Pinilla et al. [19], who used 0.1 mg estradiol benzoate for 1-day-old rats. We wanted to maintain the potency of the estrogen but increase the half-life time to avoid repetitive doses, as has been published by others [13, 20]. Animals were killed at 61 days of age or at the nearest day when control rats were in estrous. For the third group, 14 newborn rats were injected with EV. When they reached 21 days of age, seven of them were denervated bilaterally by surgical section of the superior ovarian nerve (SON), which provides the main source of sympathetic nerves to the ovary [16, 17, 21]. The other seven rats were used as controls of denervation. To avoid reinnervation of the ovary [22], rats were killed 21 days after surgery, and we studied the age of vaginal opening, estrous cycling activity, and follicular development. The animal minimal number and procedures followed the protocols approved by the Institutional Ethic Committee of Faculty of Medicine and Faculty of Chemistry and Pharmaceutical Sciences, Universidad de Chile. All experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction. All efforts were made to minimize the number of animals and their suffering.

In each experimental series, litters (control and EV treated) were made up of five randomly selected female pups. After injection, pups were raised with a lactating mother until 21 days of age. Animals were kept under a 12L:12D cycle with food and water ad libitum. To study the long-term effect of neonatal EV administration in adults or young rats on reproductive function after denervation, the timing of vaginal opening was determined. Length and frequency of estrous cycles were recorded from the time of vaginal opening to the end of the study (either 42 or 61 days of age). Estrous cyclicity was assessed by analyzing with a light microscope the level of the relative proportion of leukocytes, epithelial cells, and cornified cells in daily vaginal lavages, which characteristically change during the various stages of the estrous cycle [23, 24]. Trunk blood also was collected from rats killed at 61 days of age to obtain serum for determination of progesterone, Δ4 androstenedione, estradiol, and insulin levels. Both ovaries, adrenal glands, celiac ganglia, and other organs from each rat were removed, immediately frozen in dry ice, and stored at −80°C for further analysis.

Morphology

One ovary from young or adult rats from control rats, EV-treated rats, or rats treated with EV plus denervation was immersed in Zamboni fixative, embedded in paraffin, cut in 8-µm sections, and stained with hematoxylin and eosin. For morphometric analysis, previously reported criteria were used [23, 24]. The presence of primordial, primary, preantral, antral, atretic, precystic, and cystic follicles was analyzed. Briefly, primordial follicles were those with one oocyte surrounded by flattened granulose cells; primary follicles were counted as primordial follicles exhibiting one or two layers of cubical granulose cells; preantral (mainly secondary) follicles had no antral cavity but had more than two layers of granulose cells; atretic follicles had more than 5% cells with pyknotic nuclei in the largest cross section and exhibited shrinkage and an occasional breakdown of the germinal vesicle; antral follicles were counted when the nucleus of the oocyte was visualized; precysts, devoid of oocytes, were large follicles containing four or five plicated layers of small, densely packed granulosa cells surrounding a very large antrum and displayed an apparently normal thecal compartment; finally, cystic follicles were devoid of oocytes and displayed a large antral cavity, well-defined thecal cell layer, and a thin (mostly monolayer) granulose cell compartment containing apparently healthy cells [23].

Noradrenaline Determination by High-Performance Liquid Chromatography Electrochemical Detection in Rat Ovaries and Celiac Ganglia

Tissue processing. The ovary was collected on dry ice and stored at −80°C for assay. Since no differences in noradrenaline (NA) concentration were detected, either right or left ovary was used for biochemical determination; the ovary was weighed and homogenized in 4 vol DPBS buffer, (KCl [2.68 mM], NaCl [136.89 mM], KH_2PO_4 [1.47 mM], Na_2HPO_4 [8.10 mM], $CaCl_2 \times 2H_2O$ [0.90 mM], and $MgCl_2 \times 6H_2O$ [0.49 mM]). The resultant homogenate was divided into two aliquots. One aliquot was stored at −80°C for NGFB determinations, and the other aliquot was precipitated with perchloric acid (PCA; 1.2 N) and centrifuged at 12 000 × *g* for 10 min at 4°C. The resultant supernatant was filtered (0.2-µm disposable filter; SM16534; Minisart NML; Sartorius) and used for NA determination. Frozen celiac ganglia were homogenized in 1 mL PCA (0.2 N) using a glass-glass homogenizer and were centrifuged at 15 000 × *g* for 15 min at 4°C. The resultant supernatant was cleaned through a filtration unit and used for NA determination.

Quantification of NA from ovaries and celiac ganglia. A total of 20 µl of each filtrate solution was injected (using a Rheodyne injector valve) into a high-performance liquid chromatography (HPLC) system (Waters; Millipore Corp., Milford, MA) with the following configuration: a pump (Waters 600-E Multisolvent), a column BAS MF-6213, Phase II ODS-3 (BAS, West Lafayette, IN), and an amperometric detector (Waters 464 Pulsed Electrochemical Detector). The HPLC mobile phase [25, 26], containing 0.1 M NaH_2PO_4, 0.86 mM octyl sulfate, 1 mM EDTA, and 0.5% acetonitrile (pH adjusted to 2.6) was pumped at a flow rate of 0.90 ml/min. The potential of the amperometric detector was set at 0.650 V. Under these experimental conditions, retention time was 4.1 min for NA. Samples were analyzed by comparing their peak area and elution times with reference standards.

NGFB Quantification

NGFB was measured using the Promega NGFB E-max Immuno Assay System. Each ELISA 96-well plate was coated overnight at 4°C with an anti-NGFB polyclonal antibody (pAb; 1:1000; 100 µl/well) in the carbonate coating buffer (0.05 M sodium bicarbonate/carbonate, pH 9.7). On the following day, the contents of the wells were removed, and the plates were washed with Tris-buffered saline with Tween (20 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.05% vol/vol Tween-20). After that, they were incubated at room temperature for 1 h in block and sample buffer (200 µl/well). Two columns on the ELISA plates were designated for the NGFB standard curve as provided by the supplier. The final concentrations within the plates were 0–500 pg/ml. The aliquot for NGFB determinations (see homogenization with DPBS in previous section) was centrifuged at 13 000 × *g* for 15 min at 4°C. The samples to be assayed were prepared as follows: a 200-µl aliquot of the tissue sample was added to the well, and three successive 1:2 dilutions were made. Sample or standard was incubated for 6 h and washed and incubated overnight at 4°C with anti-NGFB mAb. At the end of the procedure, plates were washed and incubated with anti-rat IgG and horseradish peroxidase conjugate for 2.5 h at room temperature. After the incubation, a tetramethylbenzidine and the peroxidase substrate solution were added to each well, and the plates were shaken for 5–10 min at room temperature; the reaction was terminated by adding 1 N hydrochloric acid to the wells. The optical density of the reaction product was read on a microplate reader at 450 nm, and values were normalized per gram of tissue assayed.

Determination of mRNA Levels of Ngfb and Ngfr by Real-Time PCR

Real-time PCR was used to determine whether the mRNA encoding *Ngfb* or the *Ngfr* changed in the ovary during the first 72 h after EV injection. Total RNA was extracted as recommended [27]. A total of 5 µg total RNA was subjected to reverse transcription at 42°C for 60 min in a 30-µl volume using 1.6 mM dinucleotide triphospates, 10 mM dithiothreitol, 176 nM random hexamers (Invitrogen, Carlsbad, CA), 25 units of RNaseOUT (Invitrogen), 125 units of reverse transcriptase SuperScriptII (Invitrogen), and first-strand buffer. The reaction was terminated by heating the samples at 75°C for 10 min. For quantifying mRNA for *Ngfb* and *Ngfr*, all samples were analyzed in triplicate in 25-µl reaction, and a standard real-time PCR reaction mix was prepared containing the following components: 12.5 µl of 2× Brilliant Platinum SYBR Green QPCR Master Mix (Stratagene), 9.5 µl nanopure and sterile water, 0.5 µl of each primer, and 2 µl cDNA. For specific gene amplification, a standard protocol of 40 cycles was used in the MJ Research PT-200 real-time PCR (MJ Research Inc., Watertown, MA.). After initial polymerase activation at 95°C for 10 min, primer-specific amplification and quantification cycles were run at 60°C for 15 sec and 72°C for 20 sec. The fluorescence intensity of the double-strand-specific SYBR Green I, reflecting the amount of actually formed PCR product, was read at the end of each elongation step after carrying out melting curve analyses to determine the melting points of the PCR products. Then, specific initial template mRNA amounts were calculated by determining the time point at which the linear increase of sample PCR product started relative to the corresponding points of a standard curve obtained by serial dilution of known copy numbers of the corresponding control tissue. To evaluate specific amplification, a final melting curve was created (72°C–85°C) under continuous fluorescent measurement.

The *Ngfb* primer was designed from data published in GenBank, access number M36589, forward 5′-GGC CCA TGG TAC AAT CTC CTT CAA-3′ and reverse 5′-GTC CGT GGC TGT GGT CTT ATC TCC-3′ that generate a

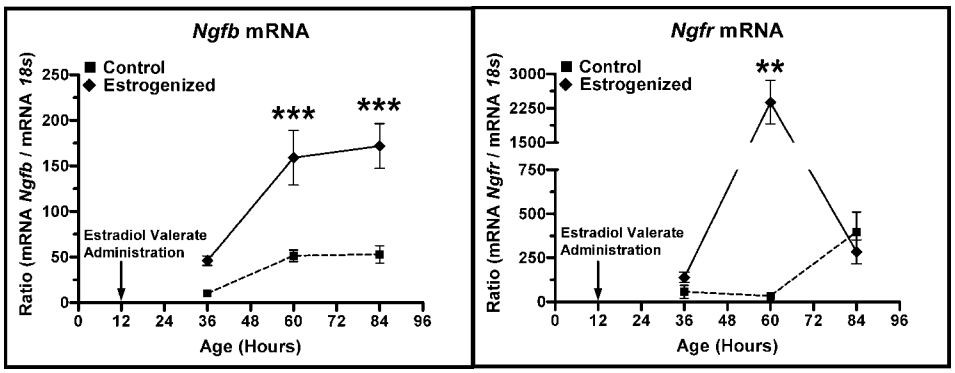

FIG. 1. Effect of EV single injection at 12 h of life on the *Ngfb* and *Ngfr* mRNA levels. The figure shows the mean values ± SEM of four independent experiments. $^{**}P < 0.01$; $^{***}P < 0.0001$.

product of 136 bp. The primer to *Ngfr* was published by Dissen et al. [28], GenBank access number X05137, forward 5′-AGC CAA CCA GAC CGT GTG TGA-3′ and reverse 5′-GTC CTG GCA GGA GAA CAC GAG-3′ that generate a product of 247 bp. To normalize the *Ngfb* and *Ngfr* mRNA amounts, ribosomal *18s* mRNA was measured in each protocol. Amounts of *18s* mRNA were determined using a commercially available RT primer pair (Ambion, Austin, TX), GenBank access number X01117. For *18s*, forward 5′-TCA AGA ACG AAA GTC GGA GG-3′ and reverse 5′-GGA CAT CTA AGG GCA TCA CA-3′. Amplification of *18s* RNA was performed in a different tube to avoid interference with the amplification of the mRNAs. Reaction tubes lacking RT enzyme were used as PCR-negative controls. To verify the products from the RT-PCR reaction, they were separated on 2.0% agarose gels, stained with ethidium bromide, and compared to a 100-bp standard (data not shown).

Determination of Total Adrenal Catecholamines

The adrenal glands were weighed and homogenized in 500 μl ice-cold 0.2 N PCA. The homogenate was centrifuged at 12 000 × *g* for 10 min at 4°C. The supernatants were used for colorimetric determination of total catecholamines. As previously reported, this method measures NA and adrenaline as a whole by the formation of noradrenochrome and adrenochrome (excitation wavelength of 540 nm) when the sample is oxidized with iodine at pH higher than 6.0 [29].

Determination of Serum Levels of Estradiol, Androstedione, and Progesterone

Estradiol (E_2) levels were determined by enzyme immunoassay (EIA) following the manufacturer's instructions (11-ESTH-430; Alpco Diagnostic, Windham, NH). Intraassay and interassay variations were less than 5%; the minimal detectable value of E_2 was 10 pg/ml serum. Androstenedione (Δ4) levels were determined by EIA following the manufacturer's instructions (11-ANDROH-208; Alpco Diagnostic). Intraassay and interassay variations were less than 6%; the minimal detectable value of Δ4 was 0.05 ng/ml serum. Progesterone levels were determined by EIA following the manufacturer's instructions (11-PROGH-305; Alpco Diagnostic). Intraassay and interassay variations were less than 5%; the minimal detectable value of progesterone was 0.1 ng/ml serum. Insulin levels were determined by EIA following the manufacturer's intructions (10-1124-01; Mercodia AB, Uppsala, Sweden). Intraassay and interassay variations were less than 5%; the minimal detectable value of insulin was 0.07 ng/ml serum.

Statistical Analysis

Data were expressed as mean ± SEM. To determine significance and when animals were compared over time or within multiple groups, we used one-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test. Differences between control and EV groups were analyzed by unpaired Student *t*-test. The comparison was statistically significant when $P < 0.05$.

RESULTS

Acute Effect of Neonatal EV Administration on mRNA for Ngfb *and* Ngfr

Figure 1 shows mRNA changes for *Ngfb* and *Ngfr* after single EV injection the morning after delivery (approximately at 12 h of life). The ovaries of estrogenized rats presented a significant increase of *Ngfb* mRNA at 36, 60, and 84 h. The mRNA for *Ngfr* increased only at 60 h.

Effect of EV Neonatal Administration on Normal Animal Growth, Uterus Weight, Vaginal Opening, Cyclicity, and Morphological Aspects of Ovaries

Neonatal EV administration increased the weight gain compared with control rats (Fig. 2). At 60 days old, rats were 30% heavier than controls. Basal levels of serum insulin of these rats did not differ from control rats (0.75 ± 0.13 ng/ml for EV rats vs. 1.06 ± 0.26 ng/ml for control rats). The effect of neonatal EV administration on uterus weights showed that the estrogenized group presented ballooned uteruses heavier than controls (425.4 ± 10.5 mg for EV rats vs. 343.8 ± 20.9 mg for controls, n = 6; $P < 0.001$). Figure 3, A and C shows the cycling activity of rats after vaginal opening and the mean day of vaginal opening for five control and five EV rats (long arrow). Mean vaginal opening occurred at 31.6 ± 0.2 days in control rats and 25.8 ± 0.2 days for neonatal estrogenized rats ($P < 0.001$). After vaginal opening, control rats maintained regular cycles throughout the study. In contrast, EV rats showed severe cyclic disruption, and most of them remained in a persistent proestrus-estrus stage, similar to a previous report of our research team [17]. On the other hand, Figure 3, B and D shows two representative ovary sections from controls and neonatal estrogenized rats, respectively. The ovarian size of neonatal EV-treated rats (Fig. 3D) was smaller than that of controls, as shown in Figure 3B (57.5 ± 2.1 mg for control rats vs. 15.3 ± 1.8 mg for EV rats, mean ± SEM, n = 5; $P <$ 0.001).

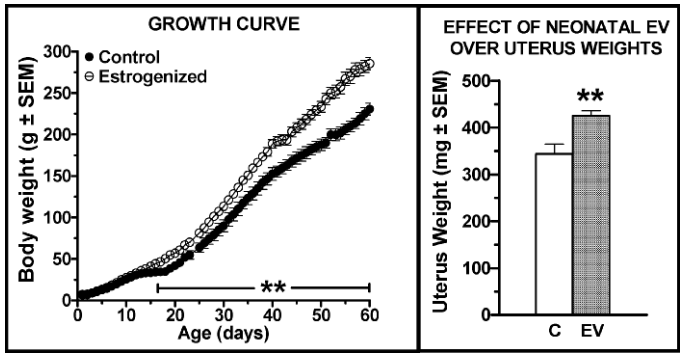

FIG. 2. The neonatal exposure to a single dose of EV (0.1 mg subcutaneously) produces a significant increase in normal growth of adult rats. This effect was observed after 16 days of age. A distinct increase in the uterus weight of neonatal estrogenized rats was also detected. Results are mean ± SEM of n = 5 experiments. $^{**}P < 0.01$. C, control.

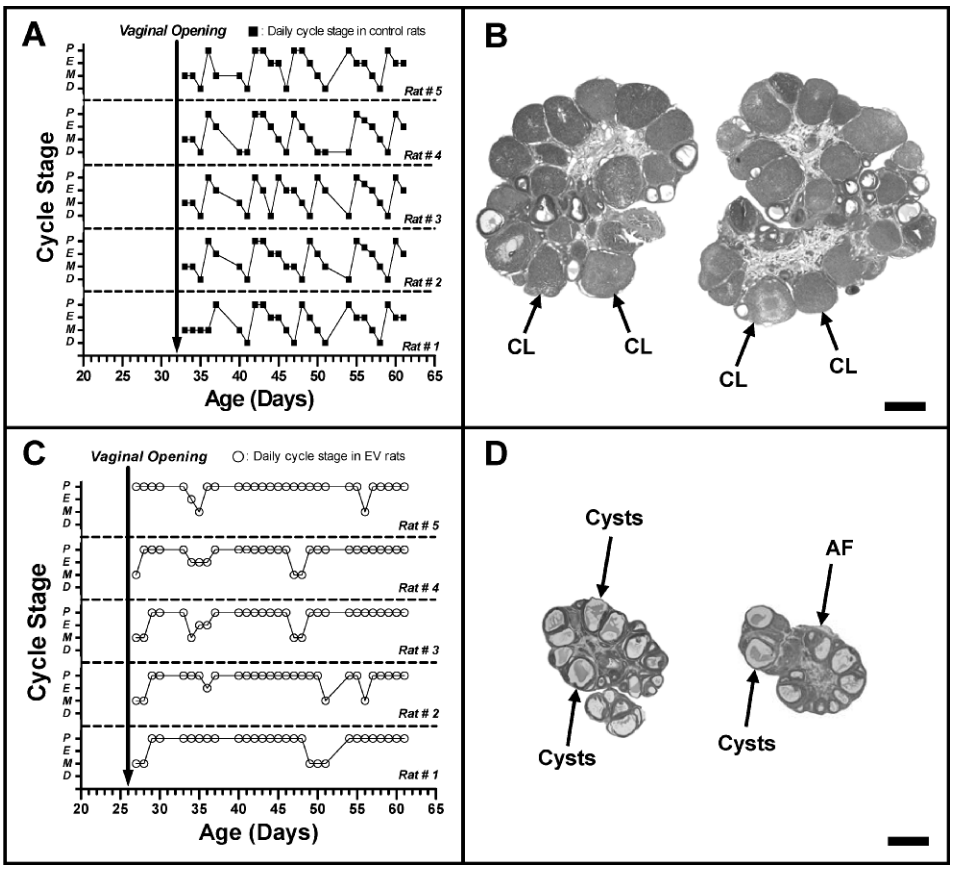

FIG. 3. **A**, **C**) The estrous cyclicity of control (**A**) and neonatal EV (**C**) rats. Arrows show the day when vaginal opening occurred in 100% of animals. **B**, **D**) The morphological aspect of the ovary from adult rats (control [**B**] and EV [**D**] rats, respectively). P, proestrus; E, estrous; M, diestrous day 1; D, diestrous day 2; CL, corpus luteum; AF, atretic follicles. Bars = 500 μm.

Effect of Neonatal EV Administration on the Follicular Dynamics of Adult Rats

Figure 4 summarizes the effects of neonatal EV administration on follicular dynamics. The total number of follicles per ovary was reduced in neonatal EV-treated rats (Fig. 4A) when compared to control rats. A diminution in the number of primordial follicles was also found (Fig. 4B). Figure 4, C and D exhibits the preantral and antral population of follicles, respectively. Both populations had a similar profile; preantral and antral follicles decreased in EV treated rats. In addition, atretic, either preantral or antral, follicles were more abundant in EV-treated rats. Neonatal estrogenization produced significant changes in precystic and cystic follicles. The mean number of cystic and precystic follicles in EV-treated rats increased with respect to the control (11.4 ± 1.1 EV rats vs. 1.6 ± 0.7 control rats; $P < 0.0001$). Corpus luteum was not found in neonatal EV-treated rats, whereas control rats presented an average of 27.4 ± 1.3 corpus lutea per ovary.

Effect of Neonatal EV Administration on NA and NGFB Concentrations from the Ovary and Celiac Ganglia and on Total Catecholamines of the Adrenal Glands in Adult Rats

To study the effect of neonatal EV administration on sympathetic nerve activation, we measured the amount of NA and NGFB in the ovary of adult rats, NA concentrations in celiac ganglia, and total content of catecholamines in adrenal glands. Figure 5 shows that neonatal EV administration increased NA concentration in the ovary. This increase also became evident when NA concentration was expressed as picograms of NA per ovary (pg/ovary: 2609.7 ± 226.7 for control rats vs. 3952.5 ± 488.7 for EV rats, mean ± SEM, n = 10; $P < 0.05$). Since NGFB is a trophic support for ovarian sympathetic nerves, its concentration served to analyze the probable association of EV-induced NGFB activation during neonatal stage, with an ovarian NGFB increase in adult rats. There was no increase in NGFB content but, rather, a 50% decrease (Fig. 5B). On the other hand, no changes were found in NA concentration in celiac ganglia, whereas in the adrenal glands, neonatal EV administration caused an increase in total catecholamines.

Effect of Neonatal EV Administration on Serum Levels of Progesterone (P_4), Δ4, and E_2 in Adult Rats

A decrease in P_4 serum levels was found in neonatal EV-treated rats (Fig. 6A). Figure 6B shows a decrease in the serum levels of Δ4 in neonatal EV-treated rats. No significant changes were found in the serum levels of E_2 (Fig. 6C).

Effect of Sympathetic Denervation on the Effect of Neonatal EV Administration on Ovarian Follicular Development

Figure 7 shows the effect of SON denervation on the age of vaginal opening, cycling activity, and morphology of the ovary. Denervation of EV-treated rats delayed mean day of vaginal opening to an age similar to control nondenervated rats (31.6 ± 0.2 days in control rats vs. 29.8 ± 0.5 days for neonatal estrogenized rats submitted to denervation; Fig. 7A). After vaginal opening, estrous cycling activity of denervated rats was not recovered to controllike regular cycles (Fig. 7, B and D). Denervation, however, strongly modified the morphological aspect of the ovary compared with the ovary of EV-treated rats (Fig. 7C). Although we did not perform a quantitative analysis of follicular population, EV treatment decreased the number of small antral follicles, and at the age of

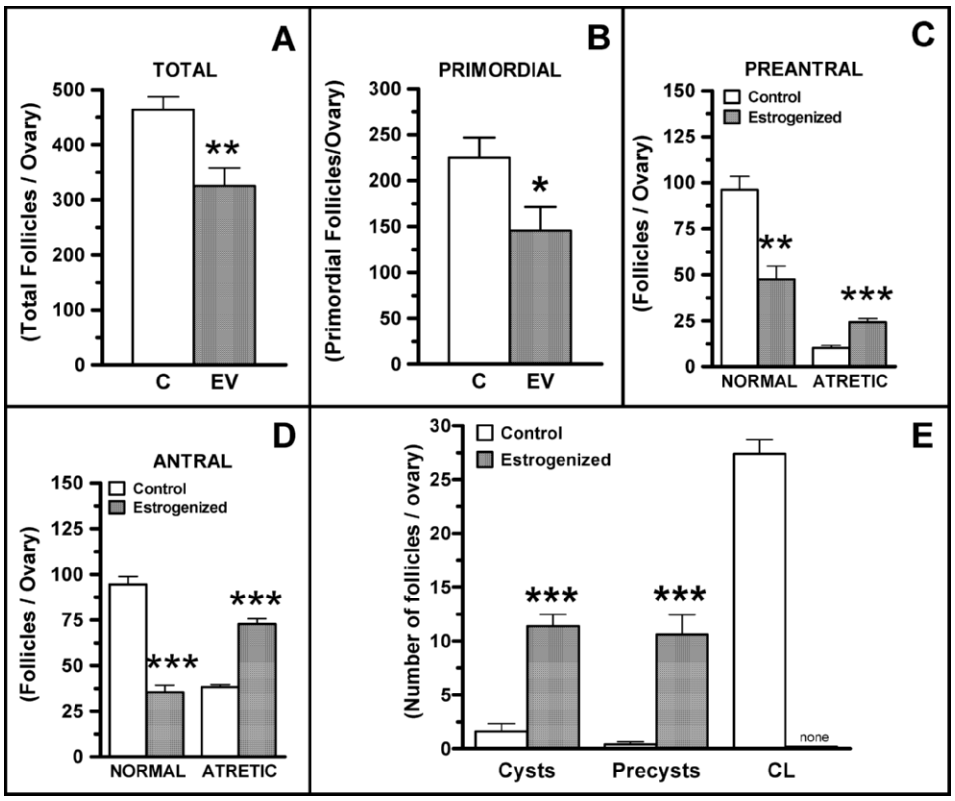

FIG. 4. Follicular quantification. **A**) Total number of follicles for control (C) and neonatal EV rats. Total number and primordial follicles decreased in neonatal EV rats (**B**). **C**, **D**) Preantral (**C**) and antral (**D**) follicles. **E**) Cysts, precysts, and corpus luteum (CL). Results are mean ± SEM of n = 5 experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$.

study (after 40 days after EV administration), clear precystic type III follicles appeared. Denervation of the ovary at 21 days of age increased the number of small antral follicles, with a concomitant decrease in antral follicles in the range of 400–600 μm, as well as type III and precystic follicles. Denervation decreased ovarian norepinephrine (from 503.1 ± 46.1 pg/mg ovary for EV rats to 128.2 ± 7.5 pg/mg ovary for denervated rats previously treated with EV (EV-SONX) rats, mean ± SEM, n = 7; $P < 0.01$) and estradiol plasma levels (118.3 ± 6.2 pg/ml for EV rats vs. 78.3 ± 4.7 pg/ml for EV-SONX rats, mean ± SEM, n = 7; $P < 0.01$).

DISCUSSION

We have found that estradiol administration to neonatal rats produced profound changes in follicular development and reproductive function in the adult rat. These effects are probably mediated by a neurotrophin-dependent activation of ovarian sympathetic nerves, as denervation reversed those changes in cyst formation and follicular development previously induced by estradiol. Although many reports on neonatal administration of compounds with estrogenic activity have described negative long-term effects on reproductive function [2, 8, 11–13], no studies have been performed thus far on the impact of neonatal estradiol administration on ovarian nerve activation and its role in the reproductive function of adult rats. Since estradiol is the endogenous steroid produced by the ovaries, it is important to study the effect that excess exposures of the hormone, either by endogenous deregulation in ovarian steroidogenesis or to abnormal exogenous levels of the steroid, would modify reproductive functions.

Short-Term Effect of Neonatal EV Administration on the Expression of Neurotrophic Components of the Ovary

We have previously reported that estradiol—through a neurotrophin-mediated mechanism—activates sympathetic neurons projecting to the ovary [22, 23]. Viral transneuronal tracing studies [30] have demonstrated that EV effect does not involve sympathetic neurons projecting from the hypothalamic paraventricular nucleus, thus supporting a local effect of estradiol on the expression of intraovarian neurotrophic factors. The rapid effect of EV on *Ngfb* and *Ngfr* mRNA expression found in our study could be the result of the estrogen action on

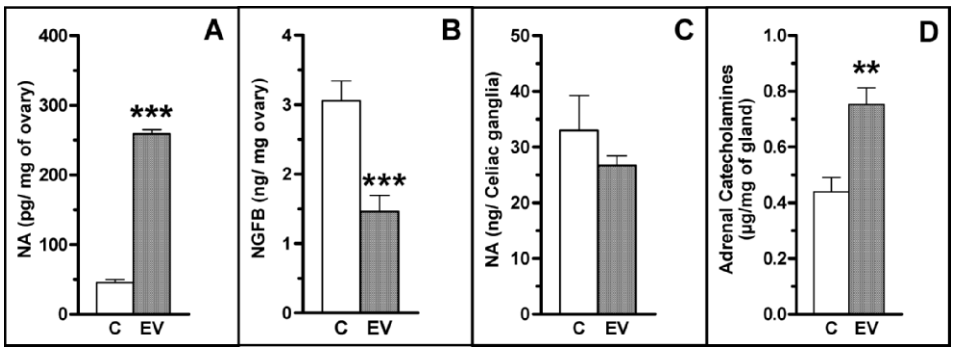

FIG. 5. **A**, **B**) Changes in the NA (**A**) and NGFB (**B**) concentrations in the ovary. **C**) NA concentration in celiac ganglia. **D**) Total catecholamines from adrenal glands. Results are mean ± SEM of n = 10. $^{**}P < 0.01$; $^{***}P < 0.0001$.

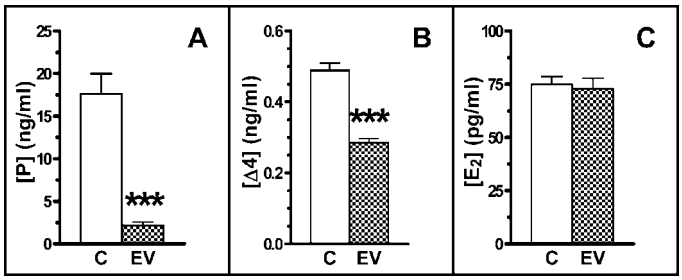

FIG. 6. The neonatal exposure to EV (0.1 mg subcutaneously) decreased serum levels of progesterone (P) and Δ4-androstenedione (Δ4), in adult rats (**A** and **B**, respectively). Serum levels of estradiol (E_2) did not change (**C**). Results are mean ± SEM of n = 5 experiments. ***$P < 0.0001$. C, control.

the estrogen response elements present in the DNA for *Ngfb* and *Ngfr* [31]. This effect on ovarian sympathetic neurons seems to be similar in the adult rat, as we had previously reported, because both *Ngfb* and *Ngfr* were the most affected by EV, with no effect on the high-affinity trkA-associated NGFB receptor [23]. Because of the similarity of the model, the *Ngfb* effect found in the present work is probably not mediated by interaction with trkA receptor. In support of this, Jefferson et al. [32] reported that genistein (a well-known inhibitor of trkA activity) exerts its estrogenic effect on the neonatal ovary acting on estrogen receptor β, independently of trkA activity, because lavendustin (a tyrosine kinase inhibitor without estrogen activity) does not cause the same effect of genistein. There exists the possibility that genistein could activate *Ngfb* and *Ngfr* mRNA transcription, as EV does in long-term effects on sympathetic activity. The acute increase in *Ngfb* mRNA found in the present study could be paralleled with a similar increase in the NGFB peptide, as we previously demonstrated to occur in the adult rat treated with EV [23]. The small size of the ovary made it impossible to get enough tissue for the EIA to measure NGFB. If it occurs as in the adult rat, the increase in NGFB and NGFR intraovarian levels is responsible for the increased levels in ovarian NA found in the rats. If this is the case, the early NGFB effect on the sympathetic neurons seems to be transient and enough to program changes in the adult sympathetic neurons.

Long-Term Effect of Neonatal EV Administration on Reproductive Function

Although the body weight increased significantly in EV-treated rats, we did not find altered basal plasma levels of insulin. These data are in agreement with a report from Stener-Victorin et al., who did not find changes in insulin resistance in EV-induced PCO rats [33]. If this was the case, the effect of estradiol found on both follicular development and sympathetic innervation is independent of the metabolic state of the animal and most likely is related to local effects on the ovary, either on follicular development or sympathetic innervation. Estradiol valerate administration in the first day of life (i.e., when the primordial follicle population has not been defined [34]) led to profound changes in sexual maturation, reproductive function, and sympathetic nerve activity in the adult rat. The anovulatory condition of the adult rat was confirmed by the total absence of corpus luteum, low levels of plasma progesterone, and the presence of bilateral polycystic ovaries.

In either case, the early changes in *Ngfb* and *Ngfr* mRNA could be responsible for increased NA level in the ovary of

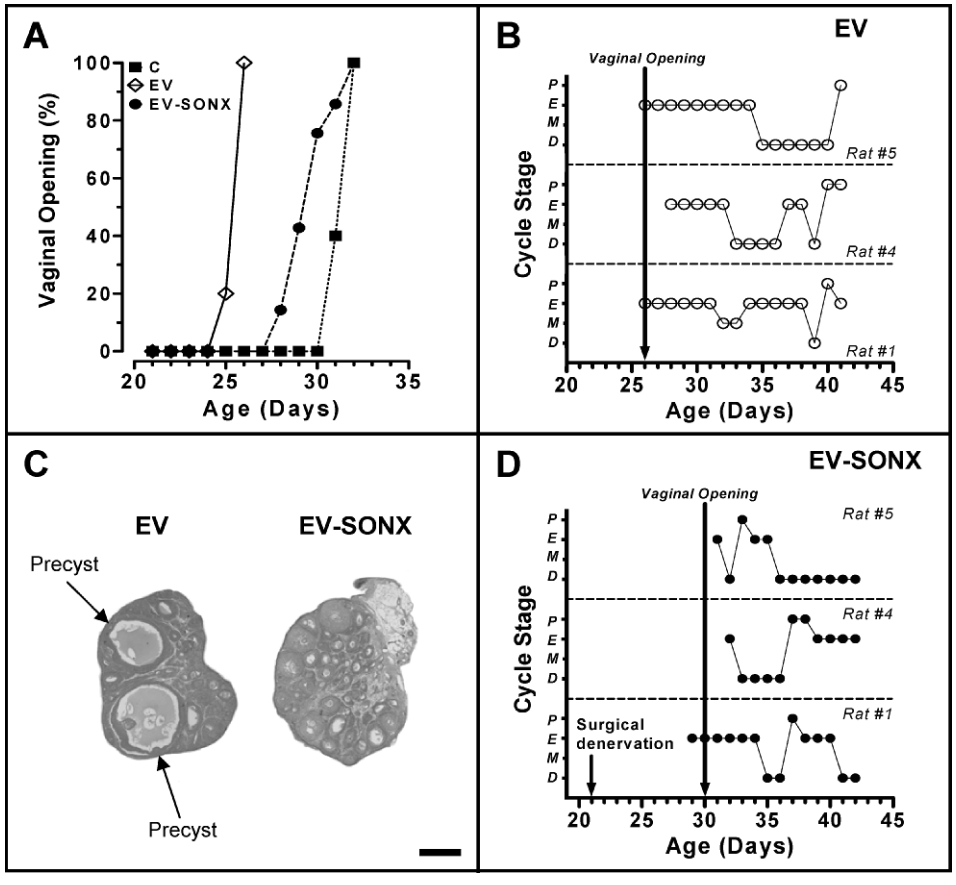

FIG. 7. Effect of sympathetic denervation (SONX) on vaginal opening and follicular development in neonatal rats treated with EV. EV was administrated before 12 h of life, and surgical denervation was done at 21 days of age (short arrow in **D**). **A**) Temporary course of vaginal opening per group (control [C], EV, and EV-SONX). **B**, **D**) The estrous cyclicity of EV and EV-SONX rats (long arrows indicate the day when vaginal opening occurred in 100% of the animals). **C**) Morphological aspect of the ovary from 42-day-old rats. Bar = 500 μm.

adult rats, as shown in the total amount of NA in the ovary or as the NA concentration per milligram of tissue to correct for changes in the weight of the ovary. It is interesting to note that the effect of EV was not found at the celiac ganglion. Probably, the estradiol effect is preferentially localized to the ovarian nerves and does not involve the central sympathetic control, as demonstrated previously [30]. Ovarian NGFB concentration in adult rats was lower than that in controls. Estradiol valerate administration to adult rats provoked a transient increase in NGFB concentration [23] just after 30 days of the administration of the steroid. If we assume a similar transient change in the adult rat, one might expect normal levels of NGFB 60 days after EV administration. We do not have an explanation for the decrease in NGFB, and it probably could be a consequence of the severe decrease in the total follicular population induced by neonatal estradiol administration and, thus, in cells producing the neurotrophic factor. The increase in adrenal catecholamines in EV rats could be the result of an increase in tyrosine hydroxylase activity in adrenal medullary cells, as has been demonstrated to occur by stimulation of catecholamine synthesis by environmental estrogenic pollutants [35, 36].

The biological effects of neonatal EV on early vaginal opening and fertility found in our work are similar to those described when *tert*-octylphenol (alkylphenol with estrogenic activity) was administrated to neonatal female rats and probably mediated through binding to estrogen receptors [37]. Studies on fetal programming demonstrated that the effects of androgen and estrogen on ovarian dysfunction are produced by interaction with estrogen receptor α (ERα) (located in theca cells and ovarian stroma) and ERβ (located in the granulosa cells of growing follicles) [38].

The decrease in the total number of follicles found in the adult rat by neonatal EV administration mainly represents a decrease in primordial follicles (Fig. 3, A and B). Since very few, if any, primordial follicles are seen within the first 24 h of birth, whereas a marked increase occurs within the next 24 h [34], the administration of EV the same day as birth affected the formation of primordial follicles, possibly by negative regulation of follicular assembly and primordial-to-primary follicle transition [39, 40]. Recently, Jefferson et al. [20] found a similar reduction in the percentage of primordial follicles after neonatal administration of genistein (phytoestrogen) to mouse. They found that genistein interacts with ERβ, causing disruption of ovarian differentiation and inhibition of oocyte nest breakdown. All of these results support a direct effect of EV during the first step of follicular development, as found in the current study. The increase in the number of preantral and antral atretic follicles could be the result of changes in NGFB availability found in this study, as we have also found in adult rats treated with a mix of NGFB antibodies in addition to an antisense oligonucleotide to *Ngfr* to block the transcription of the receptor and the effect of NGFB [23]. NGFR could mediate the apoptotic process in cells when the ratio between NGFB and the receptor favors the receptor [41].

Effect of Sympathetic Denervation on the Effect of Neonatal EV Administration on Follicular Development

To determine whether the changes induced by EV administration on the sympathetic nerves are causally related to the cystic ovary phenotype, we denervated a group of EV-treated rats. We chose 21 days of age to denervate rats because at this stage pups are separated from their mothers and it would be easy to take care of the operated rats. Also, we have previously described that denervated ovaries rapidly reinnervate [22]; thus, by denervating the ovaries at 21 days of age we have a time window of 27 days before the ovary is fully innervated. During this window, we can follow vaginal opening and a short time of cycling activity before the ovary reinnervates. The ovaries of EV-treated rats killed at 42 days old presented the morphology of a precystic ovary similar to the one we found in adult rats 30 days after EV administration (i.e., loss of small antral follicles, no cyst but the presence of type III precystic follicles [23]). In addition, the EV-treated, nondenervated rats did not present estrous cycling activity, and they had early age of vaginal opening. All of these changes, with the exception of estrous cycling activity, were reversed after surgical denervation of the ovary. The age of vaginal opening was delayed up to that of control rats not treated with EV. Morphology of the ovary was very different to that of EV-treated rats; there were no type III precystic follicles and no preovulatory follicles. These data strongly support the concept that sympathetic activity is a functional component of the response of the ovary to EV treatment. It was also expected that the animal recovered estrous cycling activity and ovulation, as we have previously found when estradiol valerate was administrated to adult or prepubertal rats [15, 17]. We have recently described that sympathetic nerves develop their functional capacity to release NA after 21 days of age [42]; it could be possible that the administration of EV at the first day of life advanced the age at the ovary developed its nerve activity. It is interesting to note that the morphological aspect of the ovary of denervated rats was almost identical to the one we described after destroying the sympathetic nerves by neonatal treatment with antibodies against NGFB [43]. Probably, the mechanism operating in both procedures is common: denervation of the ovary (either by surgical or immunological methods) delays follicular development and, in the present case, reverses the formation of cysts induced by estradiol-increased sympathetic activity. In the immunosympathectomized rats, we found a decreased production of estradiol by the ovary [43], in agreement with our present results. The significance of the decrease in estradiol levels is unclear at present.

In summary, the fact that neonatal exposure to a single dose of estradiol during a critical period of follicular development led to ovarian reproductive failure in adult animals together with an NGFB-dependent increase in ovarian sympathetic activity strongly suggest that modifications in the sympathetic tone either by stress, exposure to steroids, or adrenergic drugs [22] during development could modify permanently the function of the ovary. Along the same line, environmental pollutants with estrogenic activity that are released into the environment as a consequence of manufacturing processes could interfere with the normal development of female reproductive systems. The temporary window in which they can cause harmful effects may be critical during the prenatal and neonatal stages and, as we have found in this study, single-dose exposures of EV could permanently modify reproductive function.

ACKNOWLEDGMENT

The authors thank Dr. Víctor D. Ramírez for help with the final text. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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