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A Role for *Trk*A Nerve Growth Factor Receptors in Mammalian Ovulation*

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ABSTRACT

Several members of the neurotrophin (NT) family, including nerve growth factor (NGF), NT-3, and NT-4/5, are expressed in the mammalian ovary. As their respective receptor tyrosine kinases are also found in the gland, the possibility exists that NTs act directly on the gonads to exert effects unrelated to their support of the ovarian innervation. We now report that *trk*A, the NGF receptor tyrosine kinase, is involved in the acute activational process that leads to the first ovulation. The *trk*A gene becomes transiently expressed in periovulatory follicles at the time of the first preovulatory surge of gonadotropins at puberty; the increase in *trk*A messenger RNA (mRNA) content is dramatic (>100-fold), but transient (~9 h). No such changes in *trk*B or *trk*C mRNA were observed; the abundance of these mRNAs, which encode the receptor tyrosine kinases for NT-4/5 and brain-derived neurotrophic factor, and NT-3, respectively, remained at very low levels throughout puberty. *In vivo* and *in vitro* experiments demonstrated that the activation of *trk*A gene expression is brought about by the proestrous discharge of LH. The increase in *trk*A mRNA levels is mainly localized to cells of the follicular wall and interstitial tissue of the ovary. NGF mRNA abundance also increases at proestrus, with peak values detected about 5 h before ovulation; as in the case of *trk*A mRNA, NGF mRNA was found in thecal-interstitial cells. Both *trk*A and NGF protein, detected by immunohistochemistry, were localized to this same ovarian compartment. Interleukin-1β (IL-1β), a putative mediator of LH action, enhances both *trk*A and NGF gene expression in ovarian cells, an effect prevented by IL-1ra, a natural IL-1β receptor antagonist. IL-1β also stimulates PGE_2 release, and this effect was inhibited by both NGF antibodies and a *trk* receptor blocker; NGF antibodies administered *in vivo* attenuated the increase in ovarian PGE_2 synthesis that antedates ovulation. Immunoneutralization of NGF action or pharmacological blockade of *trk* tyrosine kinase activity targeted to one ovary resulted in the ipsilateral inhibition of ovulation. The remarkably narrow time frame of *trk*A gene activation at the completion of follicular growth suggests that NGF acting as a neuroendocrinotrophic factor in a developmentally restricted manner contributes to the acute cytodifferentiation process that leads to the first ovulation in mammals. (*Endocrinology* **137:** 198–209, 1996)

NERVE GROWTH factor (NGF) is recognized by two different membrane-spanning receptor molecules, one displaying rapid dissociation kinetics, known as low affinity NGF receptor or p75 NGFR, and another with a slow dissociation rate, known as *trk*A (1, 2). Although p75 NGFR binds all other neurotrophins (NTs), including brain-derived neurotropic factor (BDNF), NT-3, and NT-4/5, with similar low affinity (1, 3), the *trk*A receptor binds NGF preferentially and with high affinity (4, 5). Consistent with the absence of known signaling motifs in its intracellular domain, p75 NGFR appears unable to mediate NGF actions on its own (6, 7). In contrast, *trk*A, which is endowed with a tyrosine kinase domain similar to that of other receptor tyrosine kinases (8, 9), has been shown to mediate the biological effects of NGF via activation of a signaling pathway similar to that activated by mitogenic receptor tyrosine kinases (10).

Although it was initially believed that expression of the *trk*A gene was restricted to the central and peripheral nervous systems (11), it is becoming increasingly evident that NGF-mediated activation of *trk*A receptors may contribute to regulating nonneural functions, most noticeably those pertaining to differentiation and proliferative events within the endocrine and immune systems. For example, NGF induces the differentiation of bipotential somatotrophs into PRL-secreting cells (12), induces the morphological differentiation of pancreatic β-cells (13, 14), and stimulates the proliferation and differentiation of lymphocytes and granulocytes (15, 16). That these actions involve the participation of *trk*A receptors is indicated by the presence of the receptors in all nonneural cell types affected by NGF (12, 14, 17, 18).

The physiological mechanisms that may lead to activation of the NGF-dependent signaling cascade in nonneural cells are unknown. At least in the case of the immune system, the activating factors may be similar to those prompting nonneural cells, such as Schwann cells, fibroblasts, and astrocytes, to synthesize NGF in response to injury of the peripheral and central nervous systems (19–21). A drawback of such an interpretation is that it would predict an involvement of NGF only in the cellular response to injury and not in normal homeostatic regulation. There is, however, a physiological series of events that resembles an inflammatory re-

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action and occurs not in response to injury but as a consequence of hormonal stimulation. This is the ovulatory process (22), which is initiated at puberty and occurs throughout the functional lifespan of the ovary in a cyclic, repetitive, and hormonally driven manner (22). In the present report, we demonstrate that the first ovulation is preceded by a remarkable, but transient, activation of *trk*A and NGF gene expression in cells of the follicular wall, and that activation of this NGF/*trk*A neurotropic complex is an integral component of the biochemical process of follicular cytodifferentiation that leads to ovulation. A preliminary report of these results has appeared (23).

Materials and Methods

Animals

Sprague-Dawley rats 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). Food (Purina laboratory chow, Ralston-Purina, St. Louis, MO) and water were provided *ad libitum*.

In vivo procedures

To characterize the pattern of *trk*A and NGF gene expression at the time of puberty, the ovaries were collected at the end of the juvenile period (postnatal days 28–29) and during the peripubertal phase (days 32–40). Peripubertal rats were classified in different phases of puberty according to criteria previously reported (24).

To accelerate ovarian development and induce a periovulatory LH surge, rats were given a single sc injection of PMSG (8 IU/rat) on days 26–27 of age, and the ovaries were collected at several intervals thereafter. In some experiments, granulosa cells and the rest of the ovary (termed residual ovary) were separated (25) before RNA measurement.

Two series of *in vivo* experiments were carried out to define the consequences of blocking NGF actions on peripubertal ovarian function. In one of them, PMSG-injected rats also received an iv injection (7 μl/g BW) of a rabbit anti-NGF serum (see below) between 1300–1400 h on the day of the expected endogenous LH surge, and the ovaries were collected at 1700 and 2100 h, *i.e.* 56 and 60 h after PMSG administration, for PGE_2 measurement. Trunk blood was collected for LH assay. Control animals were injected with preimmune serum or normal rabbit serum. The dose of NGF antiserum used has been previously shown to prevent the development of ovarian innervation when injected into neonatal rats (26). In the other series, PMSG-treated rats received, on the morning of the day of the expected endogenous surge of LH, an intrabursal injection of either NGF antibodies (10 μl) or K-252a, a *trk* tyrosine kinase blocker (27) (10 μl of a 100 μM solution in saline-5% dimethylsulfoxide; Kamiya Biomedical Co., Thousand Oaks, CA). As fluids injected intrabursally leak through slits located near the uterotubal junction (28), the bursa was ligated between the ovary and the fallopian tube, as previously described (28). Leakage was monitored by including trypan blue (0.08%) in the injected solutions. Animals in which leakage was noted were not included in the study. For each animal, one ovary received the test substance, and the other was injected with either preimmune serum or the K-252a diluent; the treatments were alternated between the left and right ovaries. Ovulatory rate was assessed the next day by counting corpora lutea under a dissecting microscope. The effects observed were verified by histological examination of 5-μm hematoxylin-eosin-stained sections.

NGF antiserum

The NGF antiserum used in the present study (K-596) was previously shown to block development of the ovarian sympathetic innervation when administered ip to newborn rats (26). Additional experiments were performed to further characterize this antiserum.

Recognition of NGF and other NTs in immunoblots. The different NTs, including NGF, BDNF, NT-3, and NT-4/5, were applied at 200 ng to PBS-moistened nitrocellulose (Schleicher and Schuell, Keene, NH) using a Schleicher and Schuell slot blotter. The membrane was then soaked in 4% paraformaldehyde for 1 h, washed three times with PBS, blocked for 1.5 h in 8% dried milk in PBS, and reacted with various dilutions (1:250 to 1:2,000) of NGF antiserum for 3 h. The membranes were then washed with PBS (three times for 15 min each time) and exposed to goat anti-rabbit horseradish peroxidase (diluted 1:20,000) for 1 h. After another washing cycle, the blots were developed with HRP Color Development Reagent (Bio-Rad Laboratories, Richmond, CA) as recommended by the manufacturer. Although the antiserum strongly reacted with NGF at all dilutions tested (Fig. 1), it also recognized antigenic determinants present in each of the other NTs, with a NGF $\gg$ BDNF > NT-3 > NT-4/5 order of potency.

Blockade of NT-induced neurite extension in PC12 cells expressing trkA or trkB receptors. To examine the ability of NGF antiserum K-596 to block NGF action, we used two variants of PC12 cells (JW and CB), with essentially identical results. Although both variants derive from the original pheochromocytoma cell line described by Greene and Tischler (29), they differ somewhat in their morphological and adhesive properties because of the different culture conditions under which they have been grown by various laboratories. CB-PC12 cells (29) were obtained from the Cell Culture Facility (Vollum Institute for Advanced Biomedical Research, Portland, OR). The JW-PC12 cells (30) were provided by Dr. John Wagner (Department of Neurology and Neurosciences, Cornell University Medical School, New York, NY). Because antiserum K-596 also recognizes, to a lesser degree, the other members of the NT family, it was important to determine whether the antiserum was able to antagonize the neurotropic effect of NT-3, BDNF, and NT-4/5 on PC12 cells. For this purpose, a PC12 cell variant engineered to stably express the rat *trk*B gene (31) (kindly provided by Dr. George Yancopoulos, Regeneron Pharmaceuticals, New Jersey) was used.

All PC12 cell variants were maintained in DMEM containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% horse serum, and 5% FBS in a 5% CO_2 environment at 37 C. For neurite extension studies, the cells were seeded at a density of 5000 cells/well in 24-well plates. After 3 days in culture, the medium was changed to DMEM containing 1% heat-inactivated horse serum and either NGF, BDNF, or NT-4/5 at 50 ng/ml each or NT-3 at 500 ng/ml. Some wells were left untreated to serve as controls, and others were treated with one of the above NTs in the presence of K-596 NGF antiserum at dilutions ranging from 1:250 to 1:2000. The cells were monitored for 4 days, at which time they were fixed in 4% paraformaldehyde and stored in PBS at 4 C until photographed to document the effect of the NGF antiserum on NT-induced neurite outgrowth. Phase contrast photomicrographs were obtained using an ICM-405 Zeiss inverted microscope (Carl Zeiss, Inc., New York, NY).

All dilutions of the antiserum tested (1:250 to 1:2000) were effective in blocking the neurite outgrowth induced by NGF in either CB cells (not shown) or JW cells. The *upper panels* of Fig. 2 show the neurotropic effect of NGF on JW cells (B) and the suppressive effect of the antiserum at a 1:2000 dilution (C). In contrast, the antiserum administered at an even

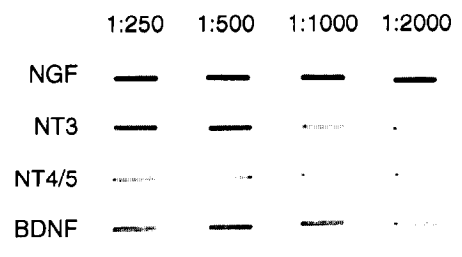

FIG. 1. Immunoblot demonstrating the preferential recognition of NGF by antiserum K-596 and the ability of this antiserum to recognize, to a lesser extent, other NTs, including BDNF, NT-3, and NT-4/5. Each purified NT was loaded on a nitrocellulose membrane at 200 ng/slot and reacted with the indicated dilutions of the antiserum. Notice the much more pronounced reactivity of the antiserum with NGF than with any of the other NTs.

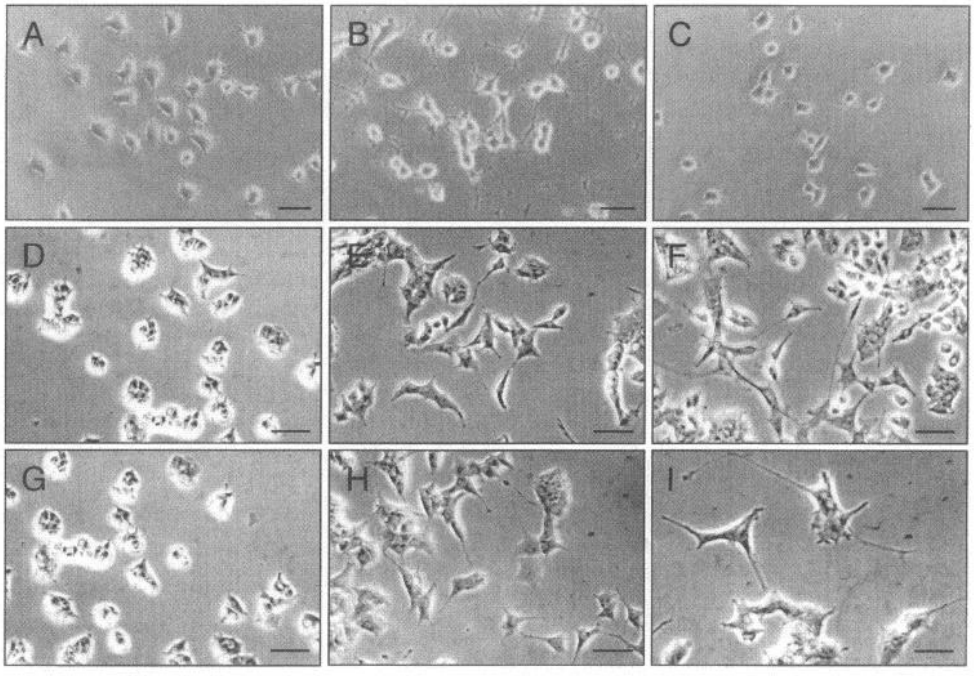

FIG. 2. Ability of antiserum K-596 to block NGF-induced neurite outgrowth in PC12 cells. A, Untreated cells (JW strain). The cells were exposed for 4 days to NGF (50 ng/ml) in the absence (B) or presence (C) of antiserum K-596 at a 1:2000 dilution. D and G (that depict slightly different fields of the same culture well) show PC12 cells stably expressing *trk*B receptors (31). E and H illustrate the stimulatory effect of BDNF and NT-4/5, respectively, on neurite outgrowth in *trk*B-expressing PC cells. F and I demonstrate the inability of antiserum K-596 (1:500) to inhibit the neurite outgrowth induced by BDNF (F; 50 ng/ml) or NT-4/5 (I; 50 ng/ml) in these cells. Notice that *trk*B-expressing PC12 cells become flatter after exposure to these NTs, giving the false impression of a higher magnification. *Bars* = 50 μm.

higher dose (1:500) failed to affect the neurite outgrowth induced by BDNF (D, E, and F) or NT-4/5 (G, H and I) on PC12/*trk*B-expressing cells. A high dose of NT-3 (500 ng/ml) did not induce neurite outgrowth (not shown), precluding an assessment of the antiserum's ability to block the neurotropic effects of NT-3. Thus, at the doses tested, antiserum K-596 can selectively block the biological activity of NGF without interfering with that of NTs acting via *trk*B receptors. Conceivably, however, higher doses of the antibody may be able to block the biological effects of NTs other than NGF.

Cell culture

Ovaries were collected from 26- to 27-day-old rats, and cell dispersates were prepared and cultured as described by Hernandez *et al.* (32). The thecal-interstitial cell-enriched suspension (which also contains some granulosa cells) was plated (2×10^6 cells/well) on six-well plates in 2 ml McCoy's 5A medium plus 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO). The cells were incubated for up to 48 h before treatment for 3 and 6 h with FSH or hCG (National Hormone and Pituitary Program, NIDDK; 50 ng/ml each). FSH was used as a control for hormone and cell specificity. As the cell dispersates used are enriched in interstitial-thecal cells, which lack FSH receptors, FSH would not be expected to affect *trk*A gene expression on these cells. On the other hand, contaminating granulosa cells in the preparation should be able to respond to FSH, if FSH is involved in eliciting the proestrous increase in ovarian *trk*A gene expression.

In other experiments, cells were treated with interleukin-1β (IL-1β; the generous gift of Dr. Eli Adashi; 10 ng/ml) alone or in the presence of a naturally occurring IL receptor antagonist (33, 34), rabbit anti-NGF serum or K252a. At the end of the incubation, the medium was collected for PGE_2 measurement, and total RNA was extracted from the cells (35) for ribonuclease (RNase) protection assay (see below).

Nucleic acid probes

The antisense RNA probes used in these studies were complementary to nucleotides (nt) 269–752 in *trk*A messenger RNA (mRNA) (36), nt 2154–2585 in *trk*B mRNA (37), nt 1897–2266 in *trk*C mRNA (38), nt 430–705 in p75 NGFR mRNA (39), nt 702-1025 in NGF mRNA (40), nt 641-1085 in BDNF mRNA (41), nt 659–880 in NT3 mRNA (42), and nt 338–469 in cyclophilin mRNA (43). They were synthesized from template complementary DNAs (cDNAs) cloned into the riboprobe vectors pGEM or pBluescript. The complementary RNA (cRNA) transcripts were radiolabeled with [^{32}P]CTP for RNA blot hybridization and RNase protection assay, and with [^{35}S]UTP for hybridization histochemistry (*trk*A and NGF). Preparation of templates for transcription and the transcription procedure itself were performed as previously reported (44, 45). Cyclophilin mRNA, which is constitutively expressed in the ovary (46), was used as an internal marker to normalize the results of RNA blot hybridization and RNase protection assay. In RNA blot hybridizations, cyclophilin mRNA was detected with a [^{32}P]deoxy-CTP-labeled (47) 644-bp DNA fragment complementary to nt 39–683 in cyclophilin mRNA, derived from the cDNA p1B15 (43).

Preparation of RNA

Total RNA was prepared by the phenol-extraction method for tissues (48) or cells (35); when needed, polyadenylated [poly(A)$^+$] RNA was isolated by a microbatch procedure (49), as described previously (44, 45).

RNA blot hybridization

The procedures used for RNA blotting and hybridization have been described in detail previously (44, 45). Autoradiographic signals were analyzed using an Agfa flatbed scanner and the computer program NIH-Image written by Dr. Wayne Rasband, NIH. An edited version was provided by Dr. Cary Mariash, University of Minnesota, which yields integrated optical densities following a user-specified method of subtraction of the background (50).

RNase protection assay

The RNase protection assay was carried out according to the method of Gilman (51), as previously described (52). Sense RNA standards were prepared using the templates described above. The sense RNAs were then transcribed, purified, and quantified according to previously described procedures (52). The ^{32}P-labeled cRNAs of interest were simultaneously hybridized to total RNA extracted from ovaries or cells. After RNase digestion, the protected species were isolated by electrophoresis in a 5% polyacrylamide-7 M urea gel. To visualize the protected fragments, the gels were dried and exposed to Kodak XAR-5 film at −85 C. RNase protection assay autoradiographic signals were analyzed as described above. When detecting *trk*A mRNA in the same gel as NGF and NT-3 mRNAs, the substantial increase in *trk*A mRNA that occurs on the afternoon of proestrus increased the background signal for NGF and

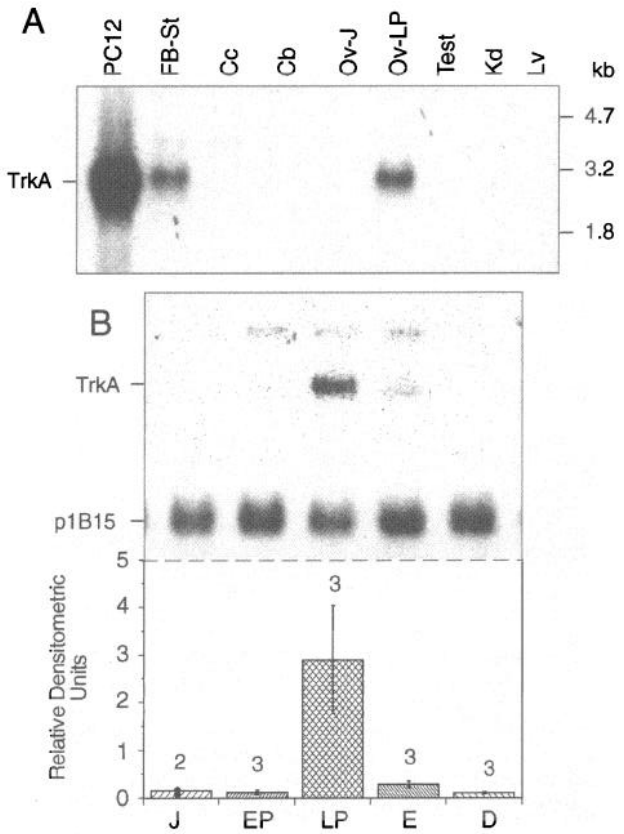

FIG. 3. Detection of *trk*A mRNA in peripubertal rat ovaries by RNA blot hybridization. A, Autoradiogram demonstrating that the message detected in the ovary of late proestrus (Ov-LP) rats is identical in size to that present in PC12 cells and basal forebrain-striatum (FB-St). Each lane contains 5 μg poly(A)$^+$ RNA. Cc, Cerebral cortex; Cb, cerebellum; Ov-J, juvenile ovary; Test, adult testis; Kd, kidney; Lv, liver. B, *Upper panel*, Autoradiogram of a representative Northern blot showing the changes in ovarian *trk*A mRNA content during the onset of rat puberty. Each lane contains 5 μg poly(A)$^+$ RNA extracted from the ovaries of 6–10 rats. B, *Lower panel*, Quantitation of the changes in *trk*A mRNA content depicted in B, *upper panel*. *Numbers above bars* represent the number of independent observations per group. J, Juvenile period; EP, early proestrus; LP, first (late) proestrus; E, first estrus; D, first diestrus.

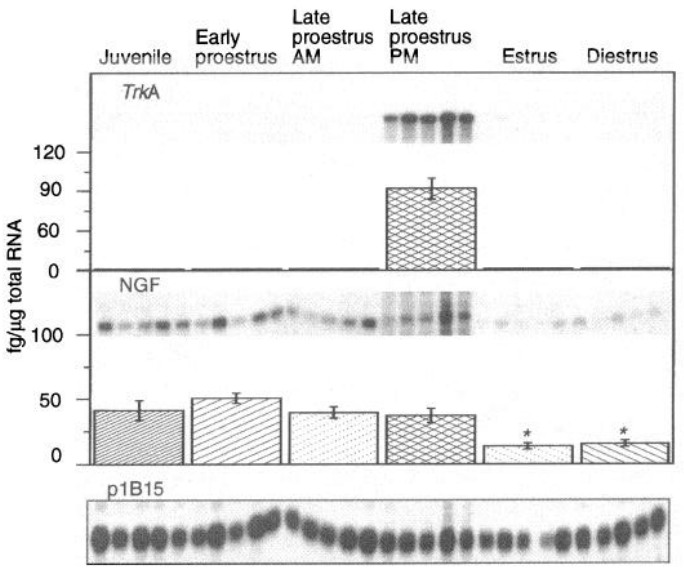

FIG. 4. Changes in *trk*A and NGF mRNA abundance in the rat ovary during the onset of puberty, as determined by RNase protection assay. *Trk*A mRNA content increased dramatically in the afternoon of late proestrus (AM = 0900 h; PM = 1700 h). NGF mRNA levels were relatively unchanged throughout puberty, but decreased after ovulation (*, $P < 0.05$; *middle panel*). As expected, juvenile and early proestrous animals had low serum levels of LH (pooled value, 38.8 ± 3.6 ng/ml; n = 7), late proestrous PM animals showed surge levels of LH (657 ± 111 ng/ml; n = 5), and estrous animals again had basal LH levels (28.0 ± 2.8 ng/ml; n = 5). Each *bar* represents the mean ± SEM of five rats per group. Each lane represents the RNase-protected fragments from 20 μg total RNA.

NT-3 mRNA measurements in those particular lanes. To correct for this difference, the background noise in these lanes was subtracted separately.

Hybridization histochemistry

The procedure employed was based on the method of Simmons *et al.* (53) with modifications as previously reported (54). Cellular expression of *trk*A and NGF mRNAs was determined in ovaries collected on the afternoon of the first proestrus or in the evening of the second day after a single PMSG injection, *i.e.* at the time of the endogenous surge of LH release triggered by the PMSG treatment. The only deviation from the reported procedure (54) was that we found the best fixation method for ovaries to be immersion in 4% paraformaldehyde-0.1 M sodium borate buffer, pH 9.5 (overnight at 4 C). Control sections were incubated with sense *trk*A or NGF RNA probes.

Immunohistochemistry

Immunohistochemical detection of *trk*A and NGF was performed in 10-μm cryostat sections from ovaries collected at 2100 h on the day of the preovulatory surge of gonadotropins induced by administration of PMSG into 27-day-old rats. The ovaries were fixed by immersion in Zamboni's fixative, as previously described (55), and processed for *trk*A and NGF immunohistochemistry using the polyclonal antisera *trk* 763 (Santa Cruz Biotechnology, Santa Cruz, CA) and K-596 (Ref. 26 and this study) to identify *trk*A and NGF, respectively. According to the manufacturer, antibody *trk* 763 specifically recognizes *trk*A without cross-reacting with other *trk* receptors. Our tests (see above) showed that antibody K-596 preferentially recognizes NGF. Tissue sections were incubated overnight at 4 C with the antibodies at 2 μg/ml (*trk* 763) or in a 1:500 dilution (K-596), and the immunoreaction was developed the next day with a fluorescein isothiocyanate-labeled affinity purified goat antirabbit γ-globulin (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). Visualization of the immunohistochemical reaction was carried out using an Axiovert Zeiss microscope equipped with epifluorescent illumination and an appropriate filter set to visualize fluorescein isothiocyanate (excitation filter, 450–490 nm; barrier filter, 510 nm; emission filter, 525 nm). The immunohistochemical procedure used was based on that reported by Gerfen and Sawchenko (56).

RIA

Serum LH was measured by RIA using kits provided by the NIDDK National Pituitary Agency. The assay was modified when the serum samples came from rats that had been treated with rabbit anti-NGF serum. In this case, the LH-antibody complex was precipitated with IgGsorb (The Enzyme Center, Malden, MA) according to the manufacturer's instructions, *i.e.* by adding 100 μl of a 10% sonicated IgGsorb suspension to each assay tube, followed by a 30-min incubation and centrifugation at 3000 rpm for 20 min to pellet the hormone-antibody complexes. IgGsorb was used to separate bound from free hormone, because the presence of NGF antiserum could interfere with the formation of the hormone-antibody-second antibody complex (57) and thereby give spurious results. Results are expressed in terms of the rat LH RP-1 standard preparations provided with the kit.

PGE_2 levels were measured as previously described (58) in whole

ovaries and culture medium from ovarian cell dispersates. Whole ovaries were homogenized in 1 M citric acid, pH 3.0, and PGE_2 was extracted twice with 5 vol ethyl acetate, as recommended (58). The organic phase of each sample was evaporated to dryness under a nitrogen stream, and the residue was resuspended in assay buffer. The recoveries of [^{3}H]PGE_2 using this procedure averaged 64%.

Data analysis

The differences in mRNA levels detected by Northern blot and RNase protection assay or in hormone concentrations were analyzed using one-way ANOVA and Student-Newman-Keuls multiple range test for individual means.

Results

Identification of ovarian trkA mRNA

The presence of *trk*A mRNA in prepubertal ovaries was first investigated by RNA blot hybridization. A 3.2-kilobase (kb) mRNA species, similar in size to that observed in PC12 cells and rat forebrain-striatum, was detected in late proestrus ovaries, *i.e.* on the day of the first preovulatory surge of gonadotropin, but not at any other phase of puberty (Fig. 3, A and B). None of the control tissues tested, including testes, kidney, liver, cerebral cortex, and cerebellum, had detectable levels of *trk*A mRNA (Fig. 3A).

Changes in ovarian trkA and NGF mRNA content during the onset of puberty

Ovarian *trk*A mRNA was almost undetectable before the afternoon of the first proestrus. At this time, and coinciding with the first preovulatory LH surge, there was a striking increase in mRNA content (Fig. 4). NGF mRNA levels did not change significantly between the juvenile period and the time of the first preovulatory LH surge, but decreased after ovulation ($P < 0.01$), *i.e.* during the first estrous and first diestrous phases of puberty (Fig. 4).

Absence of changes in ovarian trkB and trkC mRNA content and in the content of the mRNAs encoding their respective ligands, BDNF and NT-3, during the onset of puberty

As some forms of *trk*C and *trk*B lack the intracellular kinase domain (37, 38), we used intracellular probes to detect the mRNAs encoding the catalytic forms of these two receptors. Some full-length forms of *trk*C, however, contain insertions of the kinase domain, which compromise the signaling capacity of the receptor (38). *Trk*C forms containing these insertions cannot mediate proliferative or differentiation responses (38). To determine whether the peripubertal ovary expresses functionally active *trk*C receptors, we used a cRNA probe that spans the site where the incapacitating insertions are located (38) (see *Materials and Methods*). RNase protection assays using this probe revealed that both functional and nonfunctional *trk*C mRNA forms are expressed in the peripubertal ovary (100-h exposure; Fig. 5A-c). The prevalence of both types of message was, however, low and did not change throughout the peripubertal period. Both mRNA forms were undetectable after a 16-h film exposure, even on the afternoon of the first proestrus (Fig. 5A-b), during which a substantial increase in *trk*A mRNA was detected (Fig. 5A-a). *Trk*B mRNA levels were similarly low and did not change appreciably at the time of puberty (not shown).

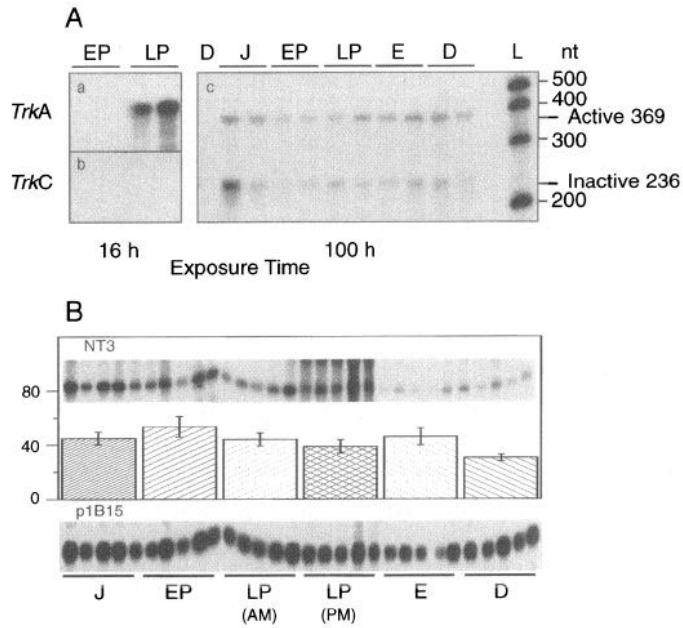

FIG. 5. A, Lack of changes in *trk*C mRNA in the rat ovary during the onset of puberty, as assessed by RNase protection assay. The cRNA probe used detects both the active and inactive (38) forms of full-length *trk*C mRNA (c). The two different film exposures used emphasize the low abundance of the *trk*C message throughout peripubertal development. Notice that no *trk*C mRNA can be detected at an exposure time (16 h) that readily allows detection of the preovulatory increase in *trk*A mRNA (a and b). B, Lack of changes in ovarian NT-3 mRNA levels during the onset of rat puberty. Each *bar* represents the mean ± SEM of five rats per group normalized to the p1B15 signal. Notice that the profile of NT-3 mRNA content is very similar, but not identical, to that of NGF mRNA (see Fig. 4), which was measured in the same RNase protection assay. Although there appears to be a decrease in signals between late proestrous and estrous groups, the apparent difference is caused by the higher background signal in the late proestrous group.

With regard to the preferred ligand for *trk*C, the NT-3 mRNA content was quantitatively similar to that of NGF mRNA and did not change appreciably at the time of puberty (Fig. 5B). On the other hand, the content of BDNF mRNA (which encodes a *trk*B-preferred ligand) was exceedingly low and remained at low values throughout puberty (not shown).

Changes in trkA and NGF mRNA content after treatment with PMSG

Treatment of juvenile animals with PMSG to induce a precocious preovulatory surge of gonadotropins resulted in significantly elevated ovarian *trk*A mRNA levels in the afternoon and evening, but not in the morning, of the day of the induced LH surge (Fig. 6). The initiation of these changes coincided with the LH surge, with peak *trk*A mRNA levels being attained by 2100 h, *i.e.* after the peak of the LH surge (Fig. 6). In contrast to *trk*A mRNA, p75 NGFR mRNA levels, which are already prominent in juvenile animals, were not affected by PMSG (not shown). Although the increase in *trk*A mRNA levels appeared to be monophasic, the NGF mRNA

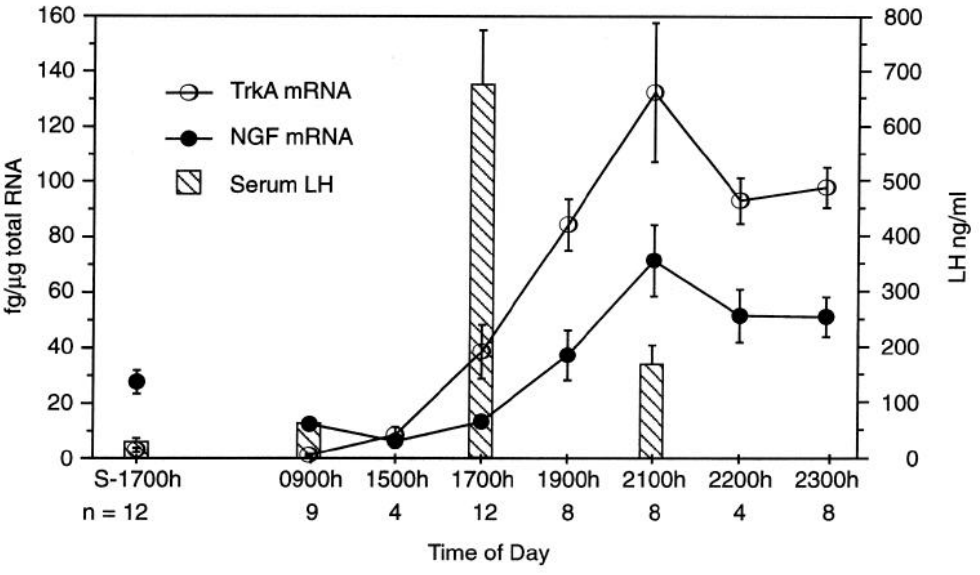

FIG. 6. Changes in *trk*A and NGF mRNA content in ovaries collected at different times on the second day after PMSG or saline injection to 26-day-old rats. The values shown are derived from three independent RNase protection assays. *Circles* represent means, *vertical lines* are the SEM, and n is the number of rats per group. Each observation derives from 20 µg total RNA extracted from the two ovaries of each rat. Serum LH levels were 30.9 ± 7.0 ng/ml in the saline (S)-treated group collected at 1700 h, 63.7 ± 6.0 ng/ml in the PMSG-treated group at 0900 h, and 675.5 ± 99.7 ng/ml in the PMSG-treated group at 1700 h. By 2100 h, LH values had declined to 170.6 ± 35.1.

content first decreased (1500 h) and then rebounded in the evening, after the LH surge, to peak levels that coincided temporally with the peak in *trk*A mRNA levels (Fig. 6).

Cellular localization of trkA and NGF mRNA

*Trk*A mRNA was localized, by hybridization histochemistry, to the thecal compartment of large preovulatory follicles and interstitial tissue of ovaries collected at 2100 h on the day of the LH surge induced by PMSG (Fig. 7, A and C). Little or no signal was detected in granulosa cells. A similar distribution was observed in the ovaries of first proestrus rats undergoing normal puberty (not shown). No hybridization was detected in sections exposed to a sense *trk*A mRNA probe (Fig. 7B). The cellular distribution of NGF mRNA, also assessed in ovaries from PMSG-treated rats (2100 h, day of the preovulatory LH surge), was similar to that of *trk*A mRNA, *i.e.* in interstitial cells and cells of the follicular wall, with little signal in granulosa cells (Fig. 7D).

RNase protection assays performed on RNA extracted from thecal-interstitial cells separated from granulosa cells confirmed this distribution, as the contents of both *trk*A and NGF mRNA were much greater in the residual ovary, consisting of thecal and interstitial cells, than in granulosa cells (data not shown). NT-3 mRNA, which has been shown by *in situ* hybridization to be present in granulosa cells (59), appeared to be equally represented in both compartments when measured by RNase protection assay as an additional control (not shown).

Immunohistochemical detection of trkA and NGF in periovulatory ovaries

In agreement with the cellular localization of their mRNAs, immunoreactive *trk*A and NGF protein were detected in the follicular wall and interstitial tissue of PMSG-treated animals (2100 h, evening of the day of the preovulatory LH surge; Fig. 8).

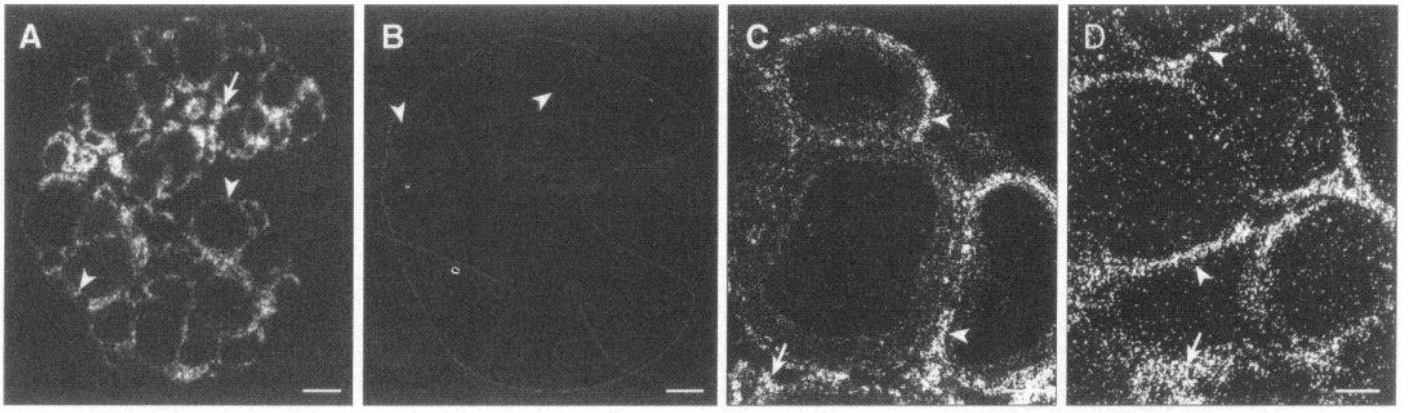

FIG. 7. Localization of *trk*A and NGF mRNAs in the ovary by hybridization histochemistry. The procedure was performed in sections derived from ovaries of PMSG-treated animals collected at 2100 h on the day of the PMSG-induced LH surge. A, *Trk*A mRNA was detected in the wall of preovulatory follicles (*arrowheads*) and in the interstitial tissue (*arrow*). *Bar* = 500 µm. B, Absence of hybridization signal after incubation of ovarian sections with a sense *trk*A RNA probe. The outline of the ovary is shown by a *dotted line*. *Arrowheads* denote two preovulatory follicles. *Bar* = 500 µm. C, Higher magnification view showing the localization of *trk*A mRNA in thecal (*arrowheads*) and interstitial (*arrow*) cells of the ovary. *Bar* = 50 µm. D, Localization of NGF mRNA in the ovary by hybridization histochemistry. As in the case of *trk*A mRNA, the mRNA encoding NGF was detected in cells of the follicular wall (*arrowheads*) and interstitial tissue (*arrow*). *Bar* = 50 µm.

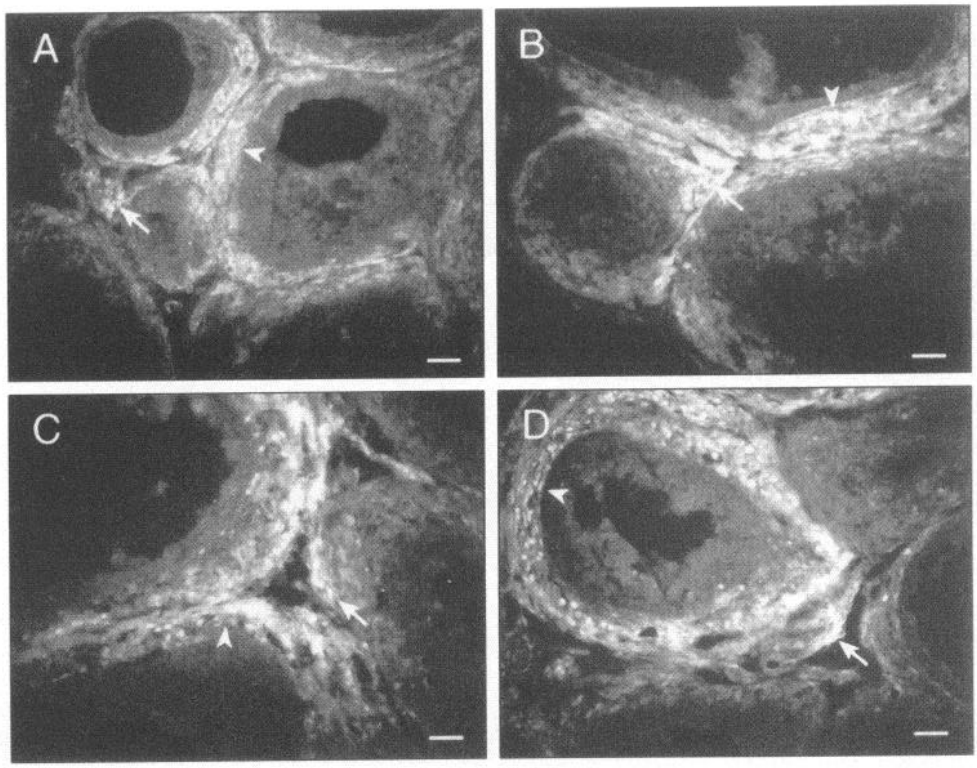

FIG. 8 Detection of *trk*A (A and B) and NGF (C and D) proteins by immunohistochemistry in preovulatory rat ovaries. The ovaries were obtained at 2100 h on the day of the LH surge induced by PMSG treatment. Both *trk*A- and NGF-immunoreactive materials are localized to cells of the follicular wall (*arrowheads*) and interstitial cells (*arrows*). *Bars* = 40 μm.

The periovulatory increase in ovarian trkA mRNA abundance is a LH-dependent phenomenon

Exposure of ovarian cell dispersates enriched in thecal-interstitial cells to hCG resulted in a 10-fold increase in progesterone and androstenedione release from the cells after 3 or 6 h of treatment (data not shown). hCG, but not FSH, increased *trk*A mRNA content after both 3 and 6 h of treatment (Fig. 9), indicating that *trk*A gene expression is not activated by FSH in contaminating granulosa cells or induced indirectly on interstitial-thecal cells by a granulosa cell-mediated effect of FSH. In contrast, NGF mRNA levels decreased after treatment with either FSH ($P < 0.05$; at 6 h) or hCG ($P < 0.05$; at 3 h; Fig. 9). NGF mRNA values had begun to rebound 6 h after hCG, as expected based on the time course of NGF mRNA expression during the PMSG-induced LH surge *in vivo* (see Fig. 6).

IL-1β and the ovarian NGF-trkA complex

IL-1β gene expression is induced by LH in preovulatory ovaries (60), and IL-1β has been shown to be a potent stimulus of NGF (19) and PGE_2 synthesis (61, 62). In view of these findings, we investigated the possibility that the changes in *trk*A mRNA and NGF mRNA observed in the ovary after exposure of the gland to preovulatory LH levels are caused at least in part by IL-1β. Ovarian cell dispersates treated with IL-1β responded within 6 h with an increase in *trk*A and NGF mRNA levels, which was inhibited by the receptor antagonist IL-1ra (Fig. 10A). Although IL-1ra completely inhibited the effect of IL-1β on *trk*A mRNA content, it only partially blocked the increase in NGF mRNA levels induced by IL-1β. As shown by others (61, 62), IL-1β stimulated PGE_2 release

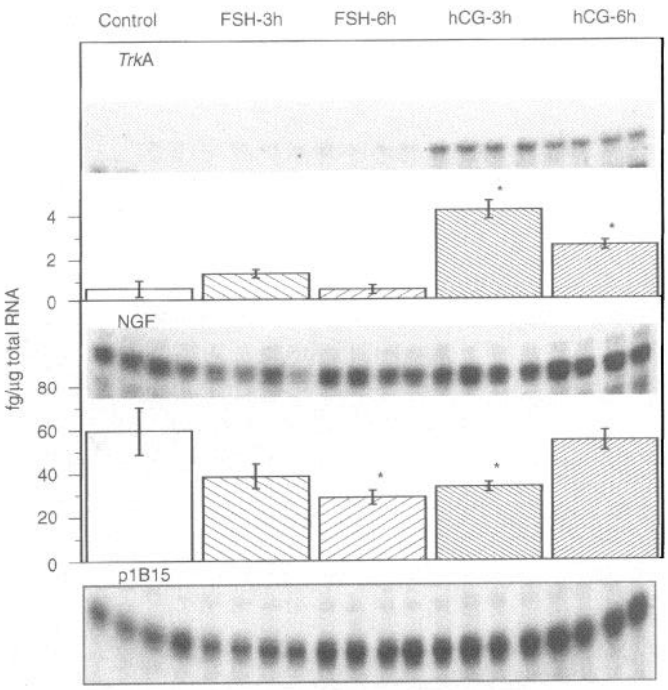

FIG. 9. Effect of gonadotropins on *trk*A and NGF mRNA levels in cultures of ovarian cell dispersates. Ovarian cells precultured for 48 h in serum-free medium responded within 3 h of hCG treatment (50 ng/ml) with an increase in *trk*A mRNA content (*, $P < 0.05$). NGF mRNA content was reduced (*, $P < 0.05$) by both FSH and hCG treatments (50 ng/ml). Each *bar* represents the mean ± SEM of four culture wells. Each lane represents the RNase-protected fragments from 20 μg total RNA.

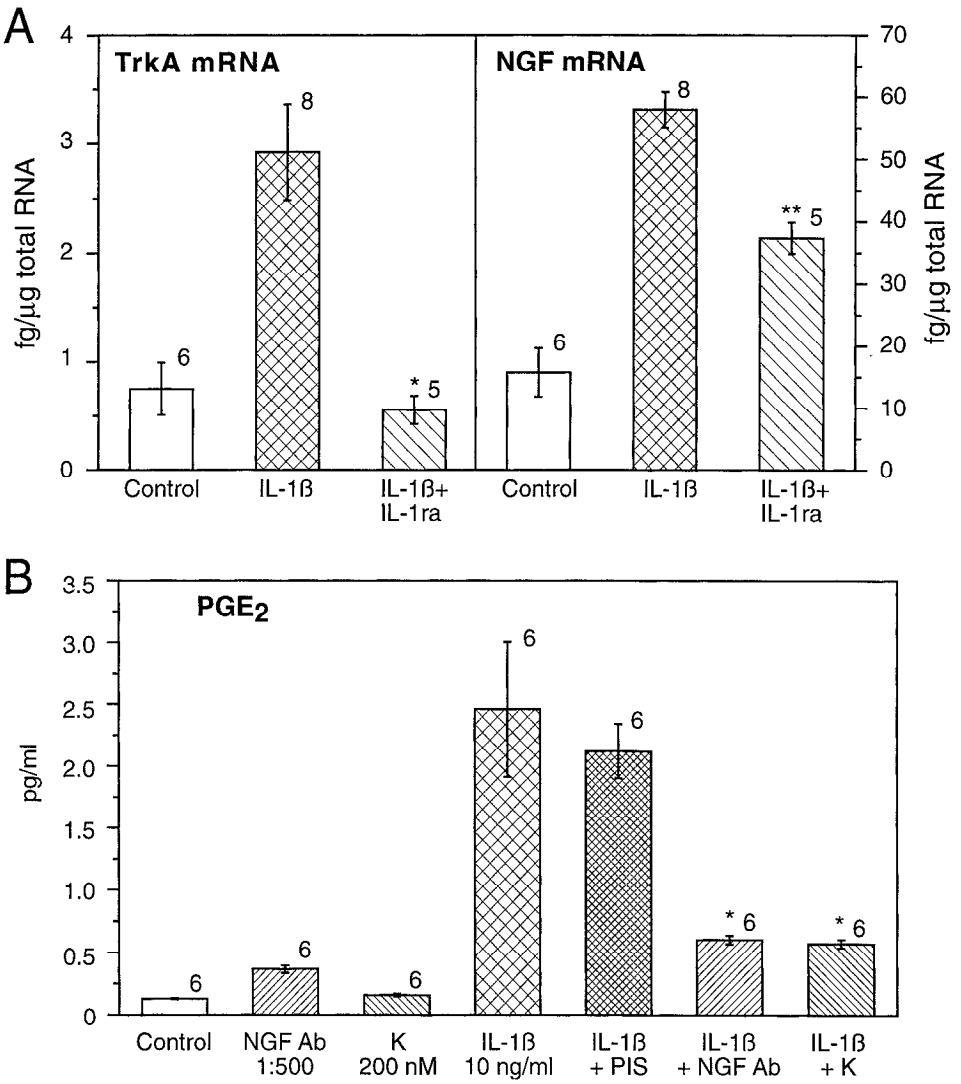

FIG. 10. A, Increase of *trk*A and NGF mRNA abundance by IL-1β in cultures of ovarian cell dispersates. The cells were treated for 6 h with IL-1β (10 ng/ml). The IL-1β receptor antagonist (IL-1ra; 5 μg/ml) blocked the effect of IL-1β on *trk*A mRNA (*, $P < 0.01$) and NGF mRNA (**, $P < 0.02$). B, IL-1β also stimulates the release of PGE_2, and this effect was blocked (*, $P < 0.01$) by both NGF antiserum (NGF Ab) and the *trk* tyrosine kinase inhibitor K-252a (K). PIS, Preimmune serum.

from ovarian cells (Fig. 10B). This increase was significantly attenuated by either NGF antiserum or K-252a (Fig. 10B), whereas neither affected basal PGE_2 release.

In vivo inhibition of ovarian PGE_2 synthesis by NGF antiserum

Treatment of 26-day-old juvenile rats with PMSG resulted in a LH surge 56 h later. Coinciding with the rise in serum LH, the ovarian PGE_2 content increased markedly (Fig. 11). This increase was significantly reduced by the administration of NGF antiserum at 1300 h, *i.e.* before initiation of the LH surge. The inhibitory effect was detected at both 1700 and 2100 h, but had statistical significance only at 1700 h, due to variability in the control group at 2100 h (Fig. 11). The antiserum did not interfere with the LH surge (Fig. 11).

Blockade of NGF biological actions inhibits ovulation

Ovarian selective inhibition of NGF biological actions by intrabursal administration of either NGF antiserum or the *trk* blocker K-252a resulted in a significant inhibition of ovulation, as evidenced by the lower number of corpora lutea detected in the treated ovary compared with that in the contralateral control gland (Fig. 12).

Discussion

A body of evidence is emerging that supports the idea that NTs may affect the survival, differentiation, and proliferation of nonneural cells. These effects, mainly studied using NGF as the ligand, do not appear to be limited to cells derived from a particular embryonic cell type, but, instead, can be demonstrated in cells of ectodermic, endodermic, and mesodermic origin. For instance, a pancreatic β-cell line of endodermic origin expresses *trk*A receptors and responds to NGF by acquiring a neuron-like phenotype (13, 14). *Trk*A receptors have also been found in islet cells of the developing and adult pancreas *in vivo* (63). On the other hand, a cell line of ectodermal origin, bipotential because it produces both GH and PRL, differentiates in the presence of NGF into PRL-producing cells, losing in the process its ability to proliferate and produce GH (12). The relevance of these phenomena to normal physiology is indicated by the recent observation that NGF promotes the proliferation and termi-

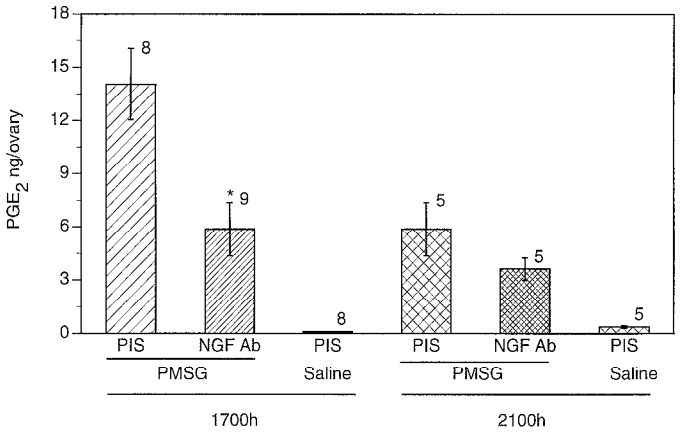

FIG. 11. Immunoneutralization of NGF action inhibits the preovulatory increase in ovarian PGE_2 content that occurs 56 h after the administration of PMSG [*, $P < 0.05$, NGF Ab-treated *vs.* PMSG-preimmune serum (PIS)-treated rats]. Rats were treated with saline or PMSG on postnatal day 26. Fifty-one hours later, they were injected iv with either PIS or NGF antiserum (NGF Ab). The ovaries were collected 56 and 60 h after PMSG or saline treatment, *i.e.* 5 or 9 h after serum injections. The NGF Ab reduced the preovulatory increase in ovarian PGE_2 content without affecting serum LH levels (PMSG-PIS, 687.8 ± 124.3 ng/ml; PMSG-NGF Ab, 970.6 ± 283.2 ng/ml; saline-PIS, 19.9 ± 10.5 ng/ml).

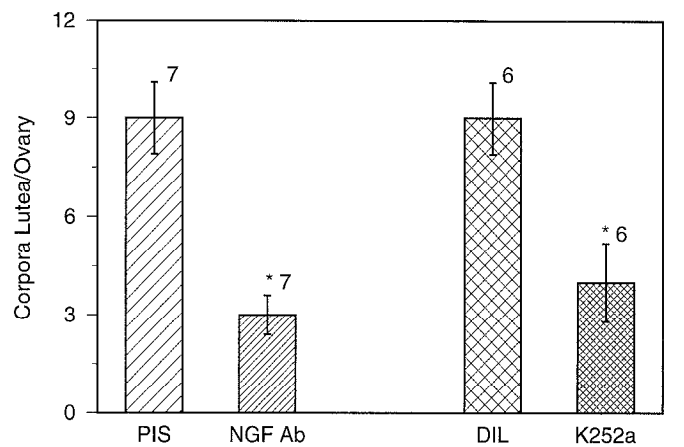

FIG. 12. Unilateral inhibition of ovulation by the intrabursal administration of either an antiserum to NGF (NGF Ab; 10 μl) or the *trk* receptor blocker K-252a (10 μl of a 100-μM solution). The inhibitors were injected in the morning of the day of the expected preovulatory surge of LH induced by the administration of PMSG 2 days earlier. PIS, Preimmune serum; DIL, diluent. *Bars* are means, and *vertical lines* are SEM. *Numbers above bars* are the number of animals per group. Corpora lutea were counted instead of ova because the ovarian bursae were tied to prevent leakage of the injected substances. Counting of ova collected from the oviducts of other PMSG-treated animals not subjected to ligation of the ovarian bursa yielded an average of 12 ± 0.84 ova/rat (n = 21), a value identical to the number of ovulations estimated by counting corpora lutea. *, $P < 0.01$ *vs.* contralateral ovary.

nal differentiation of PRL-secreting cells during postnatal pituitary maturation (64). As innervation of the anterior pituitary is scant, these findings support the view that the actions and expression of NTs are not restricted to neuronal target fields (65), but, instead, can be localized to a broader, hitherto unsuspected, range of biological sites.

Of considerable interest in this context are the observations that NGF facilitates inflammatory and immune responses (15, 18, 20, 21, 66), and that certain cytokinins, in particular IL-1β, are potent stimulators of NGF synthesis (19, 21, 67). The ability of NGF to stimulate the proliferation of B and T lymphocytes and to promote the differentiation of B cells into antibody-producing cells (16) suggests that NGF may act within the immune system as a regulatory cytokinin. The recent finding that mitogen- and antigen-presenting cell activation of T cells results in the expression of both NGF and *trk*A in the activated cells (17) further supports this concept and suggests that paracrine/autocrine effects of NGF are important mechanisms underlying the actions of NTs within the immune system. These observations and the rapid increase in NGF synthesis induced by mediators of inflammation within both the central and peripheral nervous systems (19–21) implicate NTs as a component of the acute process of inflammation itself. Indeed, NGF has been shown to induce the proliferation of lymphocytes (16, 68), the degranulation of mast cells (66), and the release of histamine and serotonin from peritoneal mast cells (18) and to promote the differentiation of granulocytes (15), all of which are important components of the acute inflammatory response.

Mammalian ovulation is a process of fundamental biological importance that resembles the inflammatory reaction, but rather than being initiated by injury, it is elicited by hormonal stimulation. Importantly, the inflammatory-like changes that characteristically precede ovulation ultimately result in tissue damage, *i.e.* the rupture of healthy antral follicles to allow release of the ovum for fertilization. A variety of factors known to be involved in inflammation, including ILs, PGs, vasoactive agents, and proteases (for review see Ref. 22) have been found to become active during the periovulatory period. The present results suggest that NGF-initiated *trk*A-mediated responses are integral components of this process. The finding that the *trk*A gene is turned on in nonneural cells of the ovarian follicle at a time when the follicle is becoming biochemically and cytologically differentiated into a new structure, namely the corpus luteum, suggests that ligand-mediated activation of *trk*A receptors contributes to these acute differentiating events. Such a view is supported by the finding that both immunoneutralization of NGF actions and pharmacological blockade of *trk* receptor-mediated intracellular signaling with K-252a lead to inhibition of ovulation. We used antibodies to NGF in addition to the receptor blocker, because of the possibility of K-252a having effects unrelated to its *trk* tyrosine kinase blocking capability (see, for instance, Ref. 69). Although the NGF antiserum used recognizes antigenic determinants in BDNF, NT-3, and NT-4, it does not prevent the biological actions of at least two of these NTs on PC12 cells, suggesting that the inhibitory effects of the antiserum on ovarian function are due to selective blockade of NGF action. The involvement of *trk*A receptors in the ovulatory process is further suggested by the remarkable fugacious nature and magnitude of the NGF/*trk*A activation; virtually no *trk*A mRNA can be detected either shortly before the preovulatory LH surge (morning of proestrus) or a few hours after ovulation, *i.e.* on the morning of the first estrus, whereas more than a 100-fold increase in mRNA levels occurs at the time of the LH surge. These periovulatory changes in *trk*A mRNA abundance appear to be specific, as neither *trk*B nor *trk*C

mRNA levels change in the ovary at the time of puberty. In fact, the prevalence of both mRNAs remains low throughout prepubertal development at levels similar to those observed for *trk*A mRNA in juvenile ovaries. Thus, even if the NGF antiserum and/or the inhibitor of tyrosine kinase used were blocking the biological actions of NT-3, BDNF, or NT-4/5, such an effect would be expected to have, at best, modest consequences. Whether similar changes in *trk*A and NGF gene expression occur in the ovary during the normal adult cycle remains to be established.

Although we have not yet identified the exact processes affected by *trk*A receptor activation, the effectiveness of IL-1β in increasing NGF and *trk*A mRNA levels in ovarian cells and the ability of both NGF antibodies and K-252a to reduce the increase in PGE_2 elicited by the cytokine suggest that NGF may mediate at least some of the actions of IL-1β in the preovulatory ovary. The synthesis of IL-1β increases at the time of the ovulatory surge of gonadotropins (60), and IL-1β has been shown to both induce ovulation (70) and stimulate the synthesis of PGs in the periovulatory ovary (62). A facilitatory effect of NGF on PG formation, although not yet described, is not unexpected, as the intracellular signaling pathway used by NGF is very similar to that used by the mitogenic peptide epidermal growth factor (10, 71), a well known stimulator of PG synthesis (72, 73). We do not know, however, if the preovulatory LH-dependent increase in *trk*A and NGF mRNA levels requires the intermediacy of IL-1β. This cytokinin is very potent in increasing NGF mRNA levels in ovarian cell dispersates as well as in other systems (19), yet a transient decrease in NGF mRNA abundance was observed at the time of the LH surge that causes an increase production of IL-1β in the ovary (60). Moreover, hCG increases *trk*A mRNA in ovarian cell dispersates, but appears unable to affect IL-1β gene expression in cultures similar to those used in the present study (Adashi, E., personal communication). Thus, LH may increase *trk*A mRNA levels via both IL-1β-dependent and independent pathways. An additional factor involved in the regulation of ovarian *trk*A expression may be estrogen, which has been shown to increase *trk*A mRNA levels in sensory neurons (74).

With regard to the potential functions of *trk*A receptor activation, it appears unlikely that the only (or most important) function of *trk*A receptors in the ovary is to mediate, via an NGF-dependent activation, the stimulatory effect of IL-1β on PG formation. It appears more plausible that *trk* receptors are involved in additional processes directly relevant to the periovulatory cytodifferentiation of mature follicles. The observation that activation of *trk*A receptors ectopically expressed in fibroblasts results in proliferative responses (75, 76) suggests that physiological acquisition of the receptors by fibroblasts engaged in specialized functions may result in similar proliferative responses. Thecal fibroblasts of the follicles in which the *trk*A gene appears to be expressed during the afternoon of proestrus, represent such specialized cells. Thecal fibroblasts undergo proliferative changes during the hour preceding ovulation, switching from a quiescent to a motile proliferative condition (22). The striking increase in *trk*A gene expression detected in the follicular wall at this time and the concomitant increase in ovarian NGF synthesis that accompanies the change in *trk*A receptors may provide the necessary conditions for the physiological manifestation of *trk*A-mediated proliferative events in the follicular wall. Support for this concept comes from recent studies showing that purified bovine thecal cells in culture proliferate in response to NGF stimulation (Dissen, G. A., M. Skinner, D. F. Hill, and S. R. Ojeda, unpublished data). While this work was near completion, the presence of *trk*A protein in rat testes was reported (77), suggesting that NGF may also contribute to the regulation of male gonadal function.

Regardless of the processes affected by *trk*A activation in periovulatory follicles, our results indicate that the acute ovary-specific suppression of NGF/*trk*A-dependent signaling has an inhibitory effect on the ovulatory process. Thus, the present study not only supports the emerging view that *trk*A receptors are physiologically involved in the control of nonneural functions, but, more importantly, it demonstrates a role for these receptors in one of the most critical developmental events signaling the initiation of reproductive competence, the first ovulation.

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