Intraovarian Excess of Nerve Growth Factor Increases Androgen Secretion and Disrupts Estrous Cyclicity in the Rat*

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ABSTRACT

A single injection of estradiol valerate induces a form of cystic ovary resembling some aspects of the human polycystic ovarian syndrome. Preceding the development of follicular cysts, there is an increase in intraovarian synthesis of nerve growth factor (NGF) and the low affinity NGF receptor (p75 NGFR). Selective blockade of NGF actions and p75 NGFR synthesis in the ovary restored estrous cyclicity and ovulatory capacity in estradiol valerate-treated rats, suggesting that an increase in NGF-dependent, p75 NGFR-mediated actions within the ovary contributes to the development of cystic ovarian disease. We have tested this hypothesis by grafting NGF-producing neural progenitor cells into the ovary of juvenile rats that have been induced to ovulate precociously by a single injection of PMSG. The NGF-producing cells, detected by their content of immunoreactive p75 NGFR material, were found scattered throughout the ovary with some of them infiltrating the granulosa cell compartment of large, precystic follicles. Ovarian NGF content was 2-fold higher than in the ovary of rats receiving control cells. Estrous cyclicity was disrupted, with the animals showing prolonged periods of persistent estrus, and an almost continuous background of vaginal cornified cells at other phases of the estrous cycle. Morphometric analysis revealed that the presence of NGF-producing cells neither reduced the total number of corpora lutea per ovary nor significantly increased the formation of follicular cysts. However, the ovaries receiving these cells showed an increased incidence of precystic, type III follicles, accompanied by a reduced number of healthy antral follicles, and an increased size of both healthy and atretic follicles. These changes in follicular dynamics were accompanied by a selective increase in serum androstenedione levels. The results show that an abnormally elevated production of NGF within the ovary suffices to initiate several of the structural and functional alterations associated with the development of follicular cysts in the rat ovary.

FOLLICULAR DEVELOPMENT is a complex process regulated by hormonal inputs, the nervous system, and—within the ovary—by a myriad of paracrine and autocrine influences (1–4). Nerve growth factor (NGF) is synthesized by, and released from, ovarian cells (5), and thus, can be considered as a component of the intragonadal system of paracrine/autocrine regulators influencing ovarian function. The presence of NGF receptors on nerve fibers innervating the ovary (6, 7), and the loss of ovarian innervation resulting from the immunoneutralization of NGF actions (8) indicate that NGF is one of the target-derived neurotrophins involved in the development and maintenance of the ovarian innervation. The additional presence of low and high affinity NGF receptors on nonneural cells of the gland (6, 7) suggests that NGF serves another, tissue-specific, role within the gland. One of the presumed functions of NGF in the ovary appears to be in the cascade of events that leads to follicular rupture at ovulation (7). NGF has been postulated to act on the follicular wall facilitating the cell-cell dissociation process that precedes the rupture of the follicle at the time of ovulation (7).

Abnormally elevated NGF levels may contribute to ovarian dysfunction as an activation of ovarian NGF synthesis and that of the low affinity NGF receptor (p75 NGFR) precedes the formation of follicular cysts induced by the administration of a single dose of estradiol valerate (EV) to rats (9). A cause-effect relationship between the elevated NGF/p75 NGFR levels and the alterations in follicular growth induced by EV was demonstrated by the restoration of normal populations of antral follicles observed after blockade of NGF actions and p75 NGFR synthesis in the ovaries of EV-treated rats (9).

Injection of a single dose of EV to cycling rats results in loss of estrous cyclicity, anovulation, and formation of follicular cysts (9–11). These changes are, in a broad sense, similar to some of the abnormalities seen in the human condition of polycystic ovarian syndrome (PCOS) (12, 13). PCOS is characterized by ovulatory failure, amenorrhea, hyperandrogenemia, and variable levels of circulating gonadotropins (12, 13) and is widely held as the most common cause of infertility

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in women (14). While the initiation and progression of the syndrome is determined by a variety of interrelated factors (12, 13), its precise etiology is unknown. The list of abnormalities associated with PCOS include defects in the hypothalamic-pituitary unit, the ovarian microenvironment, the adrenal gland and the insulin/IGF-I metabolic regulatory system (14–16). The unifying feature to most forms of PCOS is the presence of multiple medium-size antral follicles containing an enlarged thecal layer, and the occurrence of elevated serum androgen levels [especially androstenedione; (17, 18)]. A recent study implicated follistatin in the etiology of the syndrome, by showing evidence for genetic linkage of the follistatin gene to PCOS and hyperandrogenemia (19).

While this and other findings make it likely that human PCOS is determined by intraovarian defects not primarily dependent on changes in gonadotropin input (14), the nature of these defects has been only partially elucidated (18, 19). Within this framework, and in view of a potential involvement of NGF in the EV-induced cystic ovarian condition in rats (9), we set out to determine whether an abnormally elevated neurotrophic tone within the ovary is by itself capable of producing some of the ovarian abnormalities associated with the hormonal induction of ovarian cysts in this species. Such an effect would implicate an excessive production of NGF as one of the intragonadal factors contributing to the development of cystic ovarian dysfunction. The present study was undertaken to examine this hypothesis. To induce an ovary-specific increase in NGF production, without first affecting the hormonal input to the gland, we grafted into the ovary neural progenitor cells genetically engineered to produce NGF (20). Because in some PCOS patients the syndrome is associated with early initiation of puberty (16, 21), sexual precocity was induced in rats by treatment with PMSG and the NGF-producing cells were grafted into the ovary on the morning of the expected preovulatory surge of gonadotropins. The results indicate that a sustained elevation in intraovarian NGF production results in morphological and functional abnormalities resembling the early phases of EV-induced PCO.

Materials and Methods

Animals

Sprague Dawley rats (B&K Universal, Fremont, CA) were housed under controlled conditions of temperature (23−25 C) and light (14 h of light, 10 h of darkness); lights on from 0500–1900 h). They were provided *ad libitum* access to food (Purina Laboratory Chow, Ralston Purina Co., St. Louis, MO) and water.

Grafting of NGF-producing neural progenitor cells

To increase NGF levels only in ovarian tissue, we used a gene transfer-cell grafting approach previously employed by us (20, 22, 23) and others (24, 25) to target gene overexpression to discrete regions of the mammalian brain. Stable incorporation of the mouse NGF gene into the genome of conditional immortalized embryonic day 16 rat hippocampus-derived neural progenitor cells [HiB5; (26)] resulted in the generation of a cell line (E8) that constitutively secretes mouse NGF (20). The HiB5 cells were conditionally immortalized by the stable expression of a temperature-sensitive mutant form of the large T-antigen from SV40 virus (26). Thus, the cells proliferate at the permissive temperature of 33 C and stop growing at 37 C. NGF overexpression was achieved by infection with a retroviral vector containing the coding region of the mouse NGF gene under the control of the long terminal repeat of Moloney Murine Leukemia Virus (Fig. 1). HiB5 cells infected with the same retrovirus construct, but that failed to incorporate the transgene into their genome (and thus, do not produce NGF) were used as controls (clone D11). NGF release from HiB5 E8 cells (3×10^6 cells) cultured for 48 h in DMEM containing 10% FBS was found to be greater than 4,000 pg/ml as measured by a specific ELISA assay (see below). In contrast, the control D11 cells did not secret detectable NGF levels into the medium (Fig. 1). Both cell lines were grown at 33 C in DMEM plus 10% FBS, 10,000 U/ml penicillin and streptomycin, and 2 mM glutamine. On the day of grafting, the cells were removed by mild trypsinization (in PBS containing 0.05% trypsin/0.02% EDTA for 5 min), transferred into a centrifuge tube, collected by low speed centrifugation, counted and diluted to a concentration of 40,000 cells per μl of DMEM.

Twenty-eight-day-old rats were injected with PMSG (8 IU/rat, sc) to induce a synchronized first preovulatory surge of gonadotropins. On the morning of the expected gonadotropin surge, the ovaries were sequentially exposed via a dorsal approach, and the cells were injected into two sites at the opposite poles of the long axis of the ovary. At each site, 2.5 μl of the cell suspension (40,000 cells/μl) were injected using a Hamilton microsyringe. The site of injection was pinched closed with fine forceps for 30 sec, and the ovary was returned to the peritoneal cavity. Following grafting, the rats were returned to their cages and their estrous cycles were monitored daily for the remainder of the experiment, starting two days after the injection of cells. Each stage of the estrous cycle was determined in the afternoon by vaginal lavage followed by microscopic examination of the recovered cells. According to the prevailing morphology of the cells, the animals were considered to be in one of four different phases of the estrous cycle: proestrus, estrus, diestrus-1, and diestrus-2. Transitional phases were also observed. They are referred to as proestrus/estrus and estrus/diestrus, wherein cornified cells indicative of an estrous condition were present in about equal numbers with either leukocytes (diestrus) or nucleated cells (proestrus). The number of animals used for each experimental procedure (NGF assay, ovarian morphology, etc.) is indicated in the legend to each figure.

NGF ELISA assay

NGF levels were measured in culture medium and whole ovaries by an ELISA assay. The assays were performed using the NGF EMAX Immunoassay system (Promega Corp., Madison, WI), according to the manufacturer instructions. Before assay, the ovaries were weighed and homogenized using a glass/glass homogenizer, in 300 μl of buffer (100 mM Tris-HCl, pH 7.0, 0.4 M sodium chloride, 0.1% sodium azide, 2% BSA, 2% gelatin, 4 mM EDTA, 1 μM PMSF, and 0.1 U/ml aprotinin). Following a rinse with an additional 50 μl buffer, the homogenates were microfuged for 5 min at 4 C, and the supernatants were transferred to a clean tube.

Immunohistochemistry

Immunohistochemical detection of the p75 NGFR was performed on 14-μm cryostat sections from ovaries collected 60 days after grafting. The ovaries were fixed by immersion in Zamboni's fixative, as described (27) and processed for p75 NGFR immunohistochemistry using the monoclonal antibody 192 IgG (28). Tissue sections were incubated overnight at 4 C with the antibody and the immunoreaction was developed the next day using the diaminobenzidine procedure previously described (6). Controls consisted of adjacent sections incubated without the p75 NGFR antibody (6).

RIA

Serum levels of ovarian steroids were measured as previously described [estradiol (29), progesterone (30), testosterone (31), and androstenedione (32)].

Histology

Sixty days after grafting, the ovaries carrying either NGF-producing or control cells were immersed in Kahle's fixative solution, embedded in paraffin, serially sectioned at 8 μm, and were stained with hematoxylin-eosin as previously reported (8). The number of antral, type III, and atretic follicles were counted in every fifth section (8). The criteria used

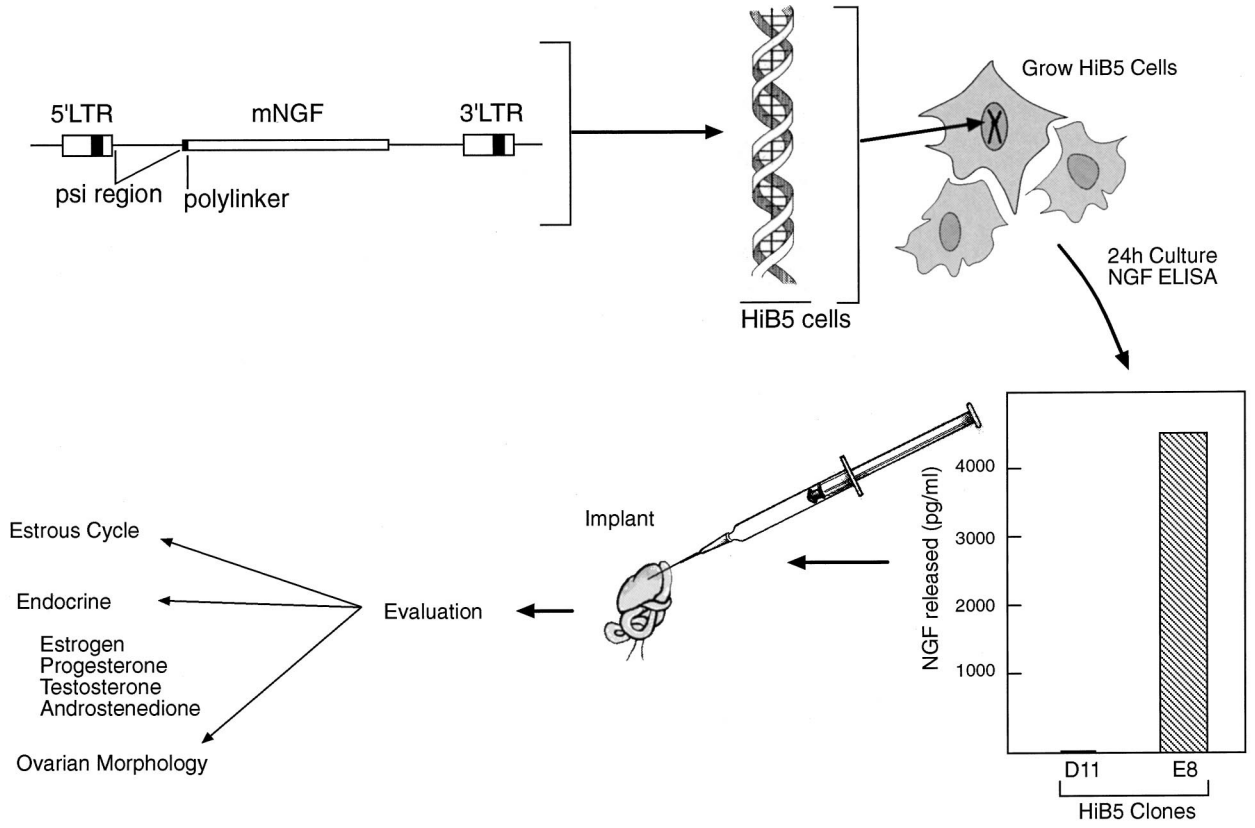


FIG. 1. Gene transfer-cell grafting procedure used to selectively increase intraovarian concentrations of NGF. The schematic drawing of the retroviral vector used to deliver NGF to the ovary has been redrawn from reference (20). Two different subclones (E8 and D11) of retrovirally infected HiB5 neural progenitor cells were compared for their ability to produce NGF. The NGF-producing cells (E8 subclone) produced NGF levels in excess of 4000 pg/ml over a 24 h period. No NGF was detected in the medium from control D11 cells.

to classify follicle types and the morphometric analysis procedure used to quantitate the changes observed are those described in detail in the companion paper (9).

Statistics

The differences among groups were analyzed using one-way ANOVA. The Student-Neuman-Keuls test was used to analyze differences between ovarian NGF levels achieved following grafting of control or NGF-producing cells, in serum levels of sex steroids between the two groups, and in the number and size of follicles and corpus lutea. The number of days spent by each animal in a particular phase of the estrous cycle was expressed as a percentage of the total days studied. The arcsine transformation was then used to normalize the percentage data before analysis by one-way ANOVA with LS means to differentiate among groups. The χ^2 test was used to analyze differences in the incidence of cysts and type III follicles between the ovaries receiving NGF-secreting or control cells.

Results

Intraovarian grafting of NGF-producing cells increases ovarian NGF content

To measure the change in ovarian NGF content resulting from the intraovarian grafting of NGF-producing cells, the total NGF content per ovary was determined by ELISA assay. Ovaries were collected 30 and 60 days after grafting the HiB5 cells. Because there were no differences in the NGF values detected at 30 and 60 days after grafting, the two groups were pooled. Ovaries receiving NGF-producing cells showed a 2-fold increase in NGF content as compared with ovaries grafted with control cells (Fig. 2). Ovarian NGF levels achieved were well above those reported in serum of rodents (33) and humans (34).

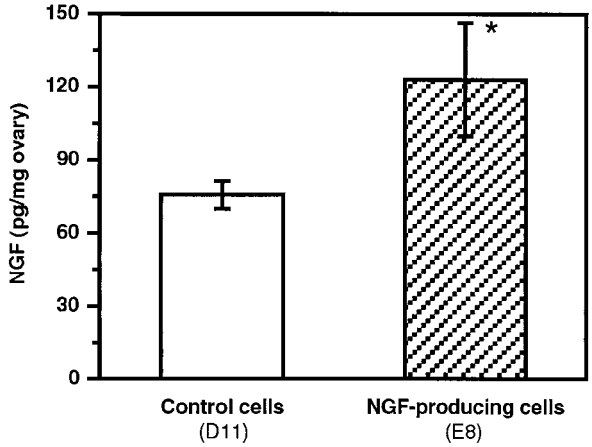
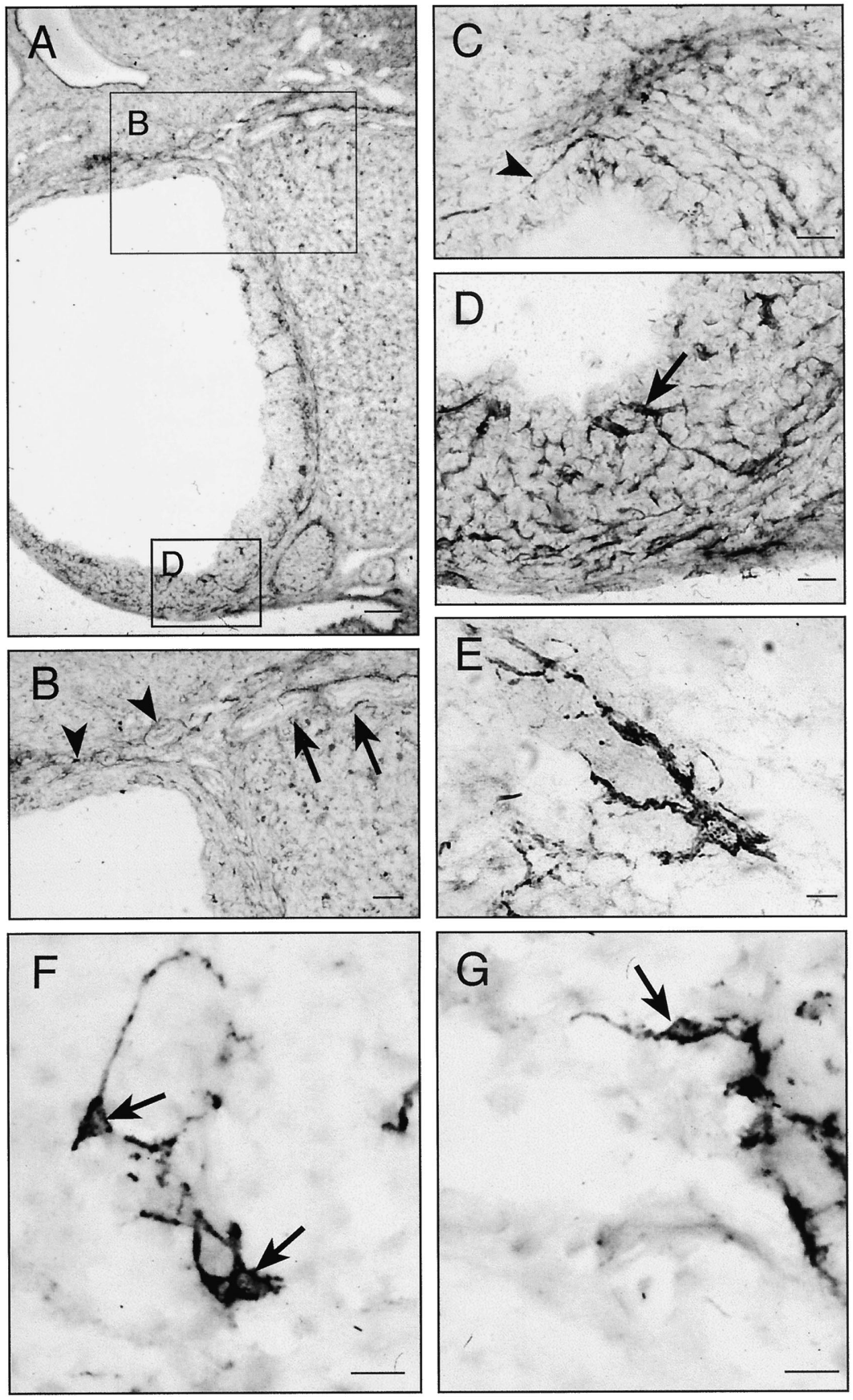


FIG. 2. Increased ovarian NGF content in ovaries receiving grafts of NGF-producing HiB5 cells (E8 subclone). Ovaries grafted with either NGF-producing cells or control (D11 subclone) cells were collected 30 or 60 days after surgery. Because there were no differences in the NGF content between these two age groups, they were pooled. One ovary from each animal was analyzed. Each *bar* represents the mean ± SEM of 13 ovaries (control, D11) and 15 ovaries (NGF-producing, E8). *, $P < 0.05$ *vs.* ovaries grafted with control cells.


A
B
D
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D
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E
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G

Localization of NGF-producing grafted cells in the ovary

Attempts to localize HiB5 cells secreting NGF by immunohistochemical detection of NGF failed, perhaps reflecting the minimal accumulation of NGF in the cytoplasm before secretion (20). Examination of HiB5 cells for expression of p75 NGFR, the low affinity neurotrophin receptor, demonstrated that they contain the messenger RNA encoding the receptor (data not shown) and can therefore, be identified by immunohistochemical detection of the receptor protein. Although the primate ovary contains p75 NGFR positive neuron-like cells (35) that could confound the search for grafted p75 NGFR-expressing cells, such endogenous cells could not be detected in the ovaries from Sprague Dawley rats (6, 36). We, therefore, identified the grafted cells by their content of p75 NGFR.

Consistent with earlier findings (6), p75 NGFR immunoreactive material was present in nerve fibers surrounding blood vessels and coursing across the interstitial tissue, as well as in fibers associated with follicles in different stages of development (Fig. 3). As before, p75 NGFR immunoreactivity was also observed in thecal cells of antral and preantral follicles. Control sections incubated without primary antibody did not show any specific staining. The ovaries grafted with NGF-producing cells exhibited cells containing p75 NGFR immunoreactive material in the interstitial tissue (Fig. 3, E–G), and—unexpectedly—infiltrating the granulosa cell compartment of large type III follicles (Fig. 3D). The morphological aspect of these cells was variable. In some cases, when located in the interstitial compartment, they exhibit a neuronal aspect with multiple, single or bipolar processes (Fig. 3, E—G). When detected in the granulosa cell layer their aspect appeared more astrocyte-like (Fig. 3D). As no such cells were detected in control nongrafted ovaries, they must represent two morphological variations of the grafted neural progenitor cells.

Estrous cyclicity is disrupted by an intraovarian excess of NGF

Rats grafted with control (D11) cells exhibited, for the most part, 4-day cycles, with only short periods of irregular cyclicity (Fig. 4). In a 4-day cycle, it is expected that 25% of the time the rats would be in proestrus, 25% in estrus, and 50% in either diestrus 1 or 2. The rats receiving the control (D11) cells exhibited cycles in which 19% of the days the rats were in proestrus, 31% of the days they were in estrus and 39% of the days the rats were in diestrus. There were a few days in which some animals were in transitional phases of either proestrus/estrus or estrus/diestrus (Fig. 5, A and B). In contrast to this profile, estrous cyclicity was rapidly interrupted in those rats grafted with NGF-producing cells (Fig. 4). These rats showed a significantly ($P < 0.05$) greater number of days in estrus than in proestrus or diestrus (Fig. 5A). In addition, their vaginal lavages persistently showed the presence of cornified epithelial cells, resulting in a greater number of transitional days (both proestrus/estrus and estrus/diestrus; Fig. 5B). Overall, the total percentage of days wherein cornified epithelial cells were present, was doubled in the rats grafted with NGF-producing cells than in control animals receiving cells unable to produce NGF ($87 \pm 2\%$ *vs.* $42 \pm 3\%$, $P < 0.0001$; Fig. 5C).

An intraovarian excess of NGF alters the dynamics of antral follicular development

Examination of the ovaries 60 days after grafting the neural progenitor cells, revealed a significant decrease in the number of healthy antral follicles in the ovaries receiving NGF-producing cells (Fig. 6B). This decrease was not accompanied by a similar reduction in the total number of antral follicles (Fig. 6A), likely because of a slightly increased number of antral atretic follicles (Fig. 6C). In addition to the selective decrease in the number of healthy antral follicles, the ovaries grafted with NGF-producing cells showed an increased size of both healthy and atretic antral follicles (Fig. 6, D and E).

Despite the striking alteration in estrous cyclicity caused by the intraovarian implantation of NGF-producing cells the total number of corpora lutea detected in these animals did not differ from that of control rats (Fig. 6F). They did, however, exhibit a significant increase in the incidence of precystic, Type III antral follicles (Table 1). While they also showed a doubling in the incidence of follicular cysts, this difference did not achieve statistical significance (Table 1). Noteworthy, the ovaries of rats injected with control cells also showed some cysts and type III follicles, a feature that may be, at least in part, related to the trauma of the grafting procedure.

Intraovarian grafting of NGF-producing cells results in elevated serum androgen levels

When measured 60 days after grafting serum androstenedione levels were significantly ($P < 0.05$) elevated in rats carrying intraovarian grafts of NGF-producing cells (Fig. 7B). Though mildly elevated, serum estradiol and testosterone levels were not statistically greater than in control rats (Fig. 7, A and B). Serum progesterone levels were also similar in the two groups.

Discussion

The present results demonstrate that an elevation of NGF levels within the ovary is sufficient to initiate some of the structural and functional alterations associated with the de-

FIG. 3. Immunohistochemical detection of HiB5 cells grafted into the ovary of peripubertal rats. The cells were identified by their content of p75 NGFR. A, p75 NGFR-immunopositive nerve fibers derived from the ovarian innervation are seen coursing through the interstitial tissue (*arrow*) and reaching a type III follicle. B, Enlarged view of area boxed in A; arrows denote p75 NGFR positive fibers innervating a blood vessel. C, Higher magnification of a section adjacent to A containing p75 NGFR immunopositive fibers (*arrowhead*) reaching the wall of the follicle depicted in A. D, High magnification view, demonstrating the presence of immunopositive neuronal/glial cell-like structures (*arrow*) within the granulosa cell layer of the follicle depicted in A. Panels E–G, p75 NGFR immunopositive cells with different morphologies detected in the interstitial tissue of an ovary grafted with neural progenitor cells producing NGF. Notice the multipolar neuronal appearance of the cell depicted in E, and the bipolar aspect of the cells shown in F and G (*arrows*). *Bars*: A, 100 μm; B, 50 μm; C and D, 25 μm; E–G, 0 μm.

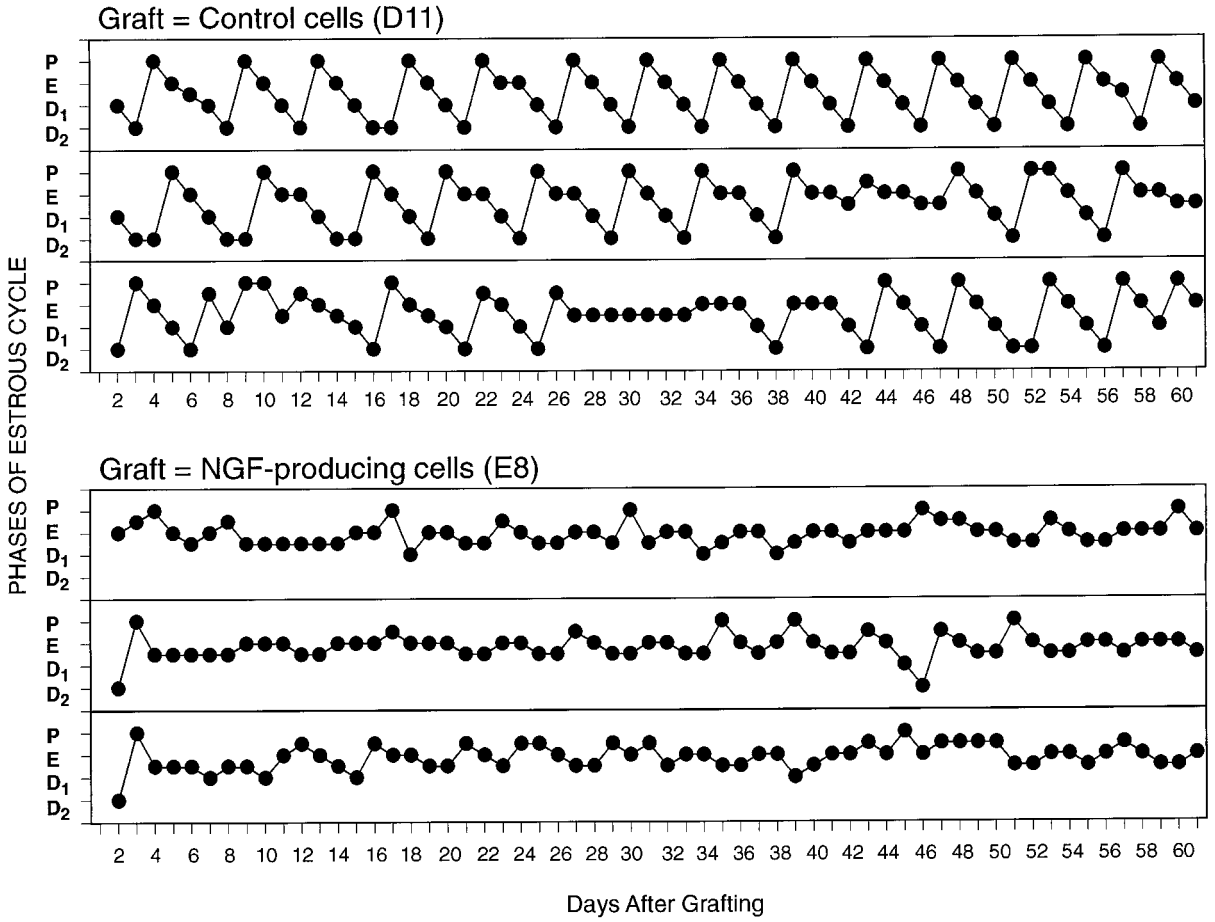

FIG. 4. Disruption of estrous cyclicity by the intraovarian implantation of NGF-producing HiB5 cells. The *upper panel* depicts the estrous cycle of three representative control rats receiving a graft of control HiB5 cells. The *lower panel* depicts three representative estrous cycle profiles of rats receiving intraovarian grafts of NGF-producing cells. The different stages of the estrous cycle (depicted on the vertical axis) were determined according to the predominant cell type present in vaginal lavages (P, proestrus; E, estrus; D_1, diestrous day 1, D_2, diestrous day 2).

velopment of follicular cysts in the rat ovary, including a reduced number of healthy antral follicles, the appearance of precystic structures, and increased serum levels of androstenedione.

One important component of the neurogenic makeup of a tissue is the regulatory system of neurotrophins and their receptors. Like in other tissues innervated by the peripheral nervous system, neurotrophins are required for the development of the ovarian innervation and for maintaining the mature function of the innervating neurons (8). Neurotrophins also enhance the production of neurotransmitters, in addition to maintaining the functional integrity of the neurons themselves (37). NGF is the prototypic member of the neurotrophin family (38). NGF signals via two receptors: one, termed p75 NGFR (also known as neurotrophin receptor), is a member of the tumor necrosis factor receptor/FAS/Apo-1/CD95 family (39); the second known as trkA (40), is a member of the trk family of tyrosine kinase receptors. The trkA receptor can by itself mediate NGF signaling via the ligand-dependent activation of its tyrosine kinase intracellular domain (41–43). The p75 NGFR on the other hand, can interact with the trkA receptor to enhance the actions initiated by low levels of NGF (44, 45). In addition, p75 NGFR can effect independent signaling as its ligand-dependent activation has been shown to stimulate sphingomyelin hydrolysis leading to an elevation in ceramide levels (46, 47), increased synthesis of the NFκ-B transcription factor (48), and apoptosis (47, 49).

A form of cystic ovary that exhibits some of the features of the human syndrome can be induced in rats by a single injection of EV (10, 11). As discussed in the companion paper (9), the EV treated rat—while not a faithful model of PCOS—has been useful to identify new attributes of the pathology in rodents. For instance, it has been shown that before the appearance of the cysts there is an activation of the sympathetic innervation to the ovary, as demonstrated by an increased release of norepinephrine in response to electrical stimulation, increased ovarian norepinephrine content and tyrosine hydroxylase enzymatic activity and an enhanced uptake of the catecholamine by the nerves of the dysfunctional gland (50). The elevated activity of the sympathetic neurons reaching the ovary was demonstrated to contribute to the maintenance of the anovulatory state in EV-treated animals, when it was shown that transection of the superior ovarian nerve [the primary source of sympathetic innerva-

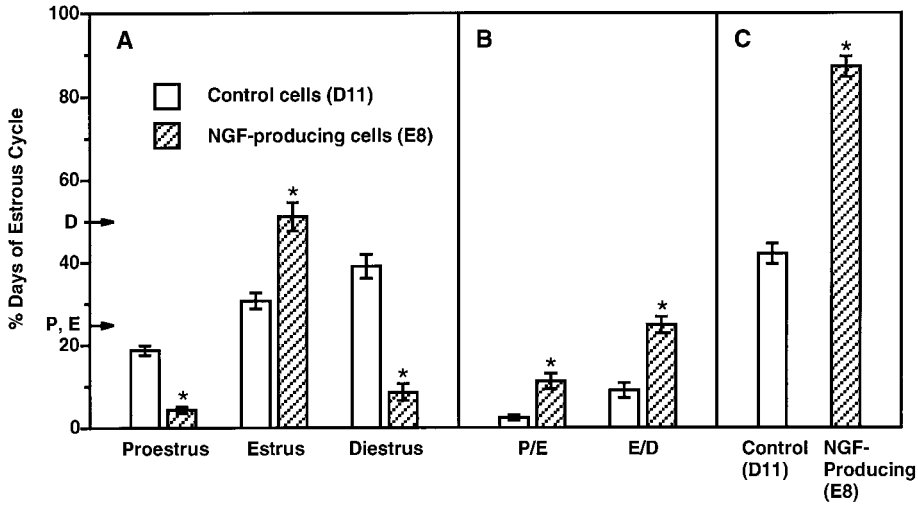

FIG. 5. Relative incidence of different stages of the estrous cycle in rats receiving intraovarian grafts of NGF-producing cells or control cells. *Bars* represent the mean values of 19 animals per group examined in five different experiments. *Vertical lines* are SEM. A, Each *bar* represents the percentage of days in which the vaginal lavages showed cells characteristic of as proestrus, estrus, or diestrus compared with the total number of observation days. If a rat showed a perfect 4-day cycle, the frequency distribution for each stage of the cycle would be: 25% proestrus (P), 25% estrus (E), and 50% diestrus (D) [*arrows* on the vertical axis mark 25% (P and E) and 50% (D)]. B, Incidence of transitional stages in animals carrying intraovarian grafts of NGF-producing cells or control cells. Such transitional stages were characterized by vaginal lavages showing equal number of cells belonging to two consecutive stages of the estrous cycle. The transitional stages were either proestrus to estrus (P/E) or estrus to diestrus (E/D). C, Percentage of days in which the vaginal lavages showed cornified epithelial cells (estrus + P/E + E/D). * $P < 0.0001$ *vs.* rats receiving control cells.

tion to the ovary (51)] resulted in prompt resumption of cyclicity and ovulatory capacity (52).

This elevated sympathetic activity is related to an intraovarian increase in the production of NGF and p75 NGFR (9). Both the increase in NGF/p75 NGFR synthesis and the activation of the sympathetic input to the ovary precede by several weeks the appearance of follicular cysts, suggesting a cause-effect relationship (9). Though inhibition of this abnormally elevated NGF tone brought ovarian morphology and function toward normalcy, these studies did not rule out the possibility that the actions of NGF/p75 NGFR (or the result of their pharmacological inhibition) require an estrogen-dependent background provided by the initial administration of EV. This is an important issue, because an EV-independent effect of NGF on ovarian function, mimicking all or part of the morphological and functional consequences of EV treatment, would implicate this neurotrophin as one of the intragonadal factors that contributes to the development of a cystic ovarian dysfunction, in the absence of primary alterations in hormonal regulatory control.

The present results provide evidence for this concept. An ovary-specific increase in NGF availability, obtained via grafting of neural progenitor cells genetically engineered to produce NGF, led to disruption of the estrous cycle, and to changes in follicular dynamics similar to some of the alterations produced by EV treatment. The most obvious similarities between the two experimental situations was the decrease in the number of healthy antral follicles and the development of precystic, type III follicles. These are large antral follicles containing four or five plicated layers of densely packed, but healthy, granulosa cells surrounding a large antrum (9, 53). Their thecal cell compartment does not appear hypertrophied. Because type III follicles contain a normal complement of LH receptors (53) and ovulate in response to LH, they may be the only follicles able to ovulate in EV-treated rats (53, 54). At the same time, however, they are considered as precystic structures, which, despite their size are unable to ovulate or luteinize in the presence of basal LH levels. Type III follicles appear to become developmentally arrested before turning into a cyst (53, 54).

Type III follicles are the first abnormal ovarian structures detected 30 days after EV injection, preceding the formation of follicular cysts (9, 53). The present results indicate that they also represent the most predominant structural follicular abnormality seen in ovaries grafted with NGF-producing cells. This finding and the elimination of type III follicles caused by the inhibition of NGF actions in EV-treated rats (9), suggest that an abnormally elevated intraovarian NGF tone contributes significantly to the genesis of these precystic structures.

The intraovarian grafting of NGF-secreting cells did not significantly increase the number of follicular cysts in the ovary. This outcome may have related to the small sample size, the relatively small (2-fold) increase in intraovarian NGF levels caused by the cells, and/or the variable and unpredictable distribution and survival of the grafted cells within the ovary. If the assumption is made that none of these factors is sufficiently important to explain the relative inability of NGF to induce cyst formation, the inevitable conclusion is that an excess of NGF alone cannot fully reproduce the ovarian abnormalities caused by EV. Intuitively, we would suspect that such is, indeed, the case. Nevertheless, it would also appear evident that the use of a transgenic approach to achieve elevated NGF levels affecting a greater fraction of the ovarian follicular population is required to provide a definitive answer to this question.

The decrease in the number of healthy antral follicles observed in ovaries grafted with NGF-producing cells is con-

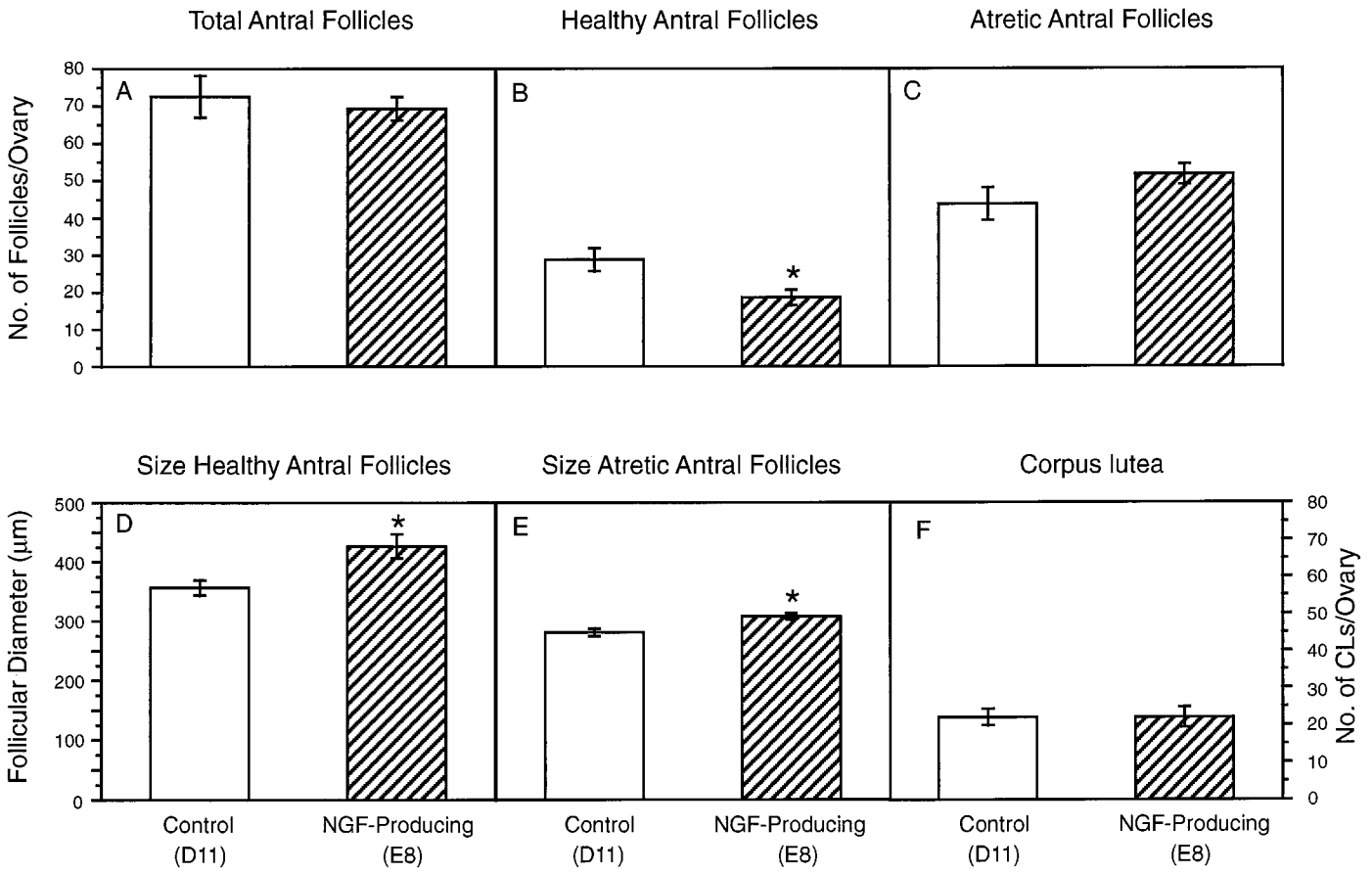

FIG. 6. Analysis of the morphological characteristics of ovaries grafted with neural progenitor cells for 60 days (control, D11; NGF producing, E8). A, Total number of antral follicles per ovary. B, Number of healthy antral follicles. C, Number of atretic antral follicles. D, Size of healthy antral follicles. E, Size of atretic antral follicles. F, Number of corpora lutea. One ovary from each animal was analyzed. Each *bar* represents the mean ± SEM of 10 ovaries (control, D11) and 10 ovaries (NGF-producing E8), from two experiments. *, $P < 0.05$ *vs.* ovaries grafted with control cells.

TABLE 1. Incidence of follicular cysts and type III follicles (Ty III F) in rats grafted with either control cells (D11) or NGF-producing cells (E8)

Ovarian structure	Groups[a] grafted with	
	Control cells	NGF-producing cells
No. cysts/no. healthy antral follicles	4/260[b]	8/186[b]
Cyst size (μm)	372.5 ± 89.7[c]	291.2 ± 50.1
No. Ty III F/no. healthy antral follicles	6/260[b]	13/186[b,d]
Ty III F size (μm)	746.7 ± 15.4	742.3 ± 36.3

[a] A total of 10 rats received intraovarian grafts of control cells and 10 rats were grafted with NGF-producing cells.
[b] Total number of follicular structures per group.
[c] Mean ± SEM.
[d] $P < 0.05$ *vs.* group grafted with control cells.

sistent with the observation that the reduction in the number of these follicles caused by EV-treatment is partially antagonized by intraovarian blockade of NGF actions (9). Noteworthy, NGF secreting cells did not reduce the number of atretic follicles as EV did, and surprisingly, caused an increase in the size of both healthy antral and atretic follicles. The most plausible explanation for these discrepancies between the two experimental paradigms is that NGF has an overall stimulatory effect on follicular development, and that this effect can only be fully manifested in the absence of those profound hormonal alterations resulting from the EV treatment. The delayed follicular development observed in rats subjected to neonatal immunoneutralization of NGF actions (8) supports this notion.

The increased size of antral follicles and the higher incidence of type III follicles observed in ovaries receiving NGF-producing cells may contribute to explaining the peculiar alterations in estrous cyclicity observed in the treated animals. A small, but persistent increase in estrogen levels by these enlarged follicles would account for both the interruption of cyclicity and the almost ever-present background of cornified cells detected in the vaginal lavages of rats carrying

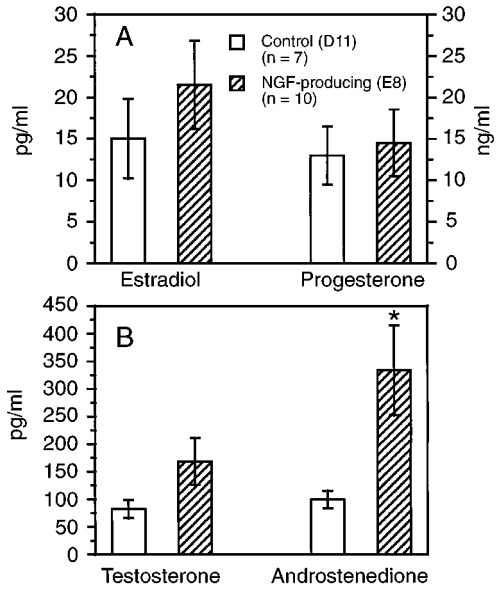

FIG. 7. Selective increase in serum androstenedione levels in rats 60 days after the intraovarian implantation of NGF-producing (E8) HiB5 cells. Control animals received grafts of HiB5 cells unable to produce NGF (control D11). A, Serum levels of estradiol (pg/ml), and progesterone (ng/ml). B, Serum levels of testosterone, and androstenedione. Bars ± vertical lines represent means ± SEM. *, $P < 0.05$ *vs.* control values.

an intraovarian graft of NGF-producing cells. Likewise, the availability of both relatively large, healthy antral follicles and LH-responsive type III follicles may have allowed the occurrence of irregular, but still functional ovulatory cycles. The presence of a similar number of corpora lutea in the ovaries from control and NGF-cell-recipient animals indicate that, this was, indeed, the case. Because we did not attempt to estimate the morphological/functional differences among corpora luteal populations, we cannot be certain of their actual age-related composition.

Rats carrying NGF-producing cells showed significantly elevated serum androstenedione levels, suggesting that an excess of NGF may significantly contribute to the hyperandrogenemia of cystic ovarian disease. NGF may enhance ovarian androgen production via both direct and indirect mechanisms. On the one hand, it may directly stimulate androgen production from thecal cells; on the other, it may do so via its trophic effects on the ovarian noradrenergic innervation. Support for the first possibility comes from the finding that NGF is able to stimulate androstenedione secretion from purified bovine thecal cells (Dissen, G. A., J. A. Parrot, M. J. Skinner, D. F. Hill, S. R. Ojeda, in preparation). An indirect, complementary effect mediated by catecholamines is supported by the well known ability of norepinephrine to stimulate ovarian androgen secretion (55, 56).

An aspect of the present study that deserves comment is the behavior of the grafted cells. Although they produce and release NGF both *in vitro* [(57), this study], and after grafting into host tissues [(57, 58), this study], it has been difficult to identify them in the host tissue by standard immunohistochemical techniques [(57), this study]. This appears to be a common feature of NGF-producing neural cells as they do not accumulate significant amounts of the peptide (57). In a previous study using the same HiB5 neural progenitor cells employed in the present experiments, they were identified by prelabeling them with bromodeoxyuridine or ^{3}H-thymidine (57). In our study, we took advantage of the finding that HiB5 cells, which contain p75 NGFR in culture, continue to express the receptor when grafted into the ovary, so they can be identified by immunohistochemistry using monoclonal antibodies to the receptor. Instead of remaining clustered near the site of injection, the cells dispersed throughout the ovary with some of them infiltrating the granulosa cells compartment of antral follicles. We do not know if this location resulted from the direct deposition of the cells inside some of these follicles at the time of injection.

Taken altogether the results demonstrate that a primary alteration in NGF availability to the peripubertal ovary results in morphological and functional abnormalities similar to some of those detected in cystic ovaries induced by EV treatment. The ability of chronically increased intraovarian levels of NGF to disrupt estrous cyclicity, induce formation of precystic follicular structures, and stimulate androgen production, suggests that an exaggerated intraovarian NGF tone may contribute to both the etiology and maintenance of cystic ovarian dysfunction in the rat. Whether these effects of NGF are due to a direct action on the ovary and/or an increase in the sympathetic outflow to the gland remains to be determined. An NGF contribution to human PCOS, and the potential relationship that may exist between this signaling system and those genes implicated in the syndrome by linkage analysis (19, 59) also need clarification.

References

1. **Richards JS** 1994 Hormonal control of gene expression in the ovary. Endocr Rev 15:725–751
2. **Dissen GA, Dees WL, Ojeda SR** 1993 Neural and neurotrophic control of ovarian development. In: Adashi EY, Leung PCK (eds) The Ovary. Raven Press, New York, pp 1–19
3. **Ojeda SR, Dissen GA, Malamed S, Hirshfield AN** 1994 A role for neurotrophic factors in ovarian development. In: Hsueh AJW, Schomberg DW (eds) Ovarian Cell Interactions: Genes to Physiology. Springer-Verlag, New York, pp 181–202
4. **Ojeda SR, Dissen GA, Junier M-P** 1992 Neurotrophic factors and female sexual development. In: Ganong WF, Martini L (eds) Frontiers in Neuroendocrinology, Vol 13. Raven Press, New York, pp 120–162
5. **Lara HE, Hill DF, Katz KH, Ojeda SR** 1990 The gene encoding nerve growth factor is expressed in the immature rat ovary: Effect of denervation and hormonal treatment. Endocrinology 126:357–363
6. **Dissen GA, Hill DF, Costa ME, Ma YJ, Ojeda SR** 1991 Nerve growth factor receptors in the peripubertal rat ovary. Mol Endocrinol 5:1642–1650
7. **Dissen GA, Hill DF, Costa ME, Dees WL, Lara HE, Ojeda SR** 1996 A role for *trk*A nerve growth factor receptors in mammalian ovulation. Endocrinology 137:198–209
8. **Lara HE, McDonald JK, Ojeda SR** 1990 Involvement of nerve growth factor in female sexual development. Endocrinology 126:364–375
9. **Lara HE, Dissen GA, Leyton V, Paredes A, Fuenzalida H, Fiedler JL, Ojeda SR** 2000 An increased intraovarian synthesis of nerve growth factor and its low affinity receptor is a principal component of steroid-induced polycystic ovary in the rat. Endocrinology 141:1059–1072
10. **Brawer JR, Naftolin F, Martin J, Sonnenschein C** 1978 Effects of a single injection of estradiol valerate on the hypothalamic arcuate nucleus and on reproductive function in the female rat. Endocrinology 103:501–512
11. **Schulster A, Farookhi R, Brawer JR** 1984 Polycystic ovarian condition in estradiol valerate-treated rats: Spontaneous changes in characteristic endocrine features. Biol Reprod 31:587–593
12. **Franks S** 1995 Polycystic ovary syndrome. N Engl J Med 333:853–861
13. **Lobo RA** 1996 A unifying concept for polycystic ovary syndrome. In: Chang RJ (ed) Polycystic Ovary Syndrome. Springer, New York, pp 334–352

14. **Strauss JF, III, Dunaif A** 1999 Molecular mysteries of polycystic ovary syndrome. Mol Endocrinol 13:800–805
15. **Ehrmann DA, Barnes RB, Rosenfield RL** 1995 Polycystic ovary syndrome as a form of functional ovarian hyperandrogenism due to dysregulation of androgen secretion. Endocr Rev 16:322–353
16. **Yen SSC** 1991 Chronic anovulation caused by peripheral endocrine disorders. In: Yen SSC, Jaffe RB (eds) Reproductive Endocrinology: Physiology, Pathophysiology and Clinical Management. Vol 3. W. B. Saunders, Co., Philadelphia, pp 576–630
17. **Erickson GF** 1991 Folliculogenesis in polycystic ovary syndrome. In: Dunaif A, Givens JR, Haseltine FP, Merriam GR (eds) Polycystic Ovary Syndrome. Blackwell Scientific, Boston, pp 111–128
18. **Nelson VL, Legro RS, Strauss III JF, McAllister JM** 1999 Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries. Mol Endocrinol 13:946–957
19. **Urbanek M, Legro RS, Driscoll DA, Azziz R, Ehrmann DA, Norman RJ, Strauss JF, III, Spielman RS, Dunaif A** 1999 Thirty-seven candidate genes for polycystic ovary syndrome: Strongest evidence for linkage is with follistatin. Proc Natl Acad Sci USA 96:8573–8578
20. **Martínez-Serrano A, Lundberg C, Horellou P, Fischer W, Bentiage C, Campbell K, McKay RDG, Mallet J, Björklund A** 1995 CNS-derived neural progenitor cells for gene transfer of nerve growth factor to the adult rat brain: Complete rescue of axotomized cholinergic neurons after transplantation into the septum. J Neurosci 15:5668–5680
21. **Garcia-Rudaz C, Armando I, Levin G, Escobar ME, Barontini M** 1998 Peripheral catecholamine alterations in adolescents with polycystic ovary syndrome. Clin Endocrinol 49:221–228
22. **Rage F, Hill DF, Sena-Esteves M, Breakefield XO, Coffey RJ, Costa ME, McCann SM, Ojeda SR** 1997 Targeting transforming growth factor a expression to discrete loci of the neuroendocrine brain induces female sexual precocity. Proc Natl Acad Sci USA 94:2735–2740
23. **Martínez-Serrano A, Björklund A** 1997 Immortalized neural progenitor cells for CNS gene transfer and repair. Trends Neurosci 20:530–538
24. **Selden RF, Skoskiewicz MJ, Howie KB, Russell PS, Goodman HM** 1987 Implantation of genetically engineered fibroblasts into mice: implications for gene therapy. Science 236:714–718
25. **Gage FH, Kawaja MD, Fisher LJ** 1991 Genetically modified cells: applications for intracerebral grafting. Trends Neurosci 14:328–333
26. **Renfranz PJ, Cunnningham MG, McKay RDG** 1991 Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. Cell 66:713–729
27. **Dissen GA, Newman Hirshfield A, Malamed S, Ojeda SR** 1995 Expression of neurotrophins and their receptors in the mammalian ovary is developmentally regulated: changes at the time of folliculogenesis. Endocrinology 136:4681–4692
28. **Taniuchi M, Johnson Jr EM** 1985 Characterization of the binding properties, and retrograde axonal transport of a monoclonal antibody directed against the rat nerve growth factor receptor. J Cell Biol 101:1100–1106
29. **Resko JA, Ploem JG, Stadelman HL** 1975 Estrogens in fetal and maternal plasma of the rhesus monkey. Endocrinology 97:425–430
30. **Resko JA, Norman RL, Niswender GD, Spies HG** 1974 The relationship between progestins and gonadotropins during the late luteal phase of the menstrual cycle in rhesus monkeys. Endocrinology 94:128–135
31. **Resko JA, Malley A, Begley D, Hess DL** 1973 Radioimmunoassay of testosterone during fetal development of the rhesus monkey. Endocrinology 93:156–161
32. **Ellinwood WE, Resko JA** 1983 Effect of inhibition of estrogen synthesis during the luteal phase on function of the corpus luteum in rhesus monkeys. Biol Reprod 28:636–644
33. **Alleva E, Aloe L, Cirulli F, Seta DD, Tirassa P** 1996 Serum NGF levels increase during lactation and following maternal aggression in mice. Physiol Behav 59:461–466
34. **Bonini S, Lambiase A, Angelucci F, Magrini L, Manni L, Aloe L** 1996 Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. Proc Natl Acad Sci USA 93:10955–10960
35. **Dees WL, Hiney JK, Schultea TD, Mayerhofer A, Danilchik M, Dissen GA, Ojeda SR** 1995 The primate ovary contains a population of catecholaminergic neuron-like cells expressing nerve growth factor receptors. Endocrinology 136:5760–5768
36. **D'Albora H, Lombide P, Ojeda SR,** Intrinsic neurons in the rat ovary: An immunohistochemical study. Cell Tissue Res, in press
37. **Badoyannis HC, Sharma SC, Sabban EL** 1991 The differential effects of cell density and NGF on the expression of tyrosine hydroxylase and dopamine b-hydroxylase in PC12 cells. Mol Brain Res 11:79–87
38. **Hallböök F, Ibañez CF, Persson H** 1991 Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6:845–858
39. **Bibel M, Hoppe E, Barde Y-A** 1999 Biochemical and funcitonal interactions between the neurotrophin receptors *trk* and $p75^{NTR}$. EMBO J 18:616–622
40. **Tessarollo L** 1998 Pleiotrophic functions of neurotrophins in development. Cytokine Growth Factor Rev 9:125–137
41. **Martin-Zanca D, Oskam R, Mitra G, Copeland T, Barbacid M** 1989 Molecular and biochemical characterization of the human *trk* proto-oncogene. Mol Cell Biol 9:24–33
42. **Kaplan DR, Martin-Zanca D, Parada LF** 1991 Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product induced by NGF. Nature 350:158–160
43. **Szeberényi J, Erhardt P** 1994 Cellular components of nerve growth factor signaling. Biochim Biophys Acta 1222:187–202
44. **Benedetti M, Levi A, Chao MV** 1993 Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci USA 90:7859–7863
45. **Hantzopoulos PA, Suri C, Glass DJ, Goldfard MP, Yancopoulos GD** 1994 The low affinity NGF receptor, p75, can collaborate with each of the trks to potentiate functional responses to the neurotrophins. Neuron 13:187–201
46. **Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA** 1994 Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265:1596–1599
47. **Dechant G BY-A** 1997 Signalling through the neurotrophin receptor $p75^{NTR}$. Curr Opin Neurobiol 7:413–418
48. **Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhäuser N, Böhm-Matthaei R, Baeuerle PA, Barde Y-A** 1996 Selective activation of NF-kB by nerve growth factor through the neurotrophin receptor p75. Science 272:542–545
49. **Bredesen DE, Rabizadeh S** 1997 $p75^{NTR}$ and apoptosis: Trk-dependent and Trk-independent effects. Trends Neurosci 20:287–290
50. **Lara HE, Ferruz JL, Luza S, Bustamante DA, Borges Y, Ojeda SR** 1993 Activation of ovarian sympathetic nerves in polycystic ovary syndrome. Endocrinology 133:2690–2695
51. **Lawrence Jr IE, Burden HW** 1980 The origin of the extrinsic adrenergic innervation to the rat ovary. Anat Rec 196:51–59
52. **Barria A, Leyton V, Ojeda SR, Lara HE** 1993 Ovarian steroidal response to gonadotropins and b-adrenergic stimulation is enhanced in polycystic ovarian syndrome. Role of the sympathetic innervation. Endocrinology 133:2696–2703
53. **Brawer J, Richard M, Farookhi R** 1989 Pattern of human chorionic gonadotropin binding in the polycystic ovary. Am J Obstet Gynecol 161:474–480
54. **Convery M, McCarthy GF, Brawer JR** 1990 Remission of the polycystic ovarian condition (PCO) in the rat following hemiovariectomy. Anat Rec 226:328–336
55. **Aguado LI, Petrovic SL, Ojeda SR** 1982 Ovarian b-adrenergic receptors during the onset of puberty: characterization, distribution, and coupling to steroidogenic responses. Endocrinology 110:1124–1132
56. **Dyer CA, Erickson GF** 1985 Norepinephrine amplifies human chorionic gonadotropin-stimulated androgen biosynthesis by ovarian theca-interstitial cells. Endocrinology 116:1645–1652
57. **Martínez-Serrano A, Fischer W, Björklund A** 1995 Reversal of age-dependent cognitive impairments and cholinergic neuron atrophy by NGF-secreting neural progenitors grafted to the basal forebrain. Neuron 15:473–484
58. **Martínez-Serrano A, Fischer W, Söderström S, Ebendal T, Björklund A** 1996 Long-term functional recovery from age-induced spatial memory impairments by nerve growth factor gene transfer to the rat basal forebrain. Proc Natl Acad Sci USA 93:6355–6360
59. **Gharani N, Waterworth DM, Batty S, White D, Gilling-Smith C, Conway GS, McCarthy M, Franks S, Williamson R** 1997 Association of the steroid synthesis gene CYP11a with polycystic ovary syndrome and hyperandrogenism. Hum Mol Genet 6:397–402