Effects of Cyclosporine on Ovarian Function in Sham-Operated and Pituitary-Grafted Young Female Rats (43868)

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Abstract. **Effects of cyclosporine (CyA) on ovarian function and the possible role of prolactin in mediating these effects were examined in young female rats. The animals were sham-operated or rendered hyperprolactinemic by transplanting pituitary glands under the renal capsule. Cyclosporine prevented the increase in plasma prolactin levels in grafted rats. However, in sham-operated animals plasma prolactin levels were increased after 8 days of CyA treatment. Plasma levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were reduced 8 days after pituitary grafting and increased by CyA at both Day 2 and Day 8 of treatment. The content of LHRH in the hypothalamus was not affected on Day 2 but was reduced on Day 8 after grafting on CyA therapy. Plasma estradiol levels were increased by CyA in sham-operated rats on Day 2 and 8 of treatment, and in pituitary-grafted rats on Day 8 of therapy. In sham-operated rats, ovarian estradiol content was reduced after 2 and after 8 days of CyA administration. In pituitary-grafted rats, the ovarian estradiol content was suppressed after 8 days, and CyA treatment prevented this effect. Ovarian estradiol release *in vitro* under basal conditions was greater in ovaries derived from 38-day-old than in those from 32-day-old animals. The ovarian estradiol response to human chorionic gonadotropin (hCG) *in vitro* was increased 2 days after pituitary transplantation. Administration of CyA for 8 days increased basal and hCG-stimulated estradiol release in both sham-operated and pituitary-grafted animals. The present findings suggest that CyA can alter ovarian function by acting directly at the gonadal level. However, a hypothalamic-hypophyseal site of action cannot be ruled out.** [P.S.E.B.M. 1995, Vol 208]

It is well known that the transplantation of an anterior pituitary to an ectopic site disrupts the usual hypothalamic control of its function, leading to a persistent stimulation of prolactin (PRL) secretion and a decrease of the release of the other adenohypophyseal hormones (1). Everett's early findings of this phenomenon have been amply confirmed in numerous subsequent studies (2). It is also well known that hyperprolactinemia has been associated with hypogonadism in almost all species studied (3), and the age of induction of hyperprolactinemia is an important factor in determining its effects on gonadal function (4). In fact, hyperprolactinemia at 30 days of age led to irreversible effects on gonadal function, which were not reversed by the normalization of circulating levels of PRL by bromocriptine treatment or surgical removal of the ectopic gland (5). However, the hypogonadism associated with hyperprolactinemia induction in adult age was reversed by normalization of plasma PRL level (6).

Cyclosporine (CyA) is a peptide of fungal origin widely used in the treatment of patients submitted to organ transplantation (7). It was recently demonstrated that CyA treatment disrupts the endocrine system. In this regard, alteration of gonadal function in male rats (8, 9) and in humans (10) was reported. Interaction between CyA and PRL on the same lympho-

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cyte receptor has been reported as a mechanism of action of CyA (11).

We have observed that disrupting the immune system by administering CyA to adult pituitary-grafted male rats prevented the increase in plasma PRL levels (12), and we subsequently observed the same effect also in young female rats (13). These findings suggest that CyA treatment may alter PRL's regulating effects on gonadal function.

This work was designed to study if CyA's effects on ovarian function are mediated by changes in PRL secretion. For that purpose, hyperprolactinemia was induced at 30 days of age, by the transplantation of a male litter-mate pituitary gland under the kidney capsule, and the animals were exposed to CyA treatment for 2 or 8 days, beginning the day of the surgery. Changes in plasma PRL, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol, the hypothalamic content of LHRH, and ovarian estradiol content were studied, as were the *in vitro* basal and human chorionic gonadotropin (hCG)-stimulated release of estradiol from the ovaries.

Materials and Methods

Animals. Female Wistar rats bred in our department were used in this study. Animals were maintained in a room with a controlled photoperiod (12:12-hr light:dark cycle, lights on at 0800 hr) and temperature (23 ± 2°C), and had constant access to food and water.

Hyperprolactinemia Induction. Half of the animals received a transplant of an anterior pituitary gland from a male litter-mate donor, under the right kidney capsule, at 30 days of age (14). Age-matched female rats were sham-operated and used as controls. Surgery was performed under tribromoethanol anaesthesia (Merck, Germany, 250 mg/kg, ip). Surgery and treatments were performed in the winter.

Cyclosporine Administration. Cyclosporine was generously provided by Sandoz (Basel, Switzerland) and was prepared as follows: 10 mg of the drug were dissolved in 0.5 ml of absolute ethanol and further diluted with olive oil to a final concentration of 1 mg/ml. Both grafted and sham-operated rats were submitted at 1300 hr to a CyA (5 mg/kg body wt/day, sc) or vehicle treatment for 2 or 8 days, beginning on Day 30 of life (day of pituitary grafting or sham operation). Animals were sacrificed by decapitation on days 32 or 38 of age, 20 hr after receiving the last injection of CyA or vehicle. The animals were observed daily to assess the day of vaginal opening. On Day 2 of treatment, all rats had closed vaginas, while on Day 8 of treatment all animals exhibited diestrous smears.

Blood, Hypothalami, and Ovaries. After decapitation, trunk blood was collected in tubes containing EDTA (60 g/liter), and the plasma was kept frozen until analyzed for PRL, LH, FSH, and estradiol levels. The hypothalami were dissected out quickly by cutting the brain from the ventral surface transversely, rostrally at the optic chiasm, caudally at the mamilliary bodies, and laterally at the hypothalamic sulci, to the depth of the anterior commissure. This dissection included the median eminence and the arcuate nuclei, which are the primary areas involved in PRL regulation. The hypothalami were sonicated in cold 2 *N* acetic acid; boiled for 5 min; centrifuged at 15000*g* at 4°C for 30 min and the supernants were kept frozen until analyzed. Ovaries from each animal from the different groups were immediately removed, dissected, and weighed. One of them was placed in tubes containing 1 ml of Krebs Ringer bicarbonate solution. The ovaries were incubated shaking for half an hour under 95% O_2 and 5% of CO_2 atmosphere, and the medium was discarded. An additional milliliter of fresh medium was added and incubated for another hour. The medium was removed and kept frozen to measure basal estradiol release. Another milliliter of fresh medium containing 20 IU of hCG, according with previous studies (15) was added to the ovaries, and the incubation was continued for an additional hour. The medium was removed and kept frozen to measure the hCG-stimulated release of estradiol. The other ovary from each animal was homogenized in 1 ml of Krebs Ringer bicarbonate solution and used to measure estradiol content.

Hormone Measurements. Plasma PRL, LH, and FSH concentrations were measured by homologous double-antibody radioimmunoassays, using materials kindly supplied by the National Hormone and Pituitary Program (National Institutes of Health, Bethesda, MD). The intra-assay coefficient of variation was 7% and the sensitivity was 0.045 μg/liter, using the r-PRL-RP3 preparation as a reference. The intra-assay coefficient of variation was 9%, and the sensitivity was 5 μg/liter, using the r-LH-RP3 preparation as a reference. The intra-assay coefficient of variation was 10%, and the sensitivity was 3.7 μg/liter, using the r-FSH-RP3 preparation as a reference.

Plasma, culture media, and ovarian content of estradiol (1,3,5[10]-estrariene-3, 17β-diol) levels were determined by a specific radioimmunoassay (RIA) with materials purchased from Sorin Biomedica (Vercelli, Italy) and previously validated in our laboratory. The intra-assay coefficient of variation was 10% and the sensitivity was 25 μg/liter.

Hypothalamic luteinizing hormone-releasing hormone (LHRH) content was also measured by a specific RIA developed in our laboratory (16). The intra-assay coefficient of variation was 0.4%, and the sensitivity of the assay was 0.25 μg/liter. All samples in each hormone determination were measured in the same assay to avoid interassay variations.

Statistics. Analysis of the data was performed by

a factorial ANOVA followed by a Scheffe test (Statview 512 (statistics program), Apple System Corp.). Mean values were considered significantly different if $P < 0.05$.

Results

Plasma PRL levels significantly increased between 32 and 38 days of life ($P < 0.05$). As expected, pituitary-grafted rats at 30 days of life had markedly increased circulating PRL levels both 2 and 8 days after the surgery, compared with sham-operated rats ($P < 0.05$). Administering cyclosporine prevented the increase in plasma PRL found 2 and 8 days after grafting in vehicle-treated animals. Cyclosporine did not significantly decrease plasma PRL levels in sham-operated rats on Day 2 and increased them on Day 8 of treatment, compared with vehicle-treated sham-operated rats ($P < 0.05$, Table I).

In sham-operated rats the expected increase ($P < 0.05$) in plasma LH levels with age was observed (Fig. 1). Plasma LH levels were decreased 8 days after pituitary grafting ($P < 0.05$) compared with sham-operating. Treatment with CyA did not change LH levels in rats bearing pituitary grafts at both times studied, compared with pituitary-grafted rats treated with vehicle. The treatment with CyA in sham-operated rats significantly decreased ($P < 0.05$ vs vehicle-treated animals) plasma LH levels at both times examined.

Plasma FSH levels significantly increased with age (38 vs 32 days, $P < 0.05$). Pituitary grafting significantly decreased plasma FSH levels on Day 8 after surgery ($P < 0.05$) compared with sham-operating. Cyclosporine therapy did not modify further plasma FSH levels in pituitary-grafted rats on Day 8 after grafting but reduced them 2 days after the transplant operation

Table I. Plasma Prolactin Levels in Sham-Operated and Pituitary-Grafted Female Rats Treated with CyA (5 mg/kg body wt/day) or Vehicle for 2 or 8 Days Beginning on the Day of Surgery

	Day 2 (μg/liter)	Day 8 (μg/liter)
Sham-operated, vehicle-treated	2.76 ± 0.62	9.4 ± 2.24[a]
Sham-operated CyA-treated	1.6 ± 0.47	15.5 ± 1.77[b]
Pituitary-grafted, vehicle-treated	15.83 ± 2.51[b]	21.25 ± 2.87[b]
Pituitary-grafted, CyA-treated	5.7 ± 1.29[c]	10.30 ± 0.78[c]

Note. Values are given as mean ± SEM (n = 10 animals per group).
[a] $P < 0.05$ vs sham-operated, vehicle-treated rats (Day 2).
[b] $P < 0.05$ vs sham-operated, vehicle-treated rats.
[c] $P < 0.05$ vs pituitary-grafted, vehicle-treated rats.

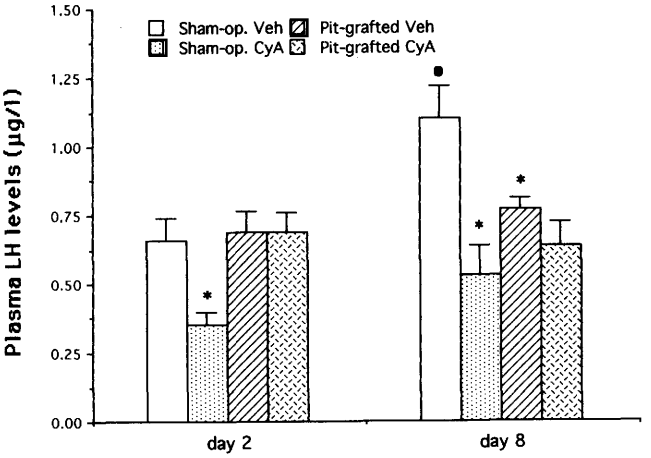

Figure 1. Plasma LH levels in sham-operated and pituitary-grafted female rats treated with CyA (5 mg/kg body wt/day) or vehicle for 2 or 8 days beginning on the day of surgery. Values are given as mean ± SEM (n = 10 animals per group).
● $P < 0.05$ vs sham-operated, vehicle-treated rats (Day 2).
* $P < 0.05$ vs sham-operated, vehicle-treated rats.

($P < 0.05$ vs vehicle-treated rats). In sham-operated rats, CyA administration decreased plasma FSH levels both on Day 2 and 8 of treatment ($P < 0.05$) compared with sham-operated rats treated with vehicle (Fig. 2).

LHRH content in the hypothalamus of pituitary-grafted rats was similar to that found in sham-operated rats at 2 days, and was decreased 8 days after the grafting ($P < 0.05$). Cyclosporine treatment for 8 days significantly decreased hypothalamic LHRH content in rats bearing pituitary grafts ($P < 0.05$ vs vehicle treated animals) and in sham-operated rats ($P < 0.05$ vs their respective vehicle-treated animals) (Fig. 3).

In sham-operated rats treated with vehicle, plasma estradiol levels increased with age ($P < 0.05$). Plasma

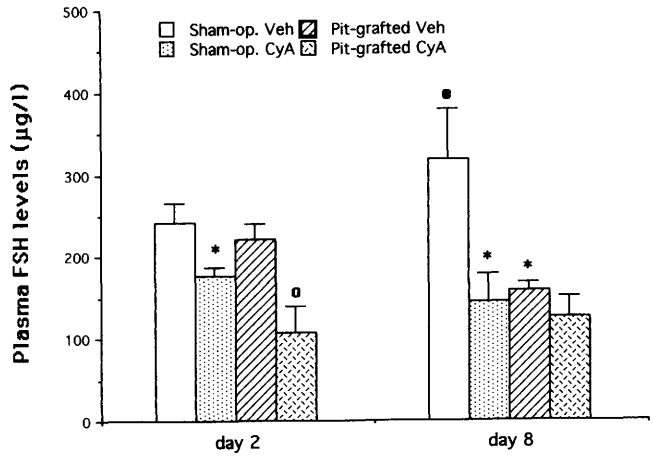

Figure 2. Plasma FSH levels in sham-operated and pituitary-grafted female rats treated with CyA (5 mg/kg body wt/day) or vehicle for 2 or 8 days beginning on the day of surgery. Values are given as mean ± SEM (n = 10 animals per group).
● $P < 0.05$ vs sham-operated, vehicle-treated rats (Day 2).
* $P < 0.05$ vs sham-operated, vehicle-treated rats.
○ $P < 0.05$ vs pituitary-grafted, vehicle-treated rats.

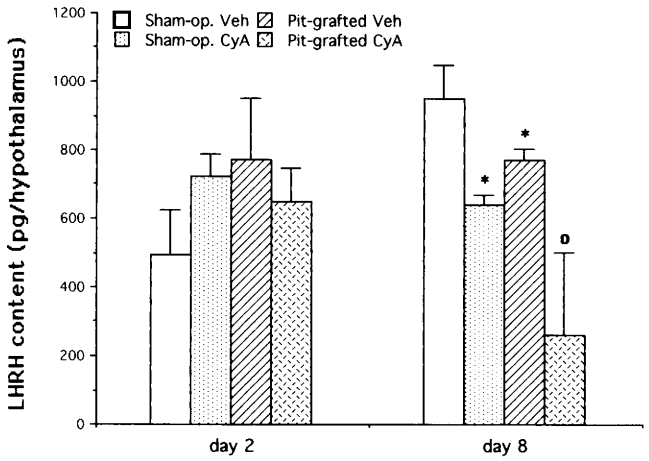

Figure 3. Hypothalamic LHRH content in sham-operated and pituitary-grafted female rats treated with CyA (5 mg/kg body wt/day) or vehicle for 2 or 8 days beginning on the day of surgery. Values are given as mean ± SEM (n = 10 animals per group).
• $P < 0.05$ vs sham-operated, vehicle-treated rats.
○ $P < 0.05$ vs pituitary-grafted, vehicle-treated rats.

estradiol levels did not change after pituitary grafting at any point studied, compared with sham-operating. The treatment with CyA to pituitary-grafted animals only increased plasma estradiol levels on Day 8 of treatment ($P < 0.05$) compared with rats of the same group treated with vehicle. There was an increase in plasma estradiol levels of sham-operated rats compared with vehicle-treated rats ($P < 0.05$), after both 2 and 8 days of CyA treatment (Fig. 4).

The grafting of one pituitary gland under the kidney capsule decreased ovarian estradiol content on Day 8 after surgery in vehicle-treated animals compared with sham-operated, vehicle-treated rats. Administration of CyA significantly diminished estradiol content in the ovaries of sham-operated rats on Day 2 and 8 of treatment, compared with vehicle-treated, sham-operated animals ($P < 0.05$), but increased it on Day 8 of treatment in pituitary-grafted rats ($P < 0.05$, Fig. 5).

In vitro basal release of estradiol was undetectable in ovaries from any group studied 2 days after the surgery and/or the beginning of the treatment with CyA (Fig. 6). When hCG was used to stimulate estradiol secretion by the ovaries, a similar estradiol release was found in ovaries coming from sham-operated animals treated either with vehicle or CyA, and from pituitary-grafted animals treated with the drug. There was also a marked increase in estradiol response to hCG in pituitary-grafted rats treated with vehicle ($P < 0.05$) compared with any studied group (Fig. 6). *In vitro* basal release of estradiol in ovaries coming from animals of 38 days of age was detectable (Fig. 7). In the ovaries from sham-operated animals treated with CyA, the basal release of estradiol was increased ($P < 0.05$) when compared with that in vehicle treated ones. Pituitary grafting did not change the basal release of estradiol, and the CyA treatment increased it ($P < 0.05$). The stimulated estradiol release after hCG administration was potentiated in ovaries coming from both sham-operated and pituitary-grafted rats treated with CyA ($P < 0.05$) compared with their respective vehicle-treated groups. In both cases, the estradiol response to hCG was similar in ovaries coming from sham-operated and pituitary-grafted animals treated with vehicle or CyA (Fig. 7).

Discussion

The foregoing results indicate that both pituitary grafting and/or chronic CyA treatment, from Day 30 of life on, were followed by inhibitory effects at the hy-

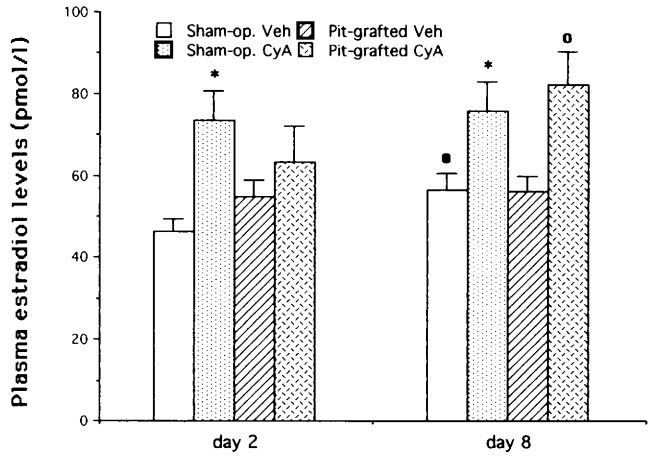

Figure 4. Plasma estradiol levels in sham-operated and pituitary-grafted female rats treated with CyA (5 mg/kg body wt/day) or vehicle for 2 or 8 days beginning on the day of surgery. Values are given as mean ± SEM (n = 10 animals per group).
● $P < 0.05$ vs sham-operated, vehicle-treated rats (Day 2).
* $P < 0.05$ vs sham-operated, vehicle-treated rats.
○ $P < 0.05$ vs pituitary-grafted, vehicle-treated rats.

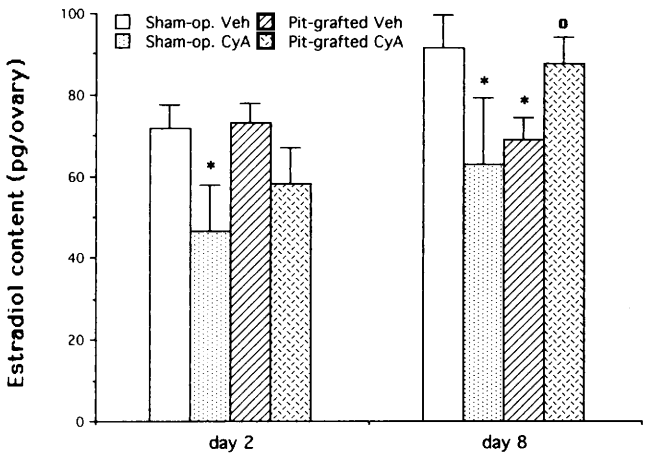

Figure 5. Ovarian estradiol content in sham-operated and pituitary-grafted female rats treated with CyA (5 mg/kg body wt/day) or vehicle for 2 or 8 days beginning on the day of surgery. Values are given as mean ± SEM (n = 10 animals per group).
• $P < 0.05$ vs sham-operated, vehicle-treated rats.
○ $P < 0.05$ vs pituitary-grafted, vehicle-treated rats.

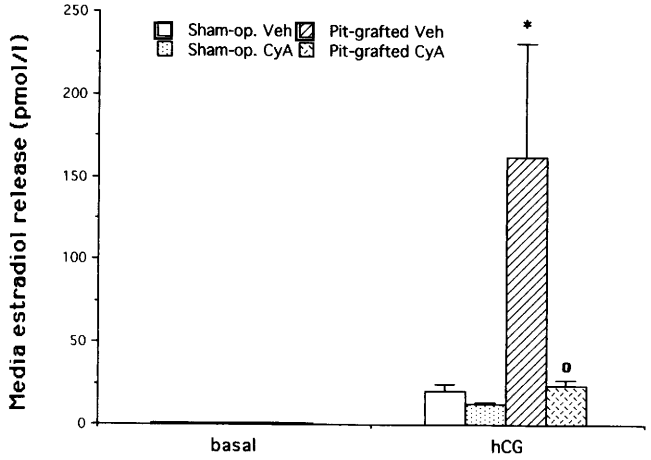

Figure 6. *In vitro* basal and hCG-stimulated estradiol release in sham-operated and pituitary-grafted female rats treated with CyA (5 mg/kg body wt/day) or vehicle for 2 days beginning on the day of surgery. Values are given as mean ± SEM (n = 10 animals per group). (Estradiol concentration in the media from the basal incubation was undetectable, <25 μg/liter).
* $P < 0.05$ vs sham-operated, vehicle-treated rats.
○ $P < 0.05$ vs pituitary-grafted, vehicle-treated rats.

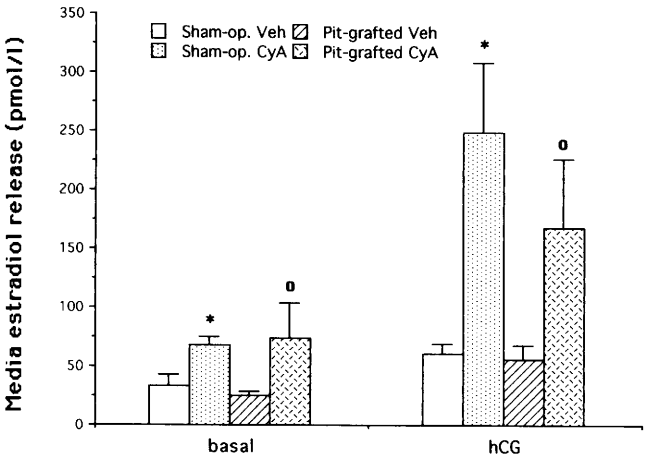

Figure 7. *In vitro* basal and hCG-stimulated estradiol release in sham-operated and pituitary-grafted female rats treated with CyA (5 mg/kg body wt/day) or vehicle for 8 days beginning on the day of surgery. Values are given as mean ± SEM (n = 10 animals per group).
* $P < 0.05$ vs sham-operated, vehicle-treated rats.
○ $P < 0.05$ vs pituitary-grafted, vehicle-treated rats.

pothalamic-pituitary axis measured by a decrease of the hypothalamic LHRH content, associated with decreased circulating levels of gonadotropins and increased PRL levels. However, the increase in plasma estradiol levels after CyA administration in both pituitary-grafted and sham-operated animals, together with an *in vitro* potentiation of estradiol secretion after hCG stimulation, suggests a stimulatory role of the drug at ovarian level. These data suggest that CyA administration is followed by a dissociation of the central and peripheral mechanisms that regulate the hypothalamic-pituitary-ovarian function. *In vitro* data suggest that CyA's effects on the ovary are not mediated by changes in PRL secretion during the chronic exposure to the drug; whereas, at the hypothalamic-hypophyseal axis, CyA's effects might by mediated by PRL changes.

In sham-operated animals, plasma LH, FSH, PRL, and estradiol increased from Day 32 to 38 of life, in agreement with previous reports in the literature (17), indicating, along with the observation of vaginal opening, that the rats used in this study were in the pubertal phase. Consequently, the changes in various parameters studied in this work after CyA treatment are complicated by the rats being prepubertal initially, but postpubertal on Day 8 of treatment. However, comparisons were all made between groups at the same stage of sexual maturation.

The changes in circulating gonadotropins shown in sham-operated rats correlated with the increase in the hypothalamic content of LHRH shown in this study. The treatment with CyA reduced plasma LH and FSH levels, and LHRH content in the hypothalamus, thus providing evidence for an inhibitory effect of the drug on the hypothalamic-pituitary axis as was suggested previously in adult male rats (18, 12) and also in other studies using higher doses of CyA (9).

Pituitary grafting was followed by the expected increase in plasma PRL levels associated with decreased circulating values of LH as long as the implant was present as in previous experiments from our (4, 19) and other (20, 21) laboratories. Surprisingly, in the present study there was a reduction in plasma values of FSH in animals of 38 days of age in disagreement with previous data from our laboratory (4). This discrepancy may be due to differences in the season in which the experiments were done, as was described in previous works from this laboratory (22). The effects on gonadotropin secretion exerted by hyperprolactinemia can be explained taking into consideration the effects of PRL on the hypothalamus decreasing the LHRH content (23).

A direct effect of CyA on the ovary has to be considered since both *in vivo* and *in vitro* release of estradiol were modified by the treatment with the immunosuppressive drug in the sham-operated group. Treatment with CyA increased plasma estradiol levels and decreased the estradiol content of the ovary, suggesting that CyA is increasing the release of estradiol into the circulation or the culture medium in contrast to the data reported in adult female rabbits (24) and for testosterone in male rats (25). The discrepancies observed might be due to the differences in the age of the animals (pubertal in this study versus adults for female rabbits). The increase in plasma estradiol levels might be explained by the direct effects of CyA on the granulosa cells modifying the aromatase activity (26). Also a potentiation of this effect could be exerted by adrenal-mediated effects of the drug (27).

The *in vitro* release of estradiol is increased in

ovaries coming from sham-operated rats of 38 days of life compared with those coming from 32 day old rats, in agreement with the increase in the circulating levels of the hormone, suggesting that an activation of the gonad is taking place and leading to a normal cyclicity pattern characteristic of adulthood (28, 29). The undetectable basal *in vitro* release of estradiol in ovaries from 32-day-old animals might be due to a quiescent activity of the ovary at that age, in agreement with previous data from literature (30). The stimulatory effect of pituitary grafting *in vivo* in 32-day-old rats on the *in vitro* estradiol response to hCG might be due to the existence of high plasma levels of PRL together with normal values of gonadotropins (31). Also, that PRL increases the number of LH receptors on rat luteal cells has been described (32). However, in 38-day-old animals hyperprolactinemia is associated with low values of gonadotropins, so that the potentiating effects of PRL on *in vitro* hCG response is not effective (31). Hence, the stimulatory effect of CyA on *in vitro* hCG estradiol responses might be due to a direct effect of the drug on the ovary, as was suggested earlier (26).

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