Prepubertal Administration of Estradiol Valerate Disrupts Cyclicity and Leads to Cystic Ovarian Morphology during Adult Life in the Rat: Role of Sympathetic Innervation

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Administration of estradiol valerate (EV) to adult rats leads to anovulation and cystic ovarian morphology. Sympathetic ovarian nerve denervation (SONX) overcomes this disruption. In this study, we determined whether EV administration to juvenile rats prevents achievement of reproductive competence, disrupts cyclicity, and whether this programming is facilitated via activation of the sympathetic nerve input to the ovary. Prepubertal rats were administered 2 mg EV in corn oil or corn oil alone. One half of the animals from each group underwent SONX on d 71 of life. Rats were euthanized on d 91 for determination of serum gonadotropins, progesterone, Δ4 androstenedione, and estradiol concentrations, ovarian norepinephrine (NE), and 3β-hydroxysteroid dehydrogenase (3β-HSD) activities and ovarian dynamics. Results revealed that EV administration during juvenile period advanced pubertal onset, suppressed circulating LH, FSH, and Δ4 androstenedione, increased ovarian NE, estradiol, and 3β-HSD activities, disrupted ovarian dynamics evidenced as absent corpus luteum and presence of ovarian cysts and culminated in anovulation. SONX restored cyclicity in these animals, normalized LH, estradiol, ovarian 3β-HSD activities, and ovarian dynamics as evidenced by the disappearance of ovarian cysts and appearance of corpus luteum and restored corpus luteum function. These findings provide evidence that EV exposure during juvenile life leads to long-lasting deleterious reproductive consequences via activation of the sympathetic ovarian nerve. (*Endocrinology* 144: 4289–4297, 2003)

REPRODUCTION, A CRITICAL physiological function, is regulated by integration of inputs from the hypothalamic-pituitary-ovarian axis. An increasing trend in infertility and reproductive disorders in humans and wild life in concert with the increasing number of industrial waste and agricultural products with estrogenic activity that are being manufactured and released into the environment (1–3) raise serious concerns regarding the impact of these environmental pollutants on reproductive health. Critical hormone sensitive periods during development are likely to be especially vulnerable to such exposures.

Several epidemiological and animal studies have shown that stimuli/insults that occur during critical hormone-sensitive periods can permanently alter the course of reproductive organ differentiation and function (4–6). Previous studies have shown that neonatal exposure of rats to testosterone, via aromatization to estradiol, disrupts ovarian morphology, induces changes in adult reproductive behavior and leads to reproductive failure in the female offspring (7, 8). Similar studies in sheep (sheep is a precocious species in terms of reproductive neuroendocrine and ovarian differentiation; Refs. 9 and 10) and monkeys have found that exposure of female fetuses to testosterone during d 30–90 of gestation leads to similar outcomes (11–16). Such findings suggest that steroid hormones and environmental contaminants with estrogenic activity can inappropriately program developmental events critical for normal reproductive function thus leading to irreversible adverse consequences in the reproductive capacity of the developing animals. Such programming can occur via resetting of hormone axes or altered gene activation. Animal models provide a means to test some of these predictions.

Our studies have shown that administration of estradiol valerate (EV) during adult reproductive life disrupts cyclicity via activation of the sympathetic ovarian nerve and increased ovarian expression of norepinephrine (NE). Sympathetic ovarian nerve denervation (SONX) overcomes this disruption (16, 17). Whether the effects of EV administered during juvenile life to rats (when ovarian differentiation is still not complete), can program severe reproductive consequences and if such consequences are also facilitated via activation of the sympathetic ovarian nerve input is unknown. The present study was undertaken to test the hypothesis that administration of EV to rats during juvenile period would program ovarian and neuroendocrine disruption culminating in reproductive failure during adulthood and that such effects are facilitated via activation of the ovarian sympathetic nerve.

Abbreviations: CL, Corpora lutea; EV, estradiol valerate; 3β-HSD, 3β-hydroxysteroid dehydrogenase; NE, norepinephrine; PCOS, polycystic ovary syndrome; SONX, sympathetic ovarian nerve denervation.

Materials and Methods

Experimental design

Fourteen-day-old female prepubertal Wistar rats, bred and raised in the University of Chile animal research facility, were used for testing the consequences of prepubertal EV administration and post pubertal SONX on cyclic changes in LH, FSH, progesterone, Δ4 androstenedione, and estradiol concentrations as well as ovarian NE concentrations during adult reproductive life. All animal procedures were performed using protocols previously approved by the Institutional Ethic Committee of Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile and Universidade de Sao Paulo (Sao Paulo, Brazil). 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 animals were maintained on a 12-h light, 12-h dark cycle and given food and water *ad libitum*. Forty-three rats were injected with a single im injection of 2 mg of EV in 0.2 ml of corn oil on d 14 of their life. Day 14 was selected for administering EV because it allows sufficient time for the EV-induced increase in the expression of tyrosine hydroxylase (the rate limiting enzyme of norepinephrine biosynthesis) and NE release from the ovary to occur before the animals achieve puberty (~28 d). In previous studies we have shown that it takes 7–15 d for EV induced increases in tyrosine hydroxylase and NE increases to occur (19). In addition, the ovaries also express FSH receptors by d 14 of life enabling them to respond to gonadotropic stimulation (20). Controls (n = 41) received vehicle only (0.2 ml of corn oil). Timing of onset of puberty was determined by monitoring the timing of vaginal opening. Frequency and length of estrous cycles were recorded from the time of vaginal opening to the end of the study (d 91). Estrous cyclicity was assessed by analysis at the light microscopy level of the relative proportion of leukocytes, epithelial and cornified cells found in daily vaginal lavages, which characteristically change during different stages of the estrous cycle (21). After establishment of reproductive competence and establishment of repetitive cycles, 24 of the control and 22 of the EV-treated animals were subjected to surgical SONX at 71 d of age (57 d after EV treatment). Details of SONX have been published earlier (18). Twenty days after SONX, rats were killed by decapitation on d 91. Trunk blood was collected from five control rats in estrus and five in diestrus for determination of LH, FSH, progesterone, and Δ4 androstenedione measurements. Because a majority of the rats treated with EV were acyclic and predominantly in estrous, only ovaries from estrus group (n = 10 rats) were processed for NE determinations. Both ovaries from each rat were removed, immediately frozen in dry ice and stored at −80 C for subsequent determination of ovarian NE concentrations. Due to blood volume limitations, a second set of rats were processed as above (n = 4/group) and trunk blood collected for measurement of circulating estradiol measurements.

Measurement of circulating levels of gonadotropins and gonadal steroids

Serum LH and FSH levels were measured using standard RIAs using kits provided through the NIDDK National Pituitary Agency. Results are expressed in terms of the rat LH RP-1 and rat FSH RP-1 standards preparations. All samples were analyzed in duplicate. The sensitivity, intra-, and interassay variability averaged 40 pg/tube, 1.8%, and 8.7%, respectively, for LH and 90 pg/tube, 5%, and 6%, respectively, for FSH. Serum levels of progesterone were measured by RIA using antibody GD-337 kindly provided by Dr. Gordon Niswender (Colorado State University, Denver, CO), as previously described (22). The sensitivity, intra and interassay coefficient of this assay averaged 25 pg/tube, 7.5% and 8.9%, respectively. Serum Δ4 androstenedione concentrations were measured by Alpco Diagnostic ELISA kits (American Lab Products Co., Windham, NH). The sensitivity, intra and interassay coefficient of the Δ4 androstenedione assay averaged 0.02 ng/ml, 6.7%, and 11.5%, respectively. Serum estradiol levels from a different group of animals treated similarly were measured by RIA after chromatographic separation in Sephadex LH-20 columns as previously described (23). The sensitivity, intra-, and interassay coefficient of the estradiol assay averaged 2.2 pg/ml, 2.7%, and 7.8%, respectively.

Measurement of ovarian NE levels

Both ovaries from each estrus rat were homogenized in 0.2 M perchloric acid. The suspensions were centrifuged at 15,000 × *g* for 10 min and the catecholamines present in the supernatant were determined by the radioenzymatic method of Saller and Zigmond (24), as we have previously described (25). Briefly, catecholamines were methylated enzymatically with a purified extract of catechol-*O*-methyl transferase obtained from rat liver and with [^{3}H-CH$_3$]*S*-adenosyl methionine (Specific activity 72.5 Ci/mmol, NEN Life Science Products Corp., Boston, MA) as a methyl donor. Methylated catecholamines were separated by thin layer chromatography and the radioactivity determined by scintillation counter. The sensitivity (twice blank cpm) was 20 pg for NE. NE concentrations are expressed as pg/mg/protein. Perchloric acid-insoluble pellets were dissolved in 1 M NaOH for determination of protein content by Lowry method (26) and used BSA as the standard.

Assessment of ovarian dynamics

Ovaries were removed from a batch of animals (control, SONX, EV-treated, EV-treated + SONX) maintained in the Animal Research Facilities of Sao Paulo for assessment of ovarian dynamics (one ovary from each animal) and ovarian 3β-HSD activity (second ovary from each animal). The experimental protocol was identical to that described above and these animals showed similar ovulatory responses (data not shown). Five estrous animals from control and EV-treated groups were used in this study. For determination of ovarian dynamics, one ovary from each control or experimental rat was cleaned of adherent fat tissue, immersed in fixative, (85% ethanol, 10% formaldehyde, and 5% acetic acid) embedded in paraffin, serially sectioned at 5 μm, and stained with trichromic acid method of Mallory as described in Ref. 27 and used for histological analysis. Ovarian dynamics was assessed from the largest section of the ovary. The number of corpora lutea (CL), number of healthy antral follicles and the number of follicular cysts as well as the total area occupied by CL, antral follicles and cysts were determined. Cystic follicles were defined according to criteria proposed previously (19) as those follicles devoid of oocytes, displaying a large antral cavity, an enlarged theca cells layer, and a thin (mostly monolayer) granulose cell compartment. Absence of oocytes was confirmed by examining serial sections.

Measurement of 3-β-hydroxysteroid dehydrogenase (3β-HSD) activity

For determination of 3β-HSD activities, the second ovary was frozen at −80 C and cryosectioned at −15 C at 6 μm thickness. The slices were mounted in glass coverslips, air-dried, and stored at −20 C. Ovarian 3β-HSD activity was measured using methods originally described by Levi *et al.* (28) and modified by Iannetta and Mello de Oliveira (29). Two different substrates were used for the reaction, namely dihydroepiandrosterone (DHEA) for the quantification of the isomerization step (Δ5 to Δ4) and epiandrosterone (EPI) for the 3-β-hydroxylation step. The slices were incubated in the presence of each substrate at 37 C for 1 h and analyzed for the appearance of the color characteristically seen by the reduction of the tetranitroblue tetrazolium present in the incubation medium. Capture of the image was performed with a light microscopy connected to a digital camera. Images stored in a computer were analyzed with the Scion Image program for Windows for determination of OD. OD values for blank tissue (without substrate) were subtracted from the experimental sample and expressed as relative activity.

Statistical analyses

Number of cycles from all four treatment groups, changes in hormonal concentrations, ovarian dynamics, enzyme activities between control, SONX, EV, EV + SONX groups were analyzed by ANOVA. To account for heterogeneity, where necessary, data were log transformed before analysis. Data are presented as mean ± SE; *post hoc* analyses were performed by Fisher's protected least significant difference test.

Results

Effect of prepubertal EV administration on onset of puberty and maintenance of estrus cyclicity during adulthood

Figure 1 summarizes percent rats showing vaginal opening as a function of age (*left panel*) and the mean timing of vaginal opening (*right panel*) for the control and EV-treated rats. Administration of EV to prepubertal rats at 14 d of age accelerated the timing of vaginal opening by 4 d as compared with control rats receiving only the vehicle (Fig. 1). Mean vaginal opening occurred at 32.6 d in controls and 28.7 d in prepubertal EV-treated animals. Administration of EV during the prepubertal period, while advancing puberty, had profound disruptive effects on the maintenance of estrous cyclicity. Cyclic activities of control and EV-treated animals between 50 and 91 d of age are shown in Fig. 2 (control, *top left*; EV-treated, *bottom left*). Control rats continued to maintain repetitive cycles throughout the study period. In contrast, EV-treated rats showed severe cyclic disruption with majority remaining in persistent estrus for prolonged periods. Overall, control rats had 5 cycles (proestrus preceding estrus) in the last 21 d consistent with recurrent 4-d cycles (Fig 2, *right panel, open bar*). Frequency of cycles was reduced by 72% in prepubertal EV-treated rats to 1.4 cycles during the last 21 d of study (Fig. 2, *right panel, closed bar*).

Effect of SONX on cyclicity

Most of the control rats that underwent SONX continued to cycle normally from beginning (Fig. 2, *top middle*). A small percentage showed irregularity during the first 4–5 d following SONX but resumed normal cyclicity soon after. Overall, 70% of the rats were cycling normally within a week after SONX (Fig. 2, *top middle*). The other 30% continued to show irregular cyclic activity, although they showed signs of recovery as evidenced by an increase in the number of incidences where a proestrus day preceded estrus. SONX restored cyclicity in prepubertal EV-treated animals (Fig. 2, *bottom middle*) and averaged three cycles during the last 21 d (Fig. 2, *right panel*).

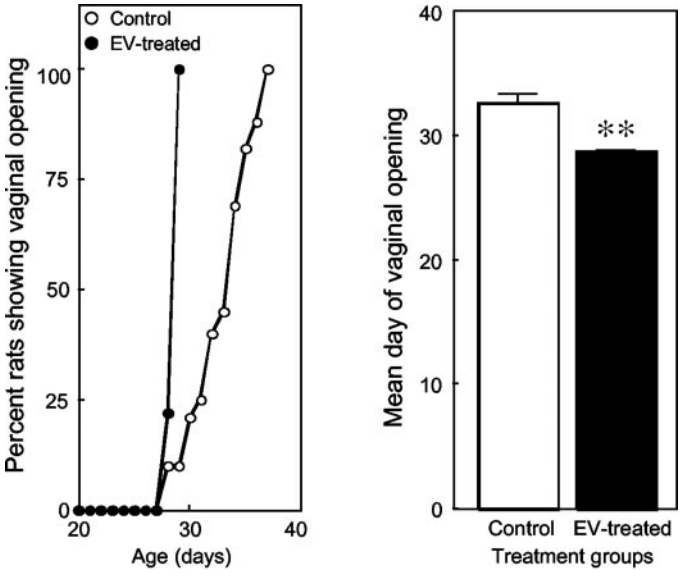

FIG. 1. Effect of prepubertal administration of EV on the timing of onset of puberty (day of vaginal opening). Shown on the *left* are percentages of animals showing vaginal opening as a function of age in the control and EV-treated animals. Shown on the *right* are mean day of vaginal opening in control (n = 41) and prepubertal EV-treated (n = 43) animals. *, Significant difference from controls ($P < 0.05$).

Effect of prepubertal EV treatment and postpubertal SONX on ovarian NE concentrations

As was reported earlier for EV treatment during adult life (17), prepubertal administration of EV resulted in a similar increase in ovarian NE concentration during adult life (measured 78 d after administration of EV) (Fig. 3). Consistent with contribution of superior ovarian nerve innervation to ovarian NE concentrations, SONX produced a parallel 40% percent decrease in NE concentration both in control and EV-treated rats.

Effect of prepubertal EV treatment and post pubertal SONX on circulating gonadotropin concentrations

As expected, circulating LH concentrations were similar between estrus and diestrus (Fig. 4, *open bars, top left* and *right panels*). Prepubertal EV administration resulted in a 60% decrease in serum LH concentrations. SONX, although having no effect on control estrus and diestrus animals, completely overcame the suppressive effects of EV on serum LH levels.

Concentrations of FSH were similar between diestrus and estrus period (Fig. 4, *open bars, middle panels*). SONX had no effect on circulating FSH during estrus but suppressed FSH during diestrus. Prepubertal EV treatment suppressed circulating FSH levels. The suppressive effects of prepubertal EV appeared to be more on LH than FSH as reflected by a decrease in LH/FSH ratio in the EV-treated animals (Fig. 4, *closed bar bottom left panel*). In contrast to the efficacy of SONX in overcoming the suppressive effects of prepubertal EV on circulating LH, SONX was not effective in overcoming the suppressive effects of prepubertal EV on circulating FSH. This resulted in an increase in LH/FSH ratio in the EV-SONX animals.

Effect of prepubertal EV treatment and postpubertal SONX on circulating progesterone, Δ4 androstenedione, estradiol, and 3β-HSD activity during adulthood. The effects of prepubertal EV administration and SONX on circulating progesterone and Δ4 androstenedione levels are shown in Fig. 5. Control rats showed the characteristic ovulatory increase in progesterone levels during the diestrus period as compared with the estrus period (Fig. 5, *top open bars*). SONX suppressed serum progesterone levels both during the estrus and diestrus periods in control animals, although levels of progesterone were higher during diestrus compared with estrus. Prepubertal-estradiol administration prevented the ovulatory increase in progesterone during diestrus to levels seen during estrus in control animals. Consistence with the cycle data presented earlier (Fig. 2), SONX overcame the inhibitory effects of prepubertal EV treatment on progesterone resulting in restoration of progesterone levels to that of the diestrus controls.

Circulating Δ4 androstenedione levels were inversely proportional to circulating progesterone levels and were lower

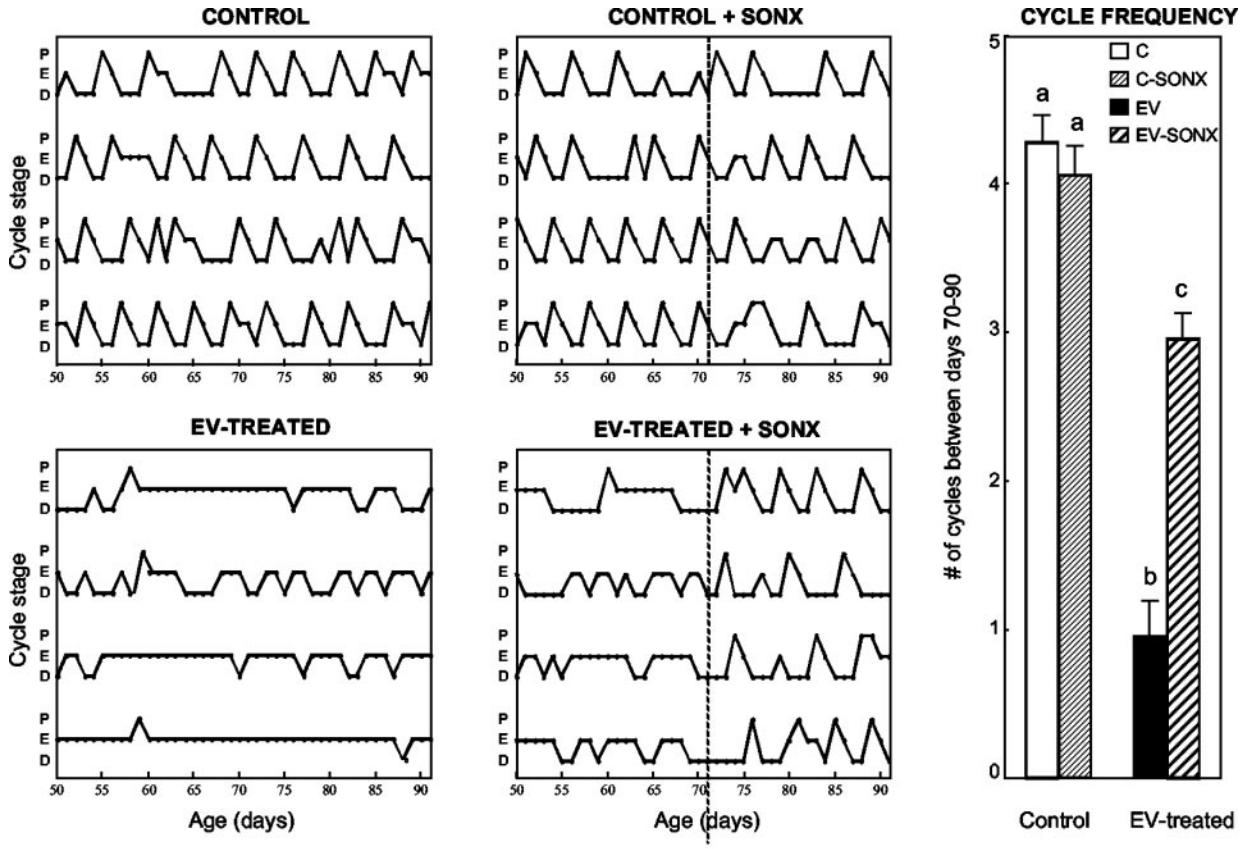

FIG. 2. Effect of prepubertal administration of EV and postpubertal SONX on the maintenance of estrous cyclicity and cycle frequency. The *two panels on the left* show representative estrous cycle patterns between d 50 and 91 of age of control (n = 24) and prepubertal EV-treated (n = 21) animals that did not undergo SONX. The *two middle panels* show representative patterns during the same time period of control (n = 17) and prepubertal EV-treated (n = 22) animals that underwent SONX on d 71. The panel on the *right* shows number of cycles between d 71 and 91 of control, control + SONX, EV-treated and EV-treated + SONX. D, Diestrus; E, estrus; P, proestrus. Significant differences ($P < 0.05$) between treatment groups are represented by *differing letters*.

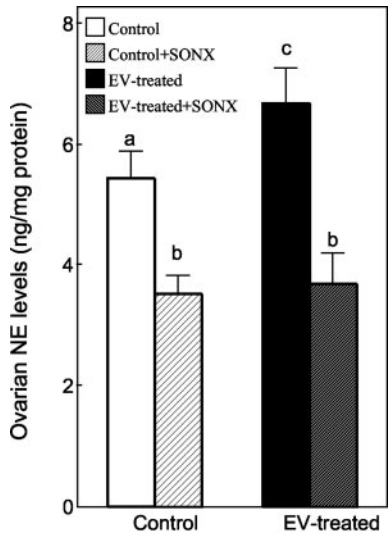

FIG. 3. Ovarian NE levels in control, control+ SONX, EV-treated and EV-treated + SONX animals. Significant differences ($P < 0.05$) between treatment groups are represented by *differing letters*.

during the diestrus as compared with the estrus controls. The effects of SONX on Δ4 androstenedione levels in control rats paralleled that of progesterone with SONX reducing circulating levels of Δ4 androstenedione both during the estrus and diestrus periods. Prepubertal EV suppressed circulating Δ4 androstenedione levels. Patterns of Δ4 androstenedione levels during estrus and diestrus were similar following SONX in the prepubertal EV-treated animals with levels of Δ4 androstenedione being lower in the diestrus than the estrus animals.

Circulating estradiol levels, measured using a different set of animals (n = 4/group), tended to be higher during diestrus than estrus in control animals (control diestrus: 21.5 ± 3.4 pg/ml, control estrus: 14.2 ± 1.0 pg/ml, $P > 0.05$, n = 4). Levels of estradiol in the EV animals, which were in persistent estrus were significantly higher compared with controls ($P < 0.05$) and averaged 43.5 ± 4.5 pg/ml (n = 4). SONX reduced levels of estradiol in EV-treated animals to control levels (25.5 ± 4.2 pg/ml).

The effects of prepubertal EV administration and SONX on 3β-HSD involved in the steroidogenic pathway are summarized in Fig. 6. The highest level of 3β-HSD activity was found in the stroma as compared with the follicular wall (theca and granulosa cell included) and corpus luteum. Prepubertal EV treatment had no effect on 3β-HSD activities in the stroma. However, SONX resulted in a decrease in 3β-HSD activities in the stroma of both control and prepubertal EV-treated animals.

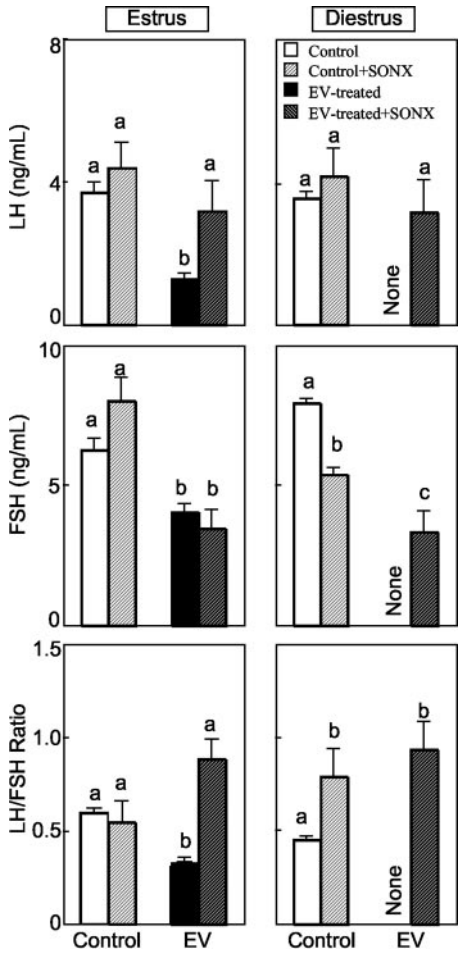

FIG. 4. Effect of prepubertal administration of EV on circulating gonadotropins and LH and FSH ratio during estrus (n = 5) and diestrus (n = 5) in control, control + SONX, EV-treated + SONX animals. Note the EV-treated animals that did not undergo SONX did not cycle and were only studied during estrus (n = 10). Significant differences ($P < 0.05$) between treatment groups are represented by *differing letters*.

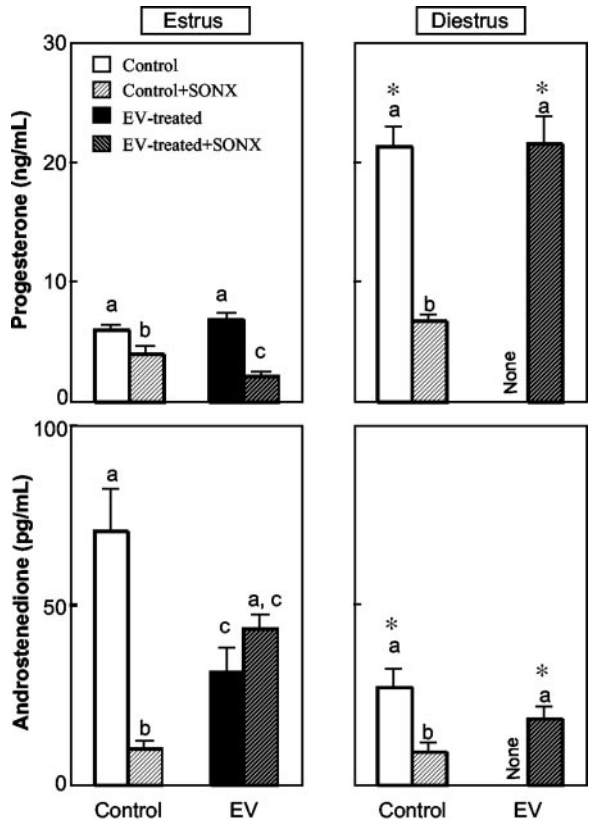

FIG. 5. Effect of prepubertal administration of EV on circulating progesterone and Δ4 androstenedione concentrations during estrus (n = 5) and diestrus (n = 5) in control, control + SONX, EV-treated + SONX animals. Note the EV-treated animals that did not undergo SONX did not cycle and were only studied during estrus (n = 10). Significant differences ($P < 0.05$) between treatment groups within estrous cycle stage are represented by *differing letters*. *, Cycle stage differences ($P < 0.05$) within a given treatment group.

The effects of prepubertal EV and SONX on 3β-HSD activities in the follicular wall were diametrically opposite to that in the stroma. During prepubertal EV-induced acyclic condition there is an increase in the enzymatic activity of 3β-HSD in the follicular wall. This increase was reversed to control levels by SONX. The effects of prepubertal EV treatment and SONX in corpus luteum was similar to that observed in stroma with EV having no effect and SONX suppressing 3β-HSD activity in both control and prepubertal EV-treated animals (Fig. 6). The suppressive effects of SONX on 3β-HSD activities in corpus luteum were more pronounced in control than prepubertal EV-treated animals.

Effect of prepubertal EV treatment and postpubertal SONX on ovarian dynamics

Figure 7 summarizes the effects of prepubertal EV administration and postpubertal SONX on the ovary. Consistent with their undergoing repetitive cycles, ovaries of control animals had several CL and follicles in various stages of development (Fig. 7A). SONX did not affect the ability of the ovary to ovulate in control rats, as evidenced by the presence of similar number of CL and follicles in various stages of development (Fig. 7C). In contrast, consistent with the acyclic condition of the prepubertal EV-treated animals, the ovaries of EV-treated rats displayed severely atretic large antral follicles, follicular cysts (well developed theca cell layer, diminished granulose cell compartment and absence of oocytes), and lack of corpus lutea (Fig. 7B). The morphology of ovaries from prepubertal EV-treated animals undergoing SONX was also consistent with the resumption of cycles in these animals (Fig. 7D). These ovaries were markedly different from the prepubertal EV-treated animals undergoing SONX but strikingly similar to that of the control rats. Numerous CL were readily apparent and there was a marked attenuation of the cystic condition (Fig. 7D), indicating that the resumption of estrous cyclicity caused by SONX was accompanied by ovulation and formation of functionally competent CL.

Analyses of ovarian dynamics shown in Table 1 further exemplify the visual morphologic attributes seen in Fig. 7. Consistent with the retention of cyclicity in control rats that underwent SONX the total number of CL, size of CL, area occupied by CL were similar between control cycling rats and control rats that underwent SONX. Prepubertal EV treatment differed markedly from controls in that there was a 93%

diminution in the number of CL (one of the treated animals showed very small CL), size of CL (87%), and the ovarian area occupied by CL (93%). The absence of CL appears to have contributed to the reduced ovarian size (reflected as reduced diameter of the largest ovarian section) in the prepubertal EV-treated animals. The restoration of cycles in prepubertal EV-treated rats that underwent SONX was substantiated by the fact that the number and size of CL and the ovarian area occupied by the CL mimicked that of the controls although the capacity of CL to produce progesterone appears to be compromised in SONX animals. Previous reports using the same experimental paradigm have demonstrated that ovarian sympathetic innervation (via SON) is important to maintain both progesterone and androgens plasma levels in rats (30). The reduced progesterone levels in SONX animals may be function of removal of sympathetic nerve input.

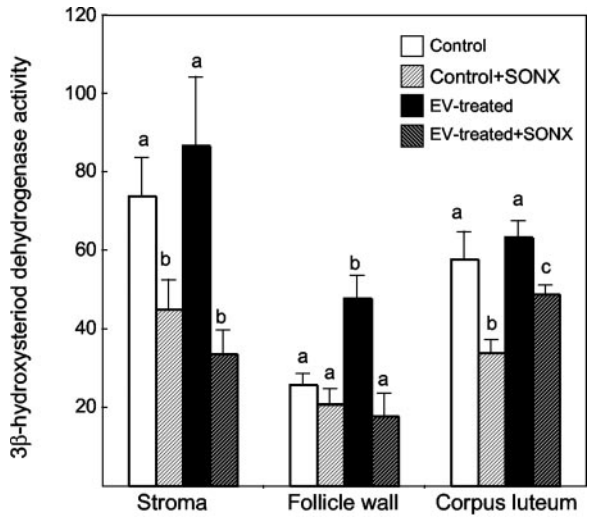

FIG. 6. Effect of prepubertal administration of EV on ovarian 3β HSD activity in control (n = 5), control+ SONX (n = 5), EV-treated (n = 5) and EV-treated + SONX animals. Because EV-treated animals that did not undergo SONX failed to cycle, to compare treatment effects 3β-HSD activity was determined only during estrus. For determination of 3β-HSD, one ovary from each animal was used. 3β-HSD levels represent the difference in optical density between experimental sample (with substrate) and blank tissue (without sustrate) expressed as arbitrary units. Significant differences ($P < 0.05$) between treatment groups are represented by *differing letters*.

There were no differences in the number and size of antral follicles or ovarian volume occupied by antral follicles between control, control + SONX, prepubertal EV-treated +SONX groups. The number of healthy ovarian follicles observed in the prepubertal EV-treated animals were reduced compared with that of controls but similar in size. There was no evidence of ovarian cysts in the controls or controls that underwent SONX. Prepubertal EV-treated rats had on the average 4.2 cysts/section occupying about 18% of ovarian volume. Interestingly, the size of the cysts was similar to that of the antral follicles although they differed from the antral follicles in not having an oocytes and a prominent granulosa cell area. SONX resulted in an 81% reduction in the number of cysts, 90% reduction in the ovarian area occupied by cysts and a 50% reduction in the size of the cyst when present.

Discussion

Our findings suggest that a single injection of EV during juvenile life, programs early pubertal onset, cessation of cyclicity in adult, neuroendocrine and ovarian deficits and development of cystic ovarian morphology. The altered programming appears to be facilitated via activation of the sympathetic ovarian nerve. Neuroendocrine and ovarian disruptions caused by EV treatment and the implications of these findings to potential threats posed by endocrine disruptors are discussed below.

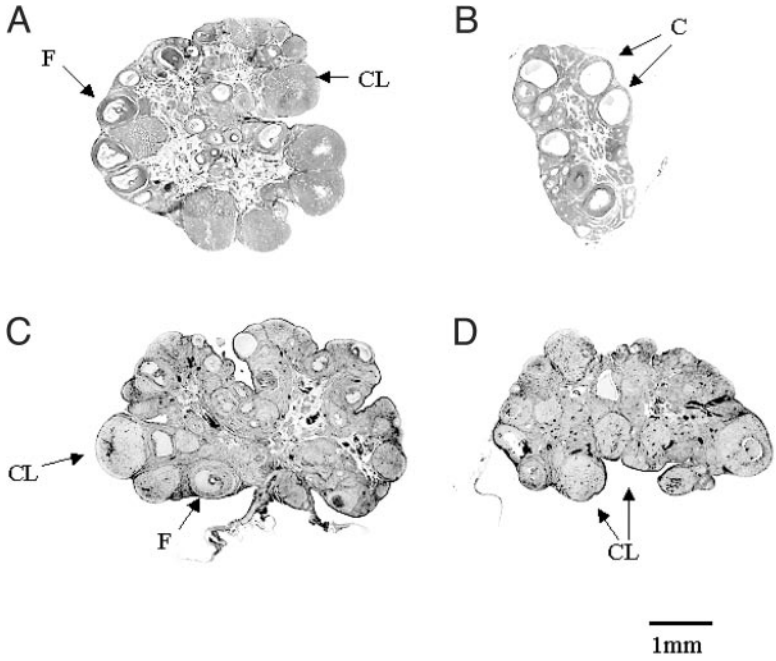

FIG. 7. Effect of prepubertal administration of EV and SONX on ovarian histology. A, Control ovary; B, EV-treated; C, control after SONX; and D, EV-treated after SONX. Micrograph correspond to the largest section of the ovary. Note the ovary from the EV-treated animals lack corpus luteum (CL), possess several cysts (C) as opposed to the EV-treated animal that underwent SONX, which is very similar to controls (presence of corpus luteum, follicles (F) and absence of cysts). Sections are 8 μm thick and were stained by the method of Mallory (27).

TABLE 1. The effect of estradiol valerate administration and superior ovarian nerve denervation on follicular development

	Control		Estradiol valerate	
	Before SONX	After SONX	Before SONX	After SONX
Number of rats analyzed	5	5	5	5
Largest diameter ($\mu m^2 \times 10^6$)	12.26 ± 1.40	11.71 ± 1.43	6.10 ± 0.98[a]	11.27 ± 1.08
CL				
Total no. of CL in largest section	8.60 ± 1.02	9.20 ± 1.32	0.60 ± 0.60[a]	8.40 ± 0.98
Total CL area ($\mu m^2 \times 10^6$)	3.39 ± 0.46	3.79 ± 0.74	0.17 ± 0.17[a]	3.29 ± 0.55
% CL area	27.40 ± 1.23	31.54 ± 2.99	2.71 ± 2.71[a]	31.22 ± 7.54
Size of CL ($\mu m^2 \times 10^6$)	0.39 ± 0.03	0.41 ± 0.04	0.05 ± 0.05[a]	0.38 ± 0.02
Antral follicles				
Total no. of antral follicles in largest section	9.2 ± 1.39	6.60 ± 0.98	4.00 ± 0.44[a]	6.60 ± 1.44
Total antral follicular area ($\mu m^2 \times 10^6$)	1.47 ± 0.39	1.37 ± 0.18	0.95 ± 0.25	1.14 ± 0.28
% antral follicular area	11.64 ± 2.90	12.50 ± 2.00	17.59 ± 5.06	9.90 ± 2.20
Size of antral follicles ($\mu m^2 \times 10^6$)	0.15 ± 0.02	0.21 ± 0.01	0.23 ± 0.05	0.17 ± 0.02
Follicular cysts				
Total no. of cysts in largest section	0	0	4.20 ± 0.37[a]	0.80 ± 0.20
Total cyst area ($\mu m^2 \times 10^6$)	0	0	1.06 ± 0.14[a]	0.63 ± 0.03
% Cyst area	0	0	17.67 ± 0.87[a]	1.12 ± 0.34
Size of cysts ($\mu m^2 \times 10^6$)			0.25 ± 0.03[a]	0.13 ± 0.03
% Estimated stromal area	60.96	55.96	62.03	57.76

Results are expressed as mean ± SE of the number of rats shown in the table. [a] $P < 0.05$.

Impact on timing of pubertal onset and maintenance of reproductive function. These studies provide evidence that juvenile administration of EV at 14 d of age [after appearance of FSH receptors in the ovary (31)] accelerates the timing of vaginal opening from 32 d in controls to 29 d in EV-treated rats. Because the rupture of vaginal membrane is sensitive to the exposed levels of estradiol levels, vaginal opening has been widely used as an index for determining the onset of puberty and achievement of reproductive competence in rats (31). These results do show that EV-treated rats are more synchronized in terms of the timing of the vaginal opening than the controls. Vaginal opening in EV-treated group occurred within a 3-d period as opposed to the 7-d period of the control group. Although the onset of puberty appears to be advanced in the prepubertal EV-treated animals, these animals failed to show repetitive cycles even 2.5 months after EV treatment. Considering that the half-life of EV is 15 d (32), the disruptive effects on reproduction seen later in life appears not to be due to the continued presence of EV in circulation. It is more likely to have originated from permanent alteration of the neuroendocrine or ovarian axis. Previous studies have shown that exposure of rats to testosterone during the neonatal period leads to reproductive and behavioral deficits (7).

Possible mechanisms programming reproductive deficits during adulthood

The reproductive failure and decreased LH activity in the prepubertal EV-treated group are likely to be facilitated via decreased hypothalamic GnRH input, reduced pituitary sensitivity to GnRH, increased ovarian estradiol production or increased pituitary sensitivity to estradiol feedback. From an ovarian perspective, in view of the fact that the activity of the enzyme using DHEA as substrate represent the capacity of the enzyme to produce androgens and the use of EPI as substrate represent the capacity to use the β-hydroxylation pathway of C_3 steroids (33), the increased isomerization activity following juvenile EV treatment suggests that the synthesis of progesterone and/or Δ4 androstenedione should be enhanced in the ovary of prenatal EV-treated rats. Paradoxically, as opposed to what the enzyme activity and the cystic appearance of the ovary predicted, but in keeping with the reduced levels of LH, circulating levels of Δ4 androstenedione and progesterone were reduced in EV-treated rats as compared with controls. Similarly, SONX of EV-treated rats, while suppressing 3β-HSD activity, had not effect on circulating Δ4 androstenedione levels. The opposing effects of prepubertal EV treatment on 3β-HSD and Δ4 androstenedione levels suggest that androgens may be rapidly converted to estradiol at the ovarian site as quickly as they are made or get peripherally converted to estrogen as quickly as they are released. The increased levels of estradiol found in EV-treated rats and the persistent estrous condition of the EV-treated rats support this premise.

Considering that the ovary communicates with the hypothalamus not only by efferent neurons but also by afferents sensory neurons that are in close proximity to the neuroendocrine hypothalamus at the paraventricular nucleus (34), a second possibility to consider is that the SONX may lead to disruption of the afferent network that may be involved in the ovarian feedback of GnRH/LH secretion.

An intriguing finding also relates to the differential effects of SONX in reversing the suppressive effects of juvenile EV treatment on LH and FSH secretion. SONX overcame the suppressive effects of EV treatment on LH, but not FSH culminating in an increase in LH/FSH ratio. The selective increase in LH release after SONX may reflect an increase in GnRH pulse generator activity stemming from recovery from persistent estrus and accompanying decrease in estradiol negative feedback. Previous studies have shown that an increase in GnRH pulse fre-

quency facilitates LH secretion preferentially over FSH (35–37).

Role of sympathetic ovarian nerve innervation in facilitating the programming action of prepubertal EV exposure

The recovery of the estrous cycling activity, rate of ovulation, and the appearance of newly formed CL that were similar in number and size to the control rats and the observed decrease in NE after 21 d of SONX suggest that the cyclic and ovarian disruption induced by EV administration during the juvenile period may be a function of a sustained increase in the sympathetic tone of the ovary as we have previously described for the adult rat (17). Interestingly, earlier studies of Farookhi *et al.* (38), found that hemiovariectomy of the rat reversed the cystic ovarian morphology that developed after EV administration to Wistar rats. Because the changes in gonadotropin, progesterone, and Δ4 androstenedione levels that followed hemiovariectomy in the EV-treated rats of Farookhi's study were similar to the ones reported in this study following SONX the decrease in the total output of steroids that follow hemiovariectomy in Farookhi's study may also be related to superior ovarian nerve input. However, earlier studies of Gerendai *et al.* (39), reported a compensatory response in the contralateral ovary to compensate for the decrease in the neural activity stemming from removal of one ovary. It is also important to point out that because superior ovarian nerve is a mixed nerve that also contains other neurotransmitters (especially VIP) (40) we cannot rule out involvement of other neurotransmitters, in addition to NE, in maintaining the polycystic condition. In this regard, VIP has been described as a neuropeptide capable of stimulating estradiol secretion from the ovary (40). More studies are required to clarify the participation of the neuropeptide as an etiologic factor in the observed cystic ovaries.

The impact of juvenile EV administration in facilitating ovarian NE levels was similar to what was evidenced following EV administration to adult rats (17). However, the magnitude of NE increase seen at 91 d of age following prepubertal EV administration (this study) was low compared with the magnitude of NE increase that followed EV administration to adult rats in our previous study. A possibility to consider is that sympathetic nerves of Wistar and Sprague Dawley rats exhibit differential sensitivities to estradiol. Using a similar approach for NE determination, we have determined that ovarian concentrations of NE are lower in the Wistar rats (this study) compared with the ovary of Sprague Dawley rats used in previous study (17). Such species differences are also evident at other levels. For example, Wistar and Sprague Dawley rats differ in the intraovarian organization of tyrosine hydroxylase-positive nerve fibers (41). An alternate possibility for the lower magnitude of NE increase seen in this study is that the stimulatory effects of juvenile EV administration on ovarian NE may be transient and decline with time due to reinnervation. Our recent findings show that ovarian NE concentrations 90 d after adult EV administration are also lower than that seen 60 d after EV administration (Venegas, M., and H. E. Lara, unpublished observation).

Furthermore, the disappearance of differences in NE concentrations between control and juvenile EV-treated rats after SONX suggests that the ovarian NE increase in the EV-treated animals originate from an increase in concentration of NE in the nerve fibers controlling the steroidogenic cells of the ovary (42). It should be noted that the time point for determining the effect of SONX (21 d) on endocrine and ovarian parameters was selected 1) to allow turn over of a complete follicular cycle in rats which normally takes 20 d to complete (43) and 2) because it represents a period when reinnervation has not occurred as determined by the low NE activity in the ovary (44). We have recently found that it takes more than 3 wk for the ovary to undergo reinnervation and restore NE concentration (44).

Implications to polycystic ovary syndrome (PCOS) in human

The findings of development of cystic ovaries could have potential implications for mechanisms underlying human infertility conditions, such as PCOS. For instance, prepubertal EV-treated rats exhibit early onset of puberty, multiple ovarian cysts and anovulation. Anovulation, multiple cysts, and hyperandrogenism are features of women with PCOS. Oligo-ovulatory adolescents who are likely to develop hyperandrogenism also manifest premature maturation of the GnRH-gonadotropin axis (45) like the EV-treated rats. On the other hand, EV-treated rats differed from women with PCOS in having low circulating levels of LH and androstenedione and oocyte-deficient cystic follicles. Whether the prepubertal EV-treated rats are insulin resistant like the majority of women with PCOS remains to be determined. The effectiveness of ovarian wedge resection (46) or laparoscopic laser cauterization (47) to increase ovulatory response in women with PCOS raise the possibility that increased superior ovarian nerve input may play a role in the development of polycystic ovarian condition observed in women with PCOS.

Implications to environmental disruptor exposure

In addition to their relevance in understanding the etiology of PCOS, our findings also bear upon our understanding of the consequences of prenatal exposure to endocrine disruptors. Existence of critical periods during development and sensitivity of the developing fetus to changes in steroid levels have raised concern about the long-lasting reproductive consequences of exposure to substances of natural and man-made origin which include phyto- and xenoestrogens (48, 49). The devastating consequences of exposure *in utero* to diethylstilbestrol (50) also bear testimonial to such concerns being a reality. More studies are required using low levels of EV and other environmentally relevant estrogen mimics to understand the impact of these findings to human health.

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