Nerve Growth Factor Induces the Expression of Functional FSH Receptors in Newly Formed Follicles of the Rat Ovary

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The neurotrophin nerve growth factor (NGF) and its two membrane-anchored receptors are expressed in the developing ovary before the organization of the first primordial follicles. In the absence of NGF, the growth of primordial follicles is retarded, indicating that NGF contributes to facilitating early follicular development. The present experiments were undertaken to determine whether NGF can also be involved in the differentiation process by which ovarian follicles become responsive to gonadotropins. Treatment of 2-d-old rat ovaries in organ culture with NGF increased FSH receptor (FSHR) mRNA within 8 h of exposure. This effect was cAMP-independent but additive to the cAMP-mediated increase in FSHR gene expression induced by either forskolin or vasoactive intestinal peptide, a neurotransmitter previously shown to induce FSHR formation in neonatal rat ovaries. After NGF treatment, the ovary acquired the capacity of responding to FSH with cAMP formation and preantral follicular growth, indicating that exposure to the neurotrophin resulted in the formation of biologically active FSHRs. Quantitative measurement of FSHR mRNA demonstrated that the content of FSHR mRNA is reduced in the ovaries of mice carrying a null mutation of the NGF gene. These results indicate that one of the functions of NGF in the developing ovary is to facilitate the differentiation process by which early growing follicles become gonadotropin-dependent during postnatal life, and that it does so by increasing the synthesis of FSHRs.

THE FORMATION OF ovarian follicles in rats and mice is a postnatal event that occurs in a temporally restricted fashion. Though, in rats, most follicles are assembled between 48 and 72 h after birth (1, 2), follicular formation in many strains of mice is initiated on the day of birth (3). After the organization of oocytes and somatic cells into primordial follicles, the newly formed follicles undergo a second (and subtler) differentiation process, in which the flattened pregranulosa cells surrounding each oocyte acquire a cuboidal morphology (4–6). The resulting primary follicles begin to grow via proliferation of these granulosa cells, a process heralded by the expression of proliferating cell nuclear antigen (PCNA) (5), a cofactor of DNA polymerase δ and cyclin-dependent kinase complexes involved in cell cycle progression (7). The activation of PCNA expression, as well as the incorporation of bromodeoxyuridine into the DNA of proliferating granulosa cells (8), precedes the initial enlargement of the oocyte that occurs when the oocytes are already surrounded by at least nine cuboidal granulosa cells (6), *i.e.* after granulosa cells have undergone at least one round of mitotic division (4, 5). As follicles reach the two-layer stage, granulosa cells undergo a third differentiation process that confers them responsiveness to pituitary gonadotropins (4, 9–12). Both this process and that of primordial follicular growth have been shown, by several lines of evidence (9, 10, 13–16) [including the genetic ablation of the genes encoding the FSH-β subunit (17), the FSH receptor (FSHR) (18, 19), and the LH receptor (20)], to occur independently of pituitary gonadotropins.

Instead, it seems that both events are controlled by signaling molecules that, produced within the ovary itself (and arriving at the gland via the extrinsic innervation) induce the formation of FSHRs, the first of the gonadotropin receptors to be expressed in the developing ovary (11). A key intracellular messenger involved in the formation of FSHR is cAMP (21, 22), which can also induce the formation of LH receptors in immature granulosa cells (23). Activation of cAMP synthesis in neonatal rat ovaries results in formation of biologically active FSHRs (24), as assessed by the ability of FSH to induce cAMP formation on its own and to facilitate follicle growth, after pretreatment of the ovary with forskolin, a stimulator of adenylate cyclase activity. Because stimulation of cAMP synthesis by vasoactive intestinal peptide (VIP, a neurotransmitter peptide) and isoproterenol (a β-adrenergic receptor agonist) also induce FSHR formation (24), it has been postulated that VIP and norepinephrine are two of the signaling molecules responsible for the activation of cAMP formation (and thus FSHR expression) in growing preantral follicles (24). Evidence also exists suggesting the existence of cAMP-independent mechanisms regulating the formation of FSHR in immature granulosa cells (25–27). Specifically, activin [a member of the TGF-β superfamily present in neonatal rat ovaries (28)] has been implicated as one of the cAMP-independent factors involved in stimulating the formation of FSHR in granulosa cells in culture (22, 25, 27) and as a factor that may cooperate with cAMP-dependent mechanisms in the induction of FSHR formation during early follicular development (25).

Abbreviations: FSHR, FSH receptor; KO, knockout; NE, norepinephrine; NGF, nerve growth factor; nt, nucleotide(s); PCNA, proliferating cell nuclear antigen; VIP, vasoactive intestinal peptide; semi-QRT, semi-quantitative RT; WT, wild-type.

In a recent study (29), we observed that the development of primary and secondary follicles is reduced in mice carrying a null mutation of the nerve growth factor (NGF) gene. This observation, coupled to the previous findings that NGF and its two membrane-anchored receptors are present in the immature rat ovary (30), and that immunoneutralization of NGF actions during neonatal life results in a subsequent delay in antral follicle growth (31), prompted us to explore the possibility of an involvement of NGF in the acquisition of FSHR by the developing ovary. A partial account of these findings has appeared (32).

Materials and Methods

Animals

Pregnant Sprague Dawley rats were purchased from B & K Universal (Fremont, CA). Upon arrival, at 14 d of gestation, they 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) and were given *ad libitum* access to food (Purina laboratory chow; Ralston Purina Co., St. Louis, MO) and tap water. Mutant C57BL/6-AB1 mice, carrying one deleted NGF allele (NGF+/−) (33), were bred to B6D2F1/J mice (The Jackson Laboratory, Bar Harbor, ME) (29). The heterozygous progeny was then employed to generate the NGF knockout (KO) (NGF−/−) mice used in this study. The pups derived from this out-crossing consistently survived to 2 wk of age, while still exhibiting the phenotype of the original −/− mutant animals (33). The ovaries of wild-type (WT) (NGF+/+) and −/− mice were collected on postnatal d 7 (day of birth = d 0) for FSHR mRNA measurement, *i.e.* after completion of follicular assembly (1–3) and at a time when follicular development has become gonadotropin-dependent (6, 34). The gonads were processed for RNA extraction after the genotype of each animal was confirmed by PCR analysis of tail DNA. Detection of NGF null mutants was accomplished using oligodeoxynucleotide primers recognizing DNA sequences contained in both the targeting cassette and the endogenous NGF gene (33).

Organ culture

The ovaries used to examine the potential effects of NGF on FSHR formation were from 2-d-old rats. As previously reported (24), ovaries of this age contain a sizable pool of newly formed follicles originated at the time of definitive ovarian histogenesis, which, in the rat, occurs between 48 and 72 h after birth (1, 2). The glands were dissected under aseptic conditions, placed on sterile lens paper, and cultured on metal grids at the interface of air/culture medium (35), under an atmosphere of 60% O_2-35% N_2-5% CO_2. This gas composition was shown earlier to maximize the survival of ovaries in organ culture (36). Two glands per well were cultured in 24-well plates; each well contained 750 μl DMEM: F-12 (50% vol/vol) medium supplemented with glucose (4.5 g/l), penicillin (100 U/ml), and streptomycin (100 μg/ml), as reported (24, 35). The time of culture was between 8 and 32 h, depending on the condition studied. In the case of short-term 8-h cultures, the ovaries were collected at the end of this period, frozen in dry ice, and stored at −85 C until RNA extraction. When the cultures were maintained for 32 h, the medium was changed at 8 and 20 h from the time the ovaries were placed in culture, and stored at −20 C for cAMP measurement. The ovaries were collected at 32 h, fixed in Kahle's fixative (8), embedded in paraffin, serially sectioned at 6 μm, and stained for morphometric analysis as described below.

Treatments

The treatment with neurotrophins and activators of cAMP synthesis were initiated at the time of placing the ovaries in culture. To study the effect of NGF on cAMP formation and/or FSHR gene expression, the glands were exposed for 8 h to NGF (Invitrogen/Life Technologies, Inc., Carlsbad, CA) at 100 ng/ml. Other ovaries were treated with forskolin (Sigma, St. Louis, MO; 1 and 20 μM) to stimulate endogenous cAMP formation, or the neuropeptide VIP (Sigma; 2 and 10 μM), previously shown to stimulate cAMP formation (35) and to be a potent inducer of FSHR synthesis in neonatal rat ovaries (24). All treatments were carried out in the presence of 0.5 mM isobutylmethylxantine (IBMX; Sigma) to inhibit phosphodiesterase activity. An 8-h treatment period was chosen because FSHR mRNA levels are maximally increased at this interval by activators of FSHR gene expression in neonatal rat ovaries (24). At the end of this 8-h period, the medium was collected for cAMP measurement; and the ovaries, for FSHR mRNA determination (see below).

Because the treatment with NGF increased FSHR mRNA content, additional experiments were performed to determine whether this activational effect was accompanied by the formation of biologically competent FSHRs. Ovaries were first incubated for 8 h in the presence of NGF or VIP (as a positive control) and then (after removing the stimulating agent) for an additional 12-h period in the presence of purified FSH (NIH-ovine FSH-S-16, 500 ng/ml). At this time, the medium was collected for cAMP determination, and the ovaries were incubated for an additional 12 h in the presence of FSH to assess the effect of the gonadotropin on follicular development. For both cAMP measurement and assessment of follicular growth after exposure to FSH, one ovary from each animal was treated, and the other served as the paired control.

Semiquantitative RT-PCR (semi-QRT-PCR)

This procedure was carried out exactly as described for the measurement of FSHR mRNA content in neonatal rat ovaries (24), using the same FSHR cDNA-containing plasmids and identical procedures to prepare RNA, generate sense FSHR RNA standards, perform the RT-PCR, and quantify the results. Fig. 1 depicts one of the standard curves generated using this system (gel in A; regression line in B). The figure also provides an example of a reaction in which FSHR mRNA content in 2-d-old ovaries was increased by an 8-h treatment with forskolin, NGF, or the combination of both agents (Fig. 1C).

Real-time RT-PCR

Availability of this new technology allowed us to more precisely determine ovarian FSHR content without the tedious processing required in semi-QRT-PCR assays. Real-time RT-PCR takes advantage of the 5′ to 3′ exonuclease activity of *Taq* polymerase to accurately detect target DNA sequences (37). In addition to regular gene-specific primers, the reaction contains a gene-specific, internal oligodeoxynucleotide probe labeled with a 5′-reporter fluorescent dye and a downstream 3′-quencher dye. When this fluorescent probe attaches to the target DNA during the annealing cycles, the reporter dye emission is quenched by the proximity of the 3′ dye. However, during each extension cycle, the probe is cleaved by the 5′-to-3′ exonuclease activity of the *Taq* enzyme as the upstream primer is extended, and the 5′ reporter dye is released from the probe. Once separated from the quencher dye, the reporter dye emits its characteristic fluorescence, which is then monitored in real time by a detector. Rather than detecting reaction products after a fixed number of cycles, the instrument detects the point in time when a given PCR product begins to accumulate. An algorithm compares the amount of reporter dye emission with the quenching dye emission every 8.5 sec during the PCR. In addition to generating an amplification plot and establishing an emission threshold, the algorithm calculates the cycle at which each PCR reaches a significant threshold (10 times the SD of the baseline). This value is directly proportional to the number of DNA copies present in the sample.

The procedure was carried out using an IBI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), equipped with a 96-well format thermal cycler. The FSHR primers used for amplification were a 5′ forward primer (5′-CAT-CAC-TGT-GTC-CAA-GGC-CA-3′) corresponding to nucleotides (nt) 1800–1819 in mouse FSHR mRNA (38), and a 3′ reverse primer (5′-TGC-GGA-AGT-TCT-TGG-TGA-AAA-3′) complementary to nt 1880–1900 in this mRNA. Both primers were from Invitrogen/Life Technologies, Inc. The internal fluorescent oligodeoxynucleotide probe (5′-TCT-GTT-CTA-CCC-CAT-CAA-TTC-TTG-TGC-CAA-3′, PE Applied Biosystems), corresponds to nt 1833–1862 in mouse FSHR mRNA and was covalently linked to the fluorescent dye 6-carboxyfluorescein at the 5′ end and the quencher dye 6-carboxy-tetramethylrhodamine at the 3′ end. The sequences of both the amplification primers and the primer probe derive from identical segments in rat (39) and mouse (38) FSHR mRNA (GenBank accession nos. L02842 and AF095642, respectively). They were selected with the

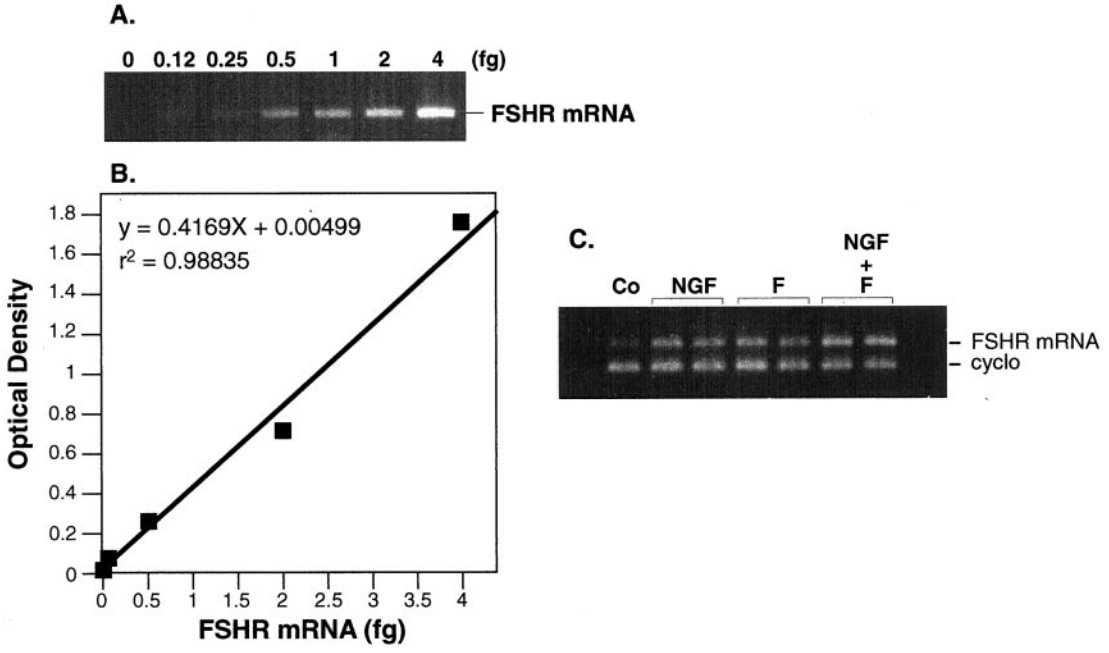

FIG. 1. Increase in steady-state levels of FSHR mRNA in 2-d-old rat ovaries induced by an 8-h exposure to NGF (100 ng/ml), forskolin (F, 1 μM), or the combination of both, as determined by semi-QRT-PCR. A, Ethidium bromide staining of a standard curve generated by RT-PCR amplification of increasing amounts of an *in vitro* transcribed polyadenylated FSHR mRNA fragment corresponding to the identical cellular mRNA sequence targeted for amplification. B, Linear regression analysis of the standard curve shown in A. C, Ethidium bromide staining of a gel illustrating the results of one experiment in which the content of FSHR mRNA-derived PCR product was increased by treatment with NGF, F, and NGF plus F. Cyclo, Fragment of cyclophilin mRNA, a constitutively expressed gene, coamplified with the target FSHR mRNA in each tube to correct the estimated FSHR mRNA values for procedural variability; Co, control, untreated ovaries.

assistance of the program Primers Express (PE Applied Biosystems) provided by Integrated DNA Technology Co. (Santa Clara, CA); 18S ribosomal RNA was used as a normalizing unit for each reaction, using a set of primers purchased as a kit (TaqMan ribosomal RNA Control Reagents kit) from PE Applied Biosystems.

To construct sense FSHR RNA standard curves, an FSHR cDNA fragment identical with that targeted in ovarian tissue for PCR amplification was generated by RT-PCR of total RNA derived from adult mouse ovaries. After extraction with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH), 1 μg total RNA was annealed (5 min at 65 C) to random hexamer primers (1 μl, 3 μg/μl stock solution; Promega Corp., Madison WI) and reverse transcribed with 1 μl SuperScript II (200 U/μl, Invitrogen/Life Technologies, Inc.) for 50 min at 42 C in a 20 μl vol. Thereafter, a 100-bp FSHR DNA fragment was PCR-amplified using the same primers (100 pmol each) designed for real-time PCR, 1 μl of the RT reaction, 0.5 μl Hot Start Tag Polymerase (5 U/μl; QIAGEN, Valencia, CA), and a touchdown PCR program (40). The resulting PCR product was cloned into the pGEM-T vector (Promega Corp.) and sequenced from both ends to verify its identity, using Amplitaq DNA polymerase and a fluorescence-based dideoxy sequencing reaction performed on a PE Applied Biosystems DNA Sequencer model 373A. The DNA template used for the generation of sense FSHR RNA standard consisted of a 426-bp *Pvu*II DNA fragment from pGEM-T containing the 100-bp FSHR cDNA sequence. The transcription reaction was carried out using 1 μl T7 polymerase (25 U/μl) and 400 ng of DNA template. After quantitation of the resulting RNA by spectrophotometry and verification of its integrity in polyacrylamide gels, different amounts (50 fg to 100 ng) were reverse-transcribed, as above, along with the experimental RNA samples (1 μg total ovarian RNA). Thereafter, the real-time PCR were carried out in a total vol of 10 μl, each reaction containing 2 μl RT reaction diluted 1:10 (samples) or 1:1,000 (standards), 5 μl TaqMan Universal PCR Master Mix (PE Applied Biosystems), 250 nM of each FSHR and ribosomal fluorescent probes, 300 nM of each FSHR primer, and 80 nM of each ribosomal primer. The sense FSHR RNA standard curves generated by this procedure contained the equivalent of 0.05–10,000 fg sense RNA input/tube. The real-time PCR program used consisted of an initial annealing period of 2 min at 50 C, followed by 10 min of denaturing at 95 C, and 40 cycles of 15 sec at 95 C and 1 min at 60 C. Each sample was run in triplicate, and the mean values obtained were compared with the FSHR mRNA standard curve to calculate their mRNA content.

cAMP RIA

Release of cAMP by the ovaries, in culture subjected to different treatments, was measured by RIA of the culture media, as described previously (24, 41). Standards and samples were acetylated before the assay to increase the sensitivity of the assay. The assay was carried out using rabbit polyclonal antibodies to cAMP (ICN Biomedicals, Inc., Costa Mesa, CA; 1:200) and ^{125}I-cAMP (Amersham Pharmacia Biotech, Arlington Heights, IL).

Morphological evaluation

Serial 6-μm sections of paraffin-embedded ovaries stained with Weigert's iron hematoxylin and counterstained with picric acid-methylene blue (8) were used for morphometric analysis of follicular development. Every third section was imaged on an Axioplan (Carl Zeiss, Jena, Germany), using a CoolSnap camera (Roper Scientific, Stillwater, MN). Follicles were counted using the manual count feature of MetaMorph (Universal Imaging Co., West Chester, PA). Only follicles in which the nucleus of the oocyte was visible were counted, as recommended (24, 42). A growing follicle was defined as one containing three rows of granulosa cells in at least one region of the cross-section in which the oocyte nucleus was visible. We have previously used this conservative approach to estimate the number of growing follicles present in neonatal ovaries in response to FSH after induction of FSHRs by activators of the cAMP-generating system (24). The total number of growing follicles per ovary was estimated by adding all follicles with three or more layers of granulosa cells present in the serial sections collected from each ovary.

Statistics

A one-way ANOVA, followed by the Student's-Neuman-Keuls test for multiple comparisons, was used to analyze the effect of different 8-h treatments on FSHR mRNA and cAMP production. The paired *t* test was used to compare the effect of treatments on FSH-induced cAMP formation and FSH-induced follicular growth between treated ovaries and their contralateral control glands. Differences in FSHR mRNA content between WT and NGF KO ovaries were analyzed using the *t* test.

Results

NGF increases FSHR mRNA content in neonatal rat ovaries

Exposure of 2-d-old rat ovaries to NGF (100 ng/ml) for 8 h in organ culture resulted in a significant ($P < 0.025$) increase in FSHR mRNA content, as determined by traditional semi-QRT-PCR (Fig. 2). The increase was similar to that produced by 1 μM forskolin. Simultaneous addition of NGF and forskolin (at 100 ng/ml and 1 μM, respectively) resulted in an additive effect ($P < 0.01$) (Fig. 2). Increasing the forskolin dose to 20 μM did not augment FSHR mRNA levels significantly more than did the 1 μM dose, and NGF did not potentiate the forskolin response to levels higher than those achieved when given in combination with the 1-μM dose (Fig. 2).

When a real-time PCR machine became available to us, we repeated part of this experiment by comparing the effect of NGF (100 ng/ml) and forskolin (20 μM) on the FSHR mRNA content of 2-d-old ovaries. Fig. 3A shows real-time amplification profiles generated in this assay using different amounts (0.05–10,000 fg/tube) of *in vitro* transcribed sense FSHR RNA. Fig. 3B depicts the linear regression analysis of the standard curve resulting from these amplifications. Fig. 3C shows real-time PCR amplification profiles demonstrating that, as detected using traditional semi-QRT-PCR, NGF is as effective as forskolin in increasing FSHR mRNA levels. As expected, the abundance of FSHR mRNA in adult ovaries was much greater (more than two orders of magnitude) than in neonatal 2-d-old glands.

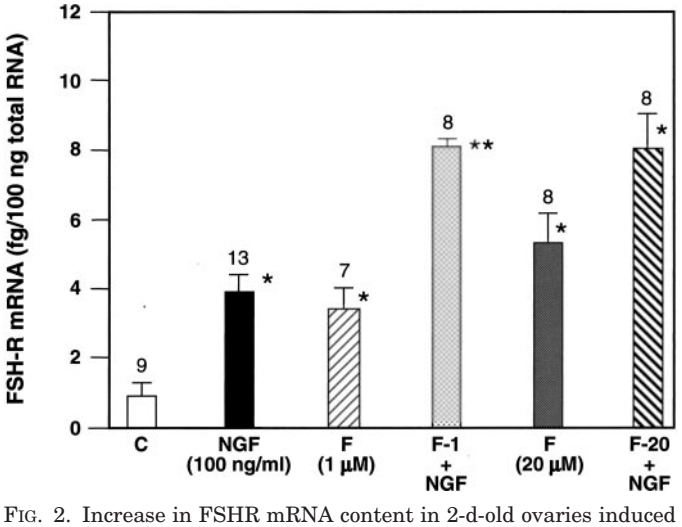

FIG. 2. Increase in FSHR mRNA content in 2-d-old ovaries induced by NGF and F (F-1, 1 μM F; F-20, 20 μM F) after 8 h of treatment in organ culture. Notice the additive effect of NGF and the low (1 μM) F dose. *Numbers above bars* are number of independent observations per group, and *vertical bars* are SEM. *, $P < 0.01$ *vs.* control (C); **, $P < 0.01$ *vs.* NGF and F (1 μM) groups.

The stimulatory effect of NGF on FSHR gene expression is not mediated by cAMP

Release of cAMP into the culture medium was not significantly increased after an 8-h exposure to NGF (Fig. 4A). In contrast, forskolin at 1 μM induced a large (7-fold) increase in cAMP levels; NGF failed to potentiate this effect. Forskolin at 20 μM produced a further, but not proportional, increase in cAMP levels; once again, NGF did not modify this response (Fig. 4A). To determine whether NGF could potentiate the cAMP response to a neuropeptide that increases FSHR gene expression in neonatal ovaries via the cAMP system (24), additional experiments were conducted in which 2-d-old ovaries were exposed to VIP (2 μM) alone or in combination with NGF (100 ng/ml). As was the case with forskolin, NGF failed to potentiate the effect of VIP on cAMP formation (Fig. 4B).

NGF, like VIP, induces the formation of biologically active FSHR in neonatal rat ovaries

To determine whether the increase in FSHR mRNA content caused by NGF was accompanied by an increase in functional FSHRs, 2-d-old ovaries were exposed to NGF for 8 h and then to purified FSH for 12 h. The ability of FSH to induce cAMP formation was then used to estimate the presence of biochemically competent FSHRs. Other ovaries were pretreated with VIP, which has been previously shown to induce the formation of such biologically active FSHRs in neonatal ovaries (24). The results demonstrated that the induction of FSHR mRNA expression by NGF, indeed, endows the ovary with the capacity of producing cAMP in response to FSH (Fig. 5). The response was similar in magnitude to that induced by VIP. As shown before (24), the increase in cAMP accumulation observed after an 8-h exposure to VIP (Fig. 4B) was no longer present after 12 h of culture in the absence of the neuropeptide (Fig. 5)

Next, a morphometric study was conducted to determine whether the newly formed FSHRs coupled to the cAMP-generating system are able to stimulate follicular growth. The same ovaries used for cAMP release were maintained in culture in the continuous presence of FSH for an additional 12 h. At the end of this period, *i.e.* after a 24-h exposure to FSH, the ovaries were serially sectioned, and the number of growing follicles (at least three, partial or complete, rows of granulosa cells) was determined. As shown in Fig. 6, pretreatment with NGF resulted in a significantly greater number of growing follicles in response to FSH, as compared with each control group, including untreated ovaries, ovaries only exposed to FSH for 24 h, and ovaries only exposed to NGF for 8 h. The microphotographs depicted in Fig. 7 illustrate these results. As previously shown (24), the ovaries treated with FSH alone, under these experimental conditions (Fig. 7B), had an aspect similar to that of untreated controls (A) or controls pretreated with NGF, but not receiving FSH afterward (C). In all cases, follicles with more than three layers of granulosa cells were rarely observed (Fig. 7, A–C). In con-

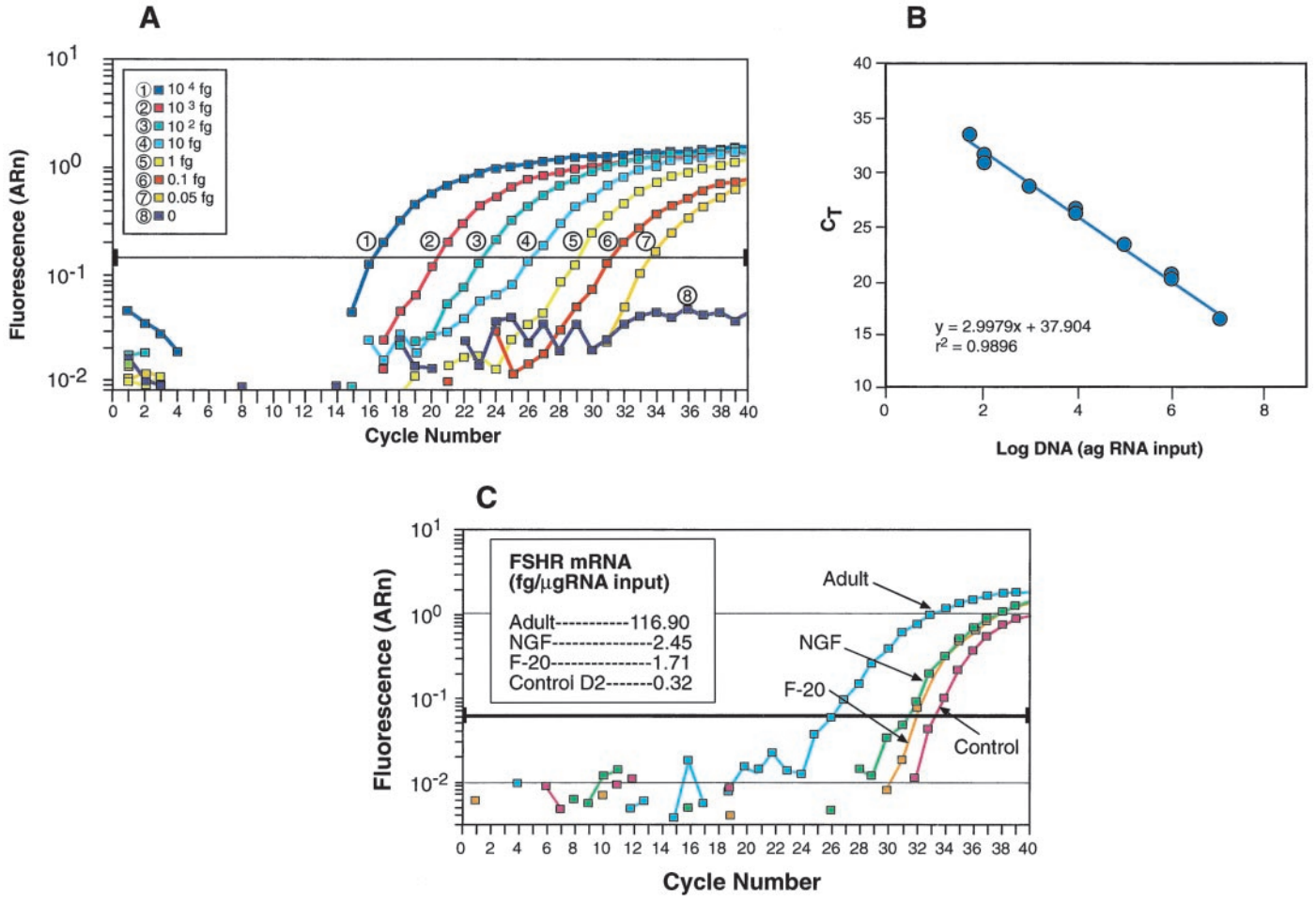

FIG. 3. *In vitro* exposure (8 h) to NGF increases ovarian FSHR mRNA content in 2-d-old rat ovaries, as assessed by real-time PCR. A, Amplification profiles generated using different amounts (0.05–10,000 fg/tube) of *in vitro* transcribed FSHR mRNA. B, Linear regression analysis of the standard curve resulting from the amplifications shown in A. C, Amplification profiles of FSHR target RNA from untreated adult ovaries and neonatal 2-d-old rat ovaries treated for 8 h in organ culture with F (20 μM) or NGF (100 ng/ml) or left untreated (control). Each curve represents the average of triplicate determinations. *Inset*, RNA values calculated by comparing the amplification profiles of FSHR target RNA to those derived from the *in vitro* transcribed sense FSH RNA standard curve shown in B. C_T, Threshold cycle number; which corresponds to the point when the fluorescence accumulates to a level 10 times greater than 1 SD from the basal values. There is a linear relationship between C_T and the logarithm of the starting amount of template. D2, Control, 2-d-old ovaries.

trast, these follicles were more readily detected in ovaries pretreated with NGF before exposure to FSH (Figs. 6 and 7D).

FSHR mRNA content is decreased in the absence of NGF

To determine whether the lack of NGF would result in reduced ovarian FSHR mRNA levels at the time when follicular development becomes gonadotropin-dependent, FSHR mRNA content was determined by real-time PCR in 7-d-old NGF KO mice and WT littermates. As shown in Fig. 8, NGF KOs had significantly ($P < 0.01$) lower levels of ovarian FSHR mRNA than their normal littermates, indicating that NGF is required for the timely formation of FSHR in the developing ovary.

Discussion

The results of this study demonstrate the unexpected involvement of NGF, a growth factor with primary neurotrophic activity, in the process underlying the acquisition of FSHRs by the developing rodent ovary. By identifying this growth factor as a facilitatory component of early ovarian development, the results further the view that acquisition of ovarian responsiveness to gonadotropins requires cell-cell signaling molecules that, operating within the confines of the developing gonad, induce the synthesis of the receptor proteins necessary for gonadotropin actions.

Earlier work, in the rat, showed that the appearance of FSHR precedes that of LH receptors (10) and that FSHR could be first detected during the second half of the first week of postnatal life (10, 12). Because follicular growth proceeds only to the secondary stage in mice lacking the FSHR gene (19), it seems that this is the phase of development during which granulosa cells [the exclusive cellular site of FSHR expression in the mammalian ovary (11, 43)] acquire functional FSHRs. Consistent with this view, a study of human ovaries showed the presence of FSHR mRNA in 50% of the primary and secondary follicles examined (44). Relatively

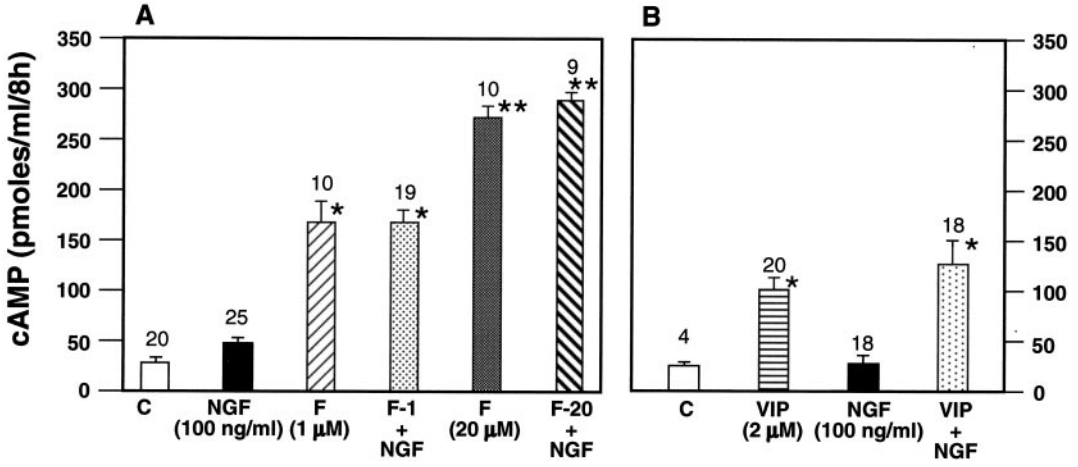

FIG. 4. Inability of NGF to increase cAMP formation in 2-d-old rat ovaries exposed to the neurotrophin for 8 h in organ culture. Notice that both F (A) and VIP (B) increased cAMP levels several fold in the culture medium, and this effect was not modified by the concomitant administration of NGF. *Vertical lines* are SEM, and *numbers above bars* are number of ovaries per group. *, $P < 0.01$ *vs.* control group; **, $P < 0.02$ *vs.* groups treated with F-1 μM.

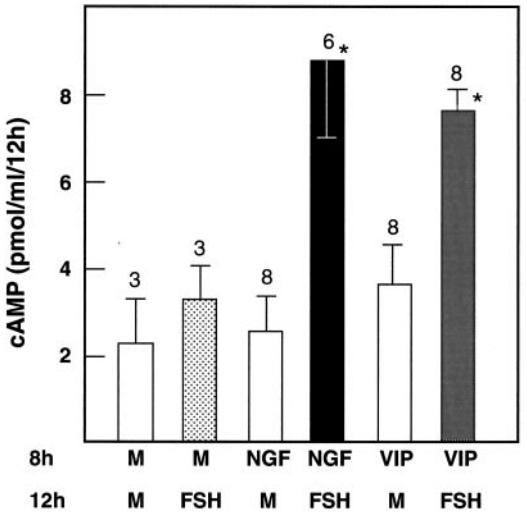

FIG. 5. NGF, like VIP, induces the appearance of functional FSHRs in neonatal 2-d-old rat ovaries, as determined by the ability of FSH to stimulate cAMP formation. After an 8-h pretreatment with NGF (100 ng/ml) or VIP (2 μM) as a positive control, the medium was replaced with fresh medium containing purified FSH (500 ng/ml). After a 12-h period, the medium was collected for cAMP RIA. *, $P < 0.025$ *vs.* groups cultured in medium alone (M) and groups not exposed to FSH regardless of pretreatment. *Vertical lines* are SEM, and *numbers above bars* are number of ovaries per group.

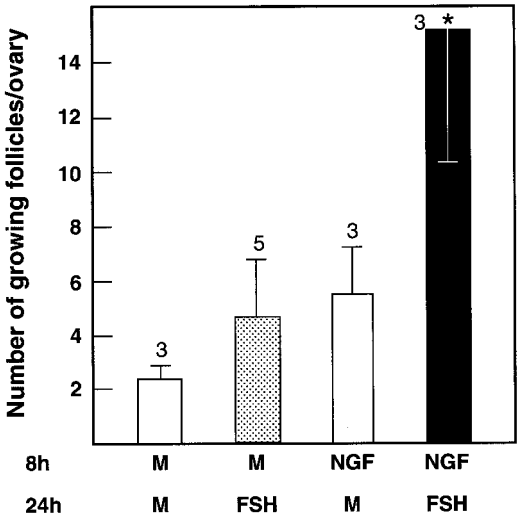

FIG. 6. NGF induces the appearance of biologically active FSHRs in neonatal rat ovaries, as determined by the ability of purified FSH (500 ng/ml) to stimulate follicular growth after a 24-h exposure period in organ culture. The ovaries were pretreated with NGF (100 ng/ml) for 8 h and then with FSH for 24 h. The number of growing follicles, defined as those with three or more layers of granulosa cells, were counted on serial 6-μm sections. M, Medium alone. *Vertical lines* are SEM, and *numbers above bars* are number of ovaries per group. *, $P < 0.05$ *vs.* control group.

little is known about the factors responsible for the activation of FSHR synthesis in immature granulosa cells (45). Administration of cAMP analogs or stimulation of endogenous cAMP formation results in FSHR formation in both isolated granulosa cells (21, 22) and neonatal ovaries in organ culture (24), indicating that factors operating via the cAMP-generating system play a role in the formation of FSHR. The identification of VIP and isoproterenol (a β-adrenergic receptor agonist) as agents able to both induce cAMP formation (24, 35) and expression of functional FSHR in neonatal rat ovaries (24) has led to the suggestion that neurotransmitter-induced cAMP formation is one of the physiological mechanisms responsible for the activation of FSHR formation in developing ovaries. Notwithstanding the potential importance of other factors acting via the cAMP pathway, the involvement of VIP and norepinephrine (NE) is inferentially supported by the fact that they are the only two ligands acting via cAMP-dependent pathways thus far shown to be present in the ovary before the growing follicles become responsive to FSH stimulation (46, 47).

The fact that responsiveness to FSH is attained *in vitro* (see, for instance, Refs. 16 and 48), in the absence of the extrinsic innervation, indicates the participation of either intragonadal sources of VIP/NE (49–51) or the involvement of additional mechanisms in the induction of FSHR synthesis. One such mechanism has been shown to involve activin, a member of

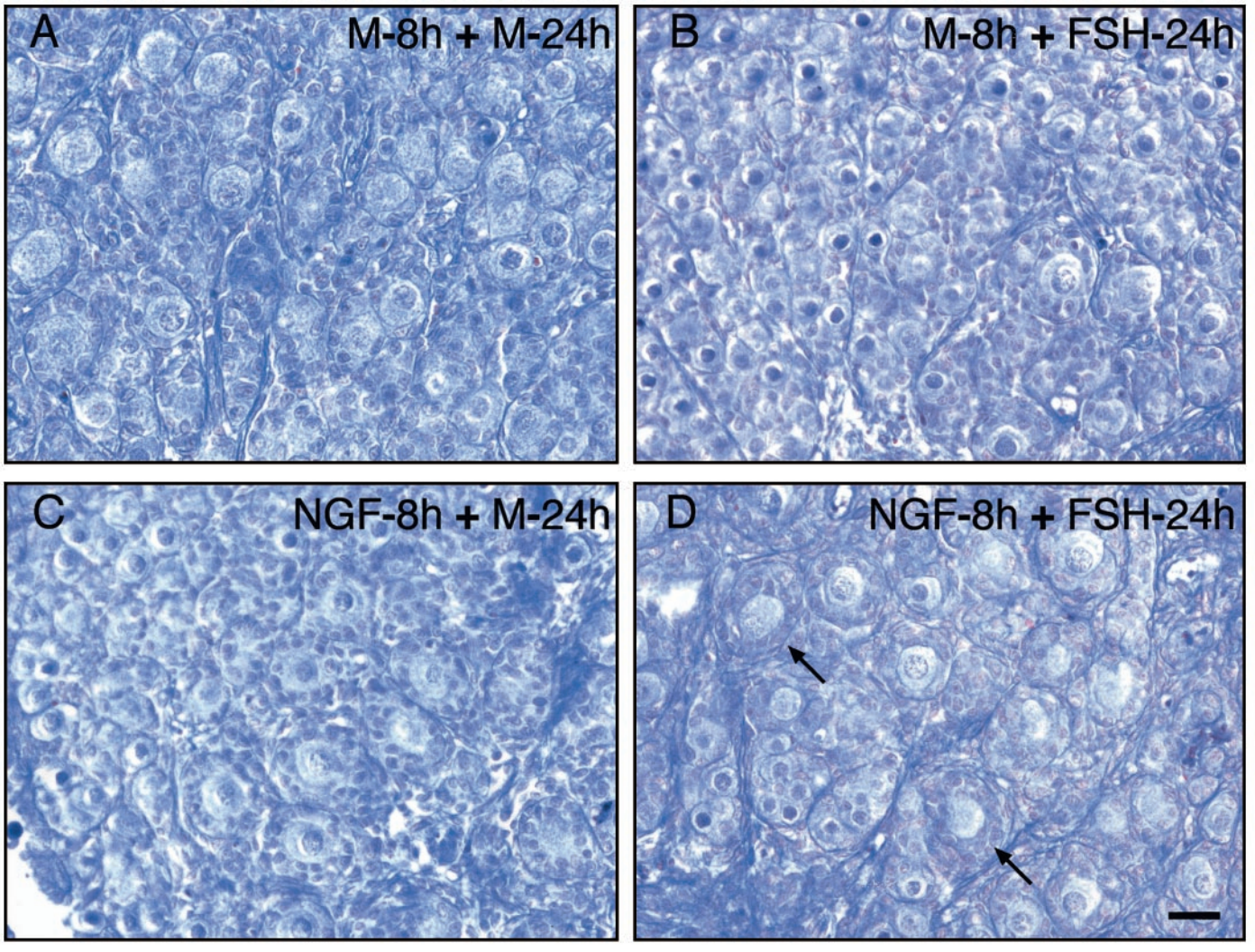

FIG. 7. Histological aspect of 2-d-old rat ovaries cultured for 32 h in medium alone (A), cultured for 8 h in medium alone followed by a 24-h treatment with FSH (500 ng/ml) (B), cultured for 8 h in medium containing NGF (100 ng/ml) followed by 24 h in medium alone (C), or treated with NGF for 8 h and then with FSH for 24 h (D). *Arrows*, Growing follicles with three or more layers of granulosa cells; *bar*, 20 μm.

the TGF-β superfamily of growth factors. Activin increases both FSHR gene expression and the number of FSHRs in immature granulosa cells (22, 25–27). The effect of activin on FSHR occurs in a cAMP-independent manner (25), suggesting that regulation of FSHR synthesis in the developing ovary is determined by both cAMP-dependent and independent mechanisms. That TGF-β itself may also contribute to the acquisition of FSHR by the immature ovary is indicated by the demonstration that TGF-β stimulates granulosa cell growth (52) and differentiation (52, 53), the latter by facilitating FSH-dependent events, such as induction of aromatase activity (52), steroidogenesis (53), and formation of LH receptors (53). As is the case of VIP and NE, activin (54) and TGF-βs (55, 56) are present in the neonatal ovary well before the acquisition of FSHRs and, thus, would be expected to be available for biological actions before the newly formed follicles become gonadotropin-dependent.

Our results identify NGF as a factor able to initiate FSHR synthesis in neonatal rat ovaries, and demonstrate the physiological importance of this effect by showing that ovarian FSHR gene expression is greatly reduced in mutant mice lacking NGF. The stimulatory action of NGF on FSHR expression is manifested after a relatively short time (8 h) and seems to be mediated by intracellular pathways independent of cAMP. Previous studies in neuronal systems have shown that although NGF, on its own, does not induce cAMP accumulation, it facilitates the effect of adenylate cyclase-stimulating agents on cellular responses (57, 58), suggesting that cAMP and NGF may act via complementary pathways to induce cellular differentiation (58). Our results are consistent with this concept because they show that, in the neonatal rat ovary, NGF neither increases cAMP formation on its own nor affects the increase in cAMP levels induced by forskolin or VIP but has an stimulatory effect on FSHR formation additive to that of cAMP.

The biological effects of NGF are mediated by two different membrane-bound receptors: one, termed p75 NGFR or p75 NTR, that recognizes all members of the neurotrophin family with similar affinity (59); and a high-affinity tyrosine kinase receptor, known as trkA, that interacts preferentially

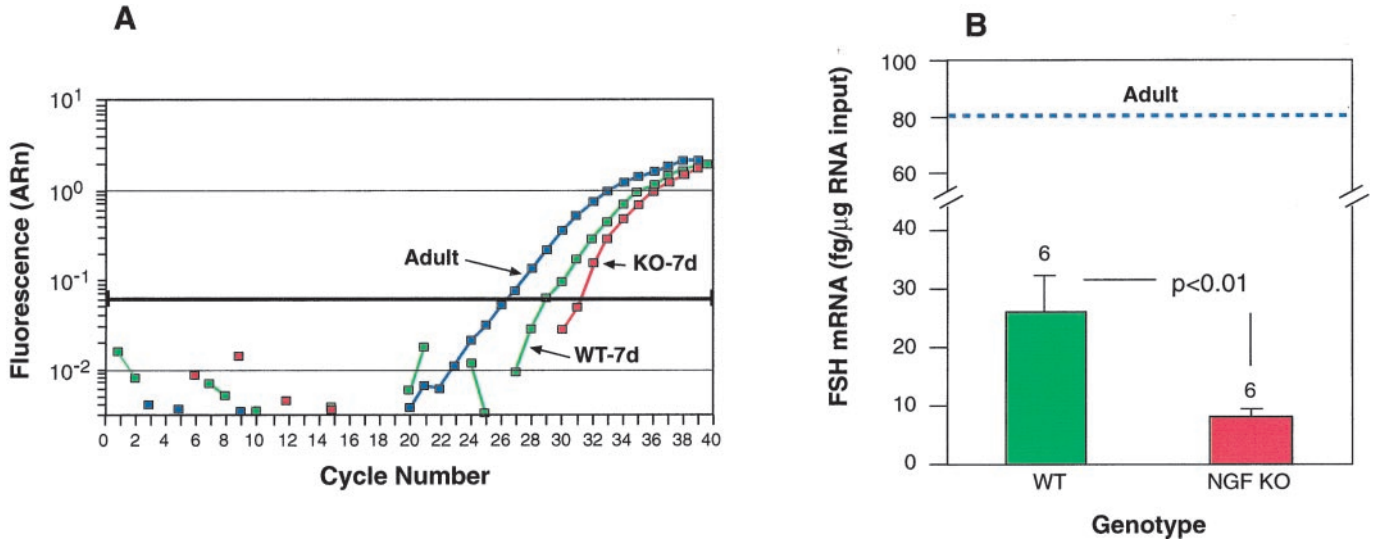

FIG. 8. Reduced content of FSHR mRNA in the ovaries of 7-d-old mutant mice lacking NGF, in comparison with WT mice, as determined by real-time PCR. A, Representative amplification profiles of FSHR target RNA from an adult ovary, and 7-d-old ovaries from an NGF KO mouse and a WT littermate. B, Mean FSHR mRNA content in ovaries from NGF KO and WT mice. *Vertical lines* are SEM, and *numbers above bars* are number of ovaries per group. The *dotted line* labeled Adult represents the FSHR mRNA content detected in a set of adult ovaries analyzed in the same assay as the infantile gonads.

with NGF (60). Both receptors can be detected in the rat ovary as early as the 18th d of gestation (30). Because the facilitatory effect of NGF on cAMP-dependent events in neural cells requires the presence of the high-affinity trkA receptors (58), we favor the idea that the stimulatory action of NGF on FSHR formation is also mediated by trkA receptors. Experiments with ovaries from trkA-deficient mice should clarify this issue.

Though the present experiments demonstrate the ability of NGF to induce the formation of functional FSHR in neonatal ovaries, they do not define the cell-cell mechanism(s) underlying this effect. In rat preovulatory follicles, trkA expression is limited to the mesenchymal (thecal-interstitial) compartments of the ovary (61, 62), but a recent examination of infantile mouse ovaries has shown the presence of both trkA mRNA and trkA immunoreactivity in granulosa cells of a subpopulation of preantral follicles (29), suggesting that at least part of the stimulatory effect of NGF on FSHR synthesis in granulosa cells of newly formed follicles results from a direct action of the neurotrophin on these cells. Because trkA receptors are also expressed in mesenchymal cells of the neonatal-infantile ovary (29), the additional participation of intermediate molecules affecting mesenchymal-to-epithelial cell communication is also plausible. A candidate for such a role may be activin itself, because, during embryonic development of the ovary, the subunits that form the activin protein are synthesized in mesonephric cells (28), the apparent precursors of ovarian mesenchymal cells (63) where trkA receptors are located. Other potential candidates are the TGF-βs, which are synthesized in ovarian mesenchymal cells and regulate granulosa cell growth and differentiation (64, 65); and the transcription of at least one of their encoding genes (TGF-β1) is directly regulated by NGF (66).

Regardless of the cell-cell signaling mechanisms mediating NGF actions on FSHR formation, our findings identify NGF as one of the intragonadal molecules involved in the acquisition of ovarian responsiveness to gonadotropins. In a broader context, the results provide strong support for the relatively new, but increasingly accepted concept that neurotrophins are required not only for neuronal development, survival, and differentiation but also for the unfolding of the very same processes in defined subsets of nonneuronal cells (67). The finding that NGF facilitates the initiation of follicular growth (29), and the present observations showing its involvement in the initial biochemical differentiation of growing follicles, define the developing ovary as one of the nonneuronal, endocrine targets of NGF actions.

Acknowledgments

We thank Ms. Maria Costa for technical help, Mr. Pablo Ojeda for morphometric analysis of follicular development, and Ms. Summer Johnson and Ms. Leela Goodspeed for maintaining our transgenic mouse colony.

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This work was supported by NIH Grants HD-24870 and RR-00163 for the operation of the Oregon Regional Primate Research Center, and by NICHD through cooperative agreement U54 HD-18185 as part of the Specialized Cooperative Centers Program in Reproduction Research. C.R. is a visiting scientist supported by a fellowship from NICHD, TW HD-00668, Fogarty International Training and Research in Population and Health grant. A.P. is a graduate student, Biochemistry Graduate Program, Faculty of Chemistry and Pharmaceutical Sciences, University of Chile, supported by a fellowship from NICHD, TW HD-00668, Fogarty International Training in Population and Health grant.

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References

1. **Malamed S, Gibney JA, Ojeda SR** 1992 Ovarian innervation develops before initiation of folliculogenesis in the rat. Cell Tissue Res 270:87–93
2. **Rajah R, Glaser EM, Hirshfield AN** 1992 The changing architecture of the neonatal rat ovary during histogenesis. Dev Dyn 194:177–192
3. **Eppig JJ, O'Brien MJ** 1996 Development *in vitro* of mouse oocytes from primordial follicles. Biol Reprod 54:197–207
4. **Hirshfield AN** 1991 Development of follicles in the mammalian ovary. Int Rev Cytol 124:43–101
5. **Oktay K, Schenken RS, Nelson JF** 1995 Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. Biol Reprod 53:295–301
6. **Lintern-Moore S, Moore GPM** 1979 The initiation of follicle and oocyte growth in the mouse. Biol Reprod 20:773–778
7. **Xiong Y, Zhang H, Beach D** 1992 D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71:505–514
8. **Hirshfield AN, DeSanti AM** 1995 Patterns of ovarian cell proliferation in rats during the embryonic period and the first three weeks postpartum. Biol Reprod 53:1208–1221
9. **Schwartz NB** 1974 The role of FSH and LH and of their antibodies on follicular growth and on ovulation. Biol Reprod 10:236–272
10. **Smith-White S, Ojeda SR** 1981 Changes in ovarian LH and FSH receptor content and in gonadotropin-induced ornithine decarboxylase activity during prepubertal and pubertal development of the rat. Endocrinology 109:152–161
11. **Richards JS, Jahnsen T, Hedin L, Lifka J, Ratoosh S, Durica JM, Goldring NB** 1987 Ovarian follicular development: from physiology to molecular biology. Recent Prog Horm Res 43:231–270
12. **Sokka T, Huhtaniemi I** 1990 Ontogeny of gonadotrophin receptors and gonadotrophin-stimulated cyclic AMP production in the neonatal rat ovary. J Endocrinol 127:297–303
13. **Peters H, Byskov AG, Lintern-Moore S, Faber M, Andersen M** 1973 The effect of gonadotrophin on follicle growth initiation in the neonatal mouse ovary. J Reprod Fertil 35:139–141
14. **Lamprecht SA, Zor U, Tsafriri A, Lindner HR** 1973 Action of prostaglandin E_2 and of luteinizing hormone on ovarian adenylate cyclase, protein kinase and ornithine decarboxylase activity during postnatal development and maturity in the rat. J Endocrinol 57:217–233
15. **Ben-Or S** 1963 Morphological and functional development of the ovary of the mouse. I. Morphology and histochemistry of the developing ovary in normal conditions and after FSH treatment. J Embryol Exp Morphol 11:1–11
16. **Wandji S-A, Srsen V, Voss AK, Eppig JJ, Fortune JE** 1996 Initiation *in vitro* of growth of bovine primordial follicles. Biol Reprod 55:942–948
17. **Kumar TR, Wang Y, Lu N, Matsuk MM** 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15:201–204
18. **Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM** 2000 The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. Endocrinology 141:1795–1803
19. **Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P** 1998 Impairing follicle-stimulating hormone (FSH) signaling *in vivo*: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci USA 95:13612–13617
20. **Zhang F-P, Poutanen M, Wilbertz J, Huhtaniemi I** 2001 Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. Mol Endocrinol 15:172–183
21. **Knecht M, Ranta T, Catt KJ** 1983 Granulosa cell differentiation *in vitro*: induction and maintenance of follicle-stimulating hormone receptors by adenosine 3′,5′-monophosphate. Endocrinology 113:949–956
22. **Tano M, Minegishi T, Nakamura K, Karino S, Ibuki Y, Miyamoto K** 1997 Transcriptional and post-transcriptional regulation of FSH receptor in rat granulosa cells by cyclic AMP and activin. J Endocrinol 153:465–473
23. **Ranta T, Knecht M, Darbon J-M, Baukal AJ, Catt KJ** 1984 Induction of granulosa cell differentiation by forskolin: stimulation of adenosine 3′,5′-monophosphate production, progesterone synthesis, and luteinizing hormone receptor expression. Endocrinology 114:845–850
24. **Mayerhofer A, Dissen GA, Costa ME, Ojeda SR** 1997 A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. Endocrinology 138:3320–3329
25. **Nakamura M, Nakamura K, Igarashi S, Tano M, Miyamoto K** 1995 Interaction between activin A and cAMP in the induction of FSH receptor in cultured rat granulosa cells. J Endocrinol 147:103–110
26. **Hasegawa T, Miyamoto K, Abe Y, Nakamura T, Sugino H, Eto Y, Shibai H, Igarashi M** 1988 Induction of follicle stimulating hormone receptor by erythroid differentiation factor on rat granulosa cell. Biochem Biophys Res Commun 156:668–674
27. **Xiao S, Robertson DM, Findlay JK** 1992 Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 131:1009–1016
28. **Kaipia A, Toppari J, Huhtaniemi I, Paranko J** 1994 Sex difference in the action of activin-A on cell proliferation of differentiating rat gonad. Endocrinology 134:2165–2170
29. **Dissen GA, Romero C, Newman Hirshfield A, Ojeda SR** 2001 Nerve growth factor is required for early follicular development in the mammalian ovary. Endocrinology 142:2078–2086
30. **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
31. **Lara HE, McDonald JK, Ojeda SR** 1990 Involvement of nerve growth factor in female sexual development. Endocrinology 126:364–375
32. **Ojeda SR, Romero C, Tapia V, Dissen GA** 2000 Neurotrophic and cell-cell dependent control of early follicular development. Mol Cell Endocrinol 163: 67–71
33. **Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD, Phillips HS** 1994 Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76: 1001–1011
34. **Peters H** 1969 The development of the mouse ovary from birth to maturity. Acta Endocrinol (Oxf) 62:98–116
35. **George FW, Ojeda SR** 1987 Vasoactive intestinal peptide enhances aromatase activity in the neonatal rat ovary before development of primary follicles or responsiveness to follicle-stimulating hormone. Proc Natl Acad Sci USA 84: 5803–5807
36. **Fainstat T** 1968 Organ culture of postnatal rat ovaries in chemically defined medium. Fertil Steril 19:317–338
37. **Gibson UEM, Heid CA, Williams PM** 1996 A novel method for real-time quantitative RT-PCR. Genome Res 6:995–1001
38. **Tena-Sempere M, Manna PR, Huhtaniemi I** 1999 Molecular cloning of the mouse follicle-stimulating hormone receptor complementary deoxyribonucleic acid: functional expression of alternatively spliced variants and receptor inactivation by a C566T transition in exon 7 of the coding sequence. Biol Reprod 60:1515–1527
39. **Sprengel R, Braun T, Nikolics K, Segaloff DL, Seeburg PH** 1990 The testicular receptor for follicle-stimulating hormone: structure and functional expression of cloned cDNA. Mol Endocrinol 4:525–530
40. **Hecker KH, Roux KH** 1996 High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. Biotechniques 20:478–485
41. **Ojeda SR, Urbanski HF, Katz KH, Costa ME** 1988 Prostaglandin E_2 releases luteinizing hormone-releasing hormone from the female juvenile hypothalamus through a Ca^{2+}-dependent, calmodulin-independent mechanism. Brain Res 441:339–351
42. **Mayerhofer A, Weis J, Bartke A, Yun JS, Wagner TE** 1990 Effects of transgenes for human and bovine growth hormones on age-related changes in ovarian morphology in mice. Anat Rec 227:175–186
43. **Hsueh AJW, Adashi EY, Jones PBC, Welsh THJ** 1984 Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr Rev 5:76–127
44. **Oktay K, Briggs D, Gosden RG** 1997 Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. J Clin Endocrinol Metab 82:3748–3751
45. **Findlay JK, Drummond AE** 1999 Regulation of the FSH receptor in the ovary. Trends Endocrinol Metab 10:183–188
46. **Advis JP, Ahmed CE, Ojeda SR** 1989 Direct hypothalamic control of vasoactive intestinal peptide (VIP) levels in the developing rat ovary. Brain Res Bull 22:605–610
47. **Ben-Jonathan N, Arbogast LA, Rhoades TA, Bahr JM** 1984 Norepinephrine in the rat ovary: ontogeny and *de novo* synthesis. Endocrinology 115:1426–1431
48. **Funkenstein B, Nimrod A, Lindner HR** 1980 The development of steroidogenic capability and responsiveness to gonadotropins in cultured neonatal rat ovaries. Endocrinology 106:98–106
49. **Gozes I, Tsafriri A** 1986 Detection of vasoactive intestinal peptide-encoding messenger ribonucleic acid in the rat ovaries. Endocrinology 119:2606–2610
50. **Lawrence Jr IE, Burden HW** 1980 The origin of the extrinsic adrenergic innervation to the rat ovary. Anat Rec 196:51–59
51. **Aguado LI, Ojeda SR** 1984 Prepubertal ovarian function is finely regulated by direct adrenergic influences. Role of the adrenergic innervation. Endocrinology 114:1845–1853
52. **Bendell JJ, Dorrington J** 1988 Rat thecal/interstitial cells secrete a transforming growth factor-β-like factor that promotes growth and differentiation in rat granulosa cells. Endocrinology 123:941–948
53. **Dodson WC, Schomberg DW** 1987 The effect of transforming growth factor-β on follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. Endocrinology 120:512–516
54. **Roberts VJ, Sawchenko PE, Vale W** 1991 Expression of inhibin/activin subunit messenger ribonucleic acids during rat embryogenesis. Endocrinology 128:3122–3129
55. **Roy SK, Hughes J** 1994 Ontogeny of granulosa cells in the ovary: lineage-specific expression of transforming growth factor β2 and transforming growth factor β1. Biol Reprod 51:821–830
56. **Levacher C, Gautier C, Saez J-M, Habert R** 1996 Immunohistochemical lo-

calization of transforming growth factor β1 and β2 in the fetal and neonatal rat ovary. Differentiation 60:45–51
57. **Berg KA, Maayani S, McKay R, Clarke WP** 1995 Nerve growth factor amplifies cyclic AMP production in the HT4 neuronal cell line. J Neurochem 64:220–228
58. **Heidemann SR, Joshi HC, Schechter A, Fletcher JR, Bothwell M** 1985 Synergistic effects of cyclic AMP and nerve growth factor on neurite outgrowth and microtubule stability of PC12 cells. J Cell Biol 100:916–927
59. **Raffioni S, Bradshaw RA, Buxser SE** 1993 The receptors for nerve growth factor and other neurotrophins. Annu Rev Biochem 62:823–850
60. **Chao MV, Hempstead BL** 1995 p75 and Trk: a two-receptor system. Trends Neurosci 18:321–326
61. **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
62. **Mayerhofer A, Dissen GA, Parrott JA, Hill DF, Mayerhofer D, Garfield RE, Costa ME, Skinner MK, Ojeda SR** 1996 Involvement of nerve growth factor in the ovulatory cascade: *Trk*A receptor activation inhibits gap-junctional communication between thecal cells. Endocrinology 137:5662–5670
63. **Makabe S, Naguro T, Nottola SA, Pereda J, Motta PM** 1991 Migration of germ cells, development of the ovary, and folliculogenesis. In: Familiari G, Makabe S, Motta PM, eds. Ultrastructure of the ovary. Norwell, MA: Kluwer Academic Publishers; 1–27
64. **Skinner MK, Keski-Oja J, Osteen KG, Moses HL** 1987 Ovarian thecal cells produce transforming growth factor-β which can regulate granulosa cell growth. Endocrinology 121:786–792
65. **Gitay-Goren H, Kim I-C, Miggans ST, Schomberg DW** 1993 Transforming growth factor β modulates gonadotropin receptor expression in porcine and rat granulosa cells differently. Biol Reprod 48:1284–1289
66. **Kim S-J, Park K, Rudkin BB, Dey BR, Sporn MB, Roberts AB** 1994 Nerve growth factor induces transcription of transforming growth factor-β1 through a specific promoter element in PC12 cells. J Biol Chem 269:3739–3744
67. **Tessarollo L** 1998 Pleiotrophic functions of neurotrophins in development. Cytokine Growth Factor Rev 9:125–137