0013-7227/96/$03.00/0
Endocrinology

Vol. 137, No. 12
Printed in U.S.A.

Involvement of Nerve Growth Factor in the Ovulatory Cascade: *trk*A Receptor Activation Inhibits Gap Junctional Communication Between Thecal Cells*

A. MAYERHOFER†, G. A. DISSEN, J. A. PARROTT, D. F. HILL, D. MAYERHOFER, R. E. GARFIELD, M. E. COSTA, M. K. SKINNER, AND S. R. OJEDA

Department of Molecular Anatomy, Anatomical Institute, Technical University (A.M.), Munich, Germany; the Division of Neuroscience, Oregon Regional Primate Research Center-Oregon Health Sciences University (G.A.D., D.F.H., D.M., M.E.C., S.R.O.), Beaverton, Oregon 97006; the Reproductive Endocrinology Center, University of California (J.A.P., M.K.S.), San Francisco, California 94143-0556; and the Department of Obstetrics and Gynecology, University of Texas Medical Branch (R.E.G.), Galveston, Texas 77555

ABSTRACT

Activation of *trk*A, the nerve growth factor (NGF) tyrosine kinase receptor, has been recently implicated in the process of mammalian ovulation. During the hour preceding follicular rupture, a marked increase in *trk*A and NGF gene expression occurs in thecal-interstitial cells of the ovary. Immunoneutralization of NGF actions or pharmacological blockade of *trk*A transducing activity inhibits ovulation, suggesting that activation of the NGF-*trk*A complex in nonneural cells of the periovulatory follicle is a physiological component of the ovulatory cascade. As thecal cells of Graafian follicles are functionally coupled by gap junctions, and the ovulatory rupture requires dissociation of thecal cell-cell communication, we sought to determine whether NGF affects the integrity of this communication. We now report that NGF-induced activation of *trk*A receptors in isolated ovarian thecal cells disrupts cell to cell communication by affecting the functional integrity of gap junctions. Bovine thecal cells expressing *trk*A receptors, but not cells lacking the receptors, respond to NGF with a reduction in the transfer of calcein, a fluorescent dye that passes through gap junctions. This effect was associated with a rapid (10–30 min) increase in serine phosphorylation of connexin-43, the main protein constituent of gap junctions in the ovary. The reduction in dye transfer was not observed when the cells were exposed to epidermal growth factor or other neurotrophins, including neurotrophin 3, neurotrophin 4, and brain-derived neurotrophic factor. Thus, cell-specific activation of *trk*A receptors in periovulatory follicles may provide one of the signals involved in inducing the cellular dissociation of the follicular wall that precedes ovulatory rupture. (*Endocrinology* **137:** 5662–5670, 1996)

NERVE GROWTH factor (NGF) belongs to a family of related target-derived protein molecules required for the survival and development of discrete neuronal populations in the central and peripheral nervous systems (1, 2). Although initial observations led to the conclusion that the biological actions of neurotrophins (NTs) are restricted to the nervous system (3, 4), new evidence suggests that they, in particular NGF, can also affect nonneural cells. The immune (5–8) and endocrine systems (9–13) appear to be the most prominent targets of these actions. NGF, via activation of the tyrosine kinase receptor *trk*A (7, 8), induces the release of inflammatory mediators from mast cells (8), promotes differentiation of specific granulocytes (6), and modulates immune responses by stimulating the growth and differentiation of B and T lymphocytes (5). In the endocrine system, NGF has been shown to regulate the differentiation of hormone-secreting cells of endodermic (pancreatic β-cells) and ectodermic (pituitary mammotrophs) origins. Thus, a β-cell line representing an early stage in pancreatic differentiation responds to NGF with extension of neurite-like processes (10). Pancreatic β-cell lines (10, 13), fetal islets in culture (13), and β-cells of the developing and adult pancreas *in vivo* (11) contain *trk*A receptors, indicating that insulin-producing cells are indeed targets for NGF action. In the pituitary, NGF promotes the differentiation of bipotential precursor cells to the mammotroph phenotype during development of the gland (9, 12), suggesting that acquisition of PRL-secreting capacity by the pituitary gland is under neurotrophic control.

A direct involvement of NGF in the regulation of gonadal function has been suggested by the detection of *trk*A receptors in the developing testis (14) and ovary (15, 16). In the rat ovary, *trk*A messenger RNA (mRNA) expression appears to be restricted to two developmental windows: before the initiation of follicular formation and during the hour preceding ovulation (15, 16). At this latter time, there is a remarkable increase in ovarian *trk*A mRNA levels. This change (brought about by the preovulatory discharge of LH) occurs in thecal-interstitial cells associated with periovulatory follicles and is accompanied by an increase in NGF mRNA content in the same ovarian compartment (16). That these changes have

Received June 6, 1996.
Address all correspondence and requests for reprints to: Dr. Sergio R. Ojeda, Division of Neuroscience, Oregon Regional Primate Research Center, 505 N.W. 185th Avenue, Beaverton, Oregon 97006. E-mail: ojedas@ohsu.edu.
* This work was supported by NIH Grants HD-24870 (to S.R.O.), Population Center Grant P30-HD-18185 and Grant RR-00163 for the operation of the Oregon Regional Primate Research Center, and a grant from the USDA (no. 1401352; to M.K.S.). This is publication 2008 of the Oregon Regional Primate Research Center (Beaverton, OR).
† Visiting Scientist at the Oregon Regional Primate Research Center and supported by a Heisenberg Senior Scientist Fellowship from the Deutsche Forschunggemeinschaft, Germany (MA 1080/4-1).

physiological importance is demonstrated by the ability of either immunoneutralization of NGF or pharmacological blockade of ovarian *trk* receptors to inhibit ovulation (16).

Ovulation is preceded by a loss in gap junctional communication among follicular cells (17). Gap junctions are small channels formed between cell membranes of juxtaposed cells that permit the exchange of ions, second messengers, and small metabolites (18, 19). Both granulosa and thecal cells of developing follicles form independent functional intercellular syncytia via gap junctions (20–22). The preovulatory surge of LH disrupts gap junctions between follicular granulosa cells (23), causing first an increase in the phosphorylation of connexin-43 (Cx43), the predominant protein constituent of gap junctions in the ovary (22–26), and later a decrease in Cx43 protein levels (23, 26). Much less is known about the periovulatory regulation of gap junctional communication in the thecal compartment, but there is immunohistochemical evidence that Cx43 is more abundant in the preovulatory theca than in thecal cells of less developed follicles (22).

The presence of NGF and its tyrosine kinase receptor in thecal-interstitial cells [which also contain the low affinity p75 NGF receptor (27)] and the striking increase in *trk*A abundance triggered by the preovulatory surge of gonadotropins in this ovarian compartment suggest that activation of *trk*A receptors may contribute to the LH-dependent changes in thecal cell homeostasis that precede ovulation. Fibroblasts ectopically expressing *trk*A receptors dissociate and proliferate in response to NGF (28), indicating that NGF is capable of affecting cell-cell communication of nonneuronal cells of mesenchymal origin. As thecal cells are also mesenchymal in origin (29), the possibility exists that NGF, acting via *trk*A receptors acquired before ovulation, contributes to the interruption of thecal cell communication that precedes the ovulatory breakdown of the follicular wall (30).

In the present study, we tested this hypothesis by reproducing *in vitro*, in the absence of gonadotropins, the preovulatory increase in *trk*A receptors caused by LH *in vivo* via transfer of the *trk*A gene into purified cultures of unstimulated thecal cells. Bovine thecal cells were used because they can be much more easily isolated from interstitial cells than their rat counterparts (31).

Materials and Methods

Thecal cell cultures

Bovine thecal cells, isolated from a mixture of small, medium, and large antral follicles, as previously reported (31), were seeded in T-75 flasks and cultured for 3 days in Ham's F-12 medium containing 10% calf serum (CS). At this time, the cells were collected by mild trypsinization, reseeded in 100-mm dishes at 1×10^6 cells/dish, and maintained for 3 more days in Ham's F-12-10% CS before using them for experiments. These cultures include cells of both the interna and external thecal layers, thus containing a mixture of thecal steroidogenic cells (31).

Transfections

Thecal cells were transfected for 5 h with an expression vector containing the entire coding region of *trk*A under the control of the cytomegalovirus (CMV) early promoter (32) or with vector alone (pCMV), each at 2 μg DNA/ml, using Lipofectamine (10 μg/ml; Life Technologies, Grand Island, NY) as the transfecting agent. After transfection, the cells were kept in Ham's F-12-10% CS for 24 h before reducing the CS concentration to 0.1%. All experiments were performed 48 h after transfection.

In situ hybridization and immunohistochemistry

The ovaries from immature rats treated with PMSG to induce a precocious preovulatory surge of gonadotropins were used to determine whether cells of the wall of periovulatory follicles coexpress Cx43 mRNA and *trk*A mRNA. The animals were injected at 27 days of age with 8 IU PMSG (sc, in 0.2 ml saline), and the ovaries were collected 2 days later at 2100 h, *i.e.* after the preovulatory LH surge and about 5–6 h before ovulation. Double *in situ* hybridization was performed as previously described (33) using an [^{35}S]UTP-labeled rat *trk*A complementary RNA (cRNA) probe complementary to nucleotides 226–752 in *trk*A mRNA (34) and a digoxygenin-UTP-labeled rat Cx43 cRNA probe complementary to nucleotides 226–564 in Cx43 mRNA (35).

For immunohistochemical detection of Cx43 in cultured bovine thecal cells, the cells from small, medium, and large follicles were isolated as described above and cultured for 8 days (with one intermediate passage on day 3) before fixation in Zamboni's fixative. The immunohistochemical reaction was performed as previously described (16) using a well characterized Cx43 antiserum (22, 36) at a 1:500 dilution.

Northern blot and ribonuclease (RNase) protection assay

The procedures used were described in detail previously (16, 37, 38). To detect Cx43 by Northern blot, we used the same cRNA probe employed for hybridization histochemistry, but labeled with [^{32}P]UTP. Detection of *trk*A mRNA by RNase protection assay also employed the same cRNA used for *in situ* hybridization, but labeled with [^{32}P]UTP.

Protein phosphorylation

To determine whether transfection of thecal cells with the *trk*A expression vector resulted in expression of biologically active receptors, thecal cells that had been transfected with either plasmid alone or with the *trk*A expression vector were treated with NGF (100 ng/ml; 5 min), lysed in RIPA buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 0.2% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonylflouride, 0.15 U/ml aprotinin, 1 mM sodium orthovanudate) (39), and immunoprecipitated for 4 h at 4 C with *trk*A antibody 203 (40), followed by overnight precipitation with protein A-Sepharose at 4 C. The immunoprecipitates were separated by SDS-PAGE (7% gel), blotted onto nitrocellulose, and visualized with the monoclonal antibody to phosphotyrosine 4G10 (39, 40) followed by enhanced chemiluminescence (41).

To detect Cx43, the cells were disrupted and centrifuged at $250 \times g$ (23) before immunoprecipitation with a Cx43-specific antibody (22, 36) bound to protein A-Sepharose (41). This antibody recognizes both phosphorylated and nonphosphorylated Cx43 species (22). After immunoprecipitation, the samples were separated by SDS-PAGE using 15% gels. After Western blotting, the nitrocellulose membrane was incubated with an antiphosphoserine monoclonal antibody (Sigma Chemical Co., St. Louis, MO; 1:500) and developed using enhanced chemiluminescence (41). Subsequently, the membrane was stripped and reprobed with a monoclonal antibody (4G10) to phosphotyrosine (39, 40).

Calcein dye transfer

Forty-eight hours after transfection, thecal cells were dye-loaded for 30 min at 37 C (42) with 10 μM DilC18 (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate) and 5 μM calcein/AM mixture (diluted in PBS containing 1 g glucose/liter from frozen 1-mM stock solutions in dimethylsulfoxide; both dyes were obtained from Molecular Probes, Eugene, OR). The cells were washed with PBS, trypsinized (0.05% trypsin and 0.02% EDTA in PBS) and seeded in six-well plates containing Ham's F-12 medium-0.1% CS together with nonloaded "naive" *trk*A-transfected thecal cells trypsinized in the same way. By seeding one sixth of a 100-mm dish of nonloaded cells per well, a confluence of approximately 80–90% was achieved. The ratio of loaded to nonloaded cells was 1:400. NGF, NT-3, NT-4, or epidermal growth factor (EGF; 100 ng/ml each) was added 30 min after seeding; at this time the cells had settled and had both started to spread and reestablish cell-cell

contacts, as evidenced by the detection of dye transfer. Dye transfer was assessed using a Nikon Diaphot inverted microscope (Nikon Corp., Melville, NY) equipped with appropriate filter sets for fluorescein isothiocyanate fluorescence (for calcein) and rhodamine fluorescence (for DilC18), interfaced with a 486DX/66 IBM-compatible personal computer (Comtrade, City of Industry, CA) equipped with a digitizing board and Metamorph Image Analysis software (Universal Imaging Corporation, West Chester, PA) and a cooled CCD video camera (Princeton Instruments, Trenton, NJ). Dye transfer from donor cells (double staining for DilC18 and calcein) to neighboring cells (positive for calcein, but not for DilC18) was evaluated between 90–120 min after seeding by counting the number of calcein-positive cells that surround one calcein/DilC18-positive donor cell. This evaluation was performed blind, *i.e.* without knowledge of the treatment.

Statistics

The differences in dye transfer between *trk*A-transfected and plasmid only-transfected cells treated with different growth factors were analyzed by ANOVA followed by the Student-Newman-Keuls test for comparison of multiple groups with unequal replications.

Results

Thecal cells of the rat ovary express Cx43 and trkA mRNA

Double *in situ* hybridization experiments were performed on rat ovaries collected at 2100 h on the day of the endogenous preovulatory LH surge induced by PMSG treatment of immature animals. As previously shown by immunohistochemical localization of the Cx43 protein (22), the mRNA encoding Cx43 was extremely abundant in granulosa cells of small and medium-sized antral follicles (Fig. 1A, *arrows*). The hybridization signal was noticeably reduced in large periovulatory follicles (Fig. 1A, *arrowheads*), suggesting that the preovulatory disruption of granulosa cell-cell communication had been initiated. Although less prominent than in granulosa cells, Cx43 mRNA expression was also detected in the thecal compartment (Fig. 1, A and B, *double arrowheads*). As previously reported (16), *trk*A mRNA was detected in thecal and interstitial cells (Fig. 1C, *arrow* and *arrowhead*, respectively), but not in the granulosa cell compartment. Detailed examination of the cells expressing Cx43 and *trk*A mRNA by double *in situ* hybridization showed coexpression of these mRNAs in a subpopulation of thecal cells (*black arrows*), but not granulosa cells, of periovulatory follicles (Fig. 2, A and B). Most of the thecal cells containing *trk*A mRNA and Cx43 mRNA appeared to be localized in the theca externa (*black arrows*), but not all *trk*A mRNA-expressing cells contained Cx43 mRNA (*white arrows*). Colocalization of the mRNAs in interstitial cells was less prominent, as many of these cells appeared to be either *trk*A mRNA or Cx43 mRNA positive (Fig. 2A, *black* and *white arrowheads*, respectively).

Bovine thecal cells in culture as a model to study potential periovulatory NGF actions on the follicular wall

In contrast to those in the rat, bovine thecal cells can be easily separated from contaminating interstitial and granulosa cells (31) and, thus, can be readily used for functional studies without the confounding influence of nonthecal paracrine inputs. Like rat cells, however, they express Cx43 mRNA and immunoreactive Cx43 protein, as shown by Northern blot analysis and immunohistofluorescence, respectively (Fig. 3, A and B).

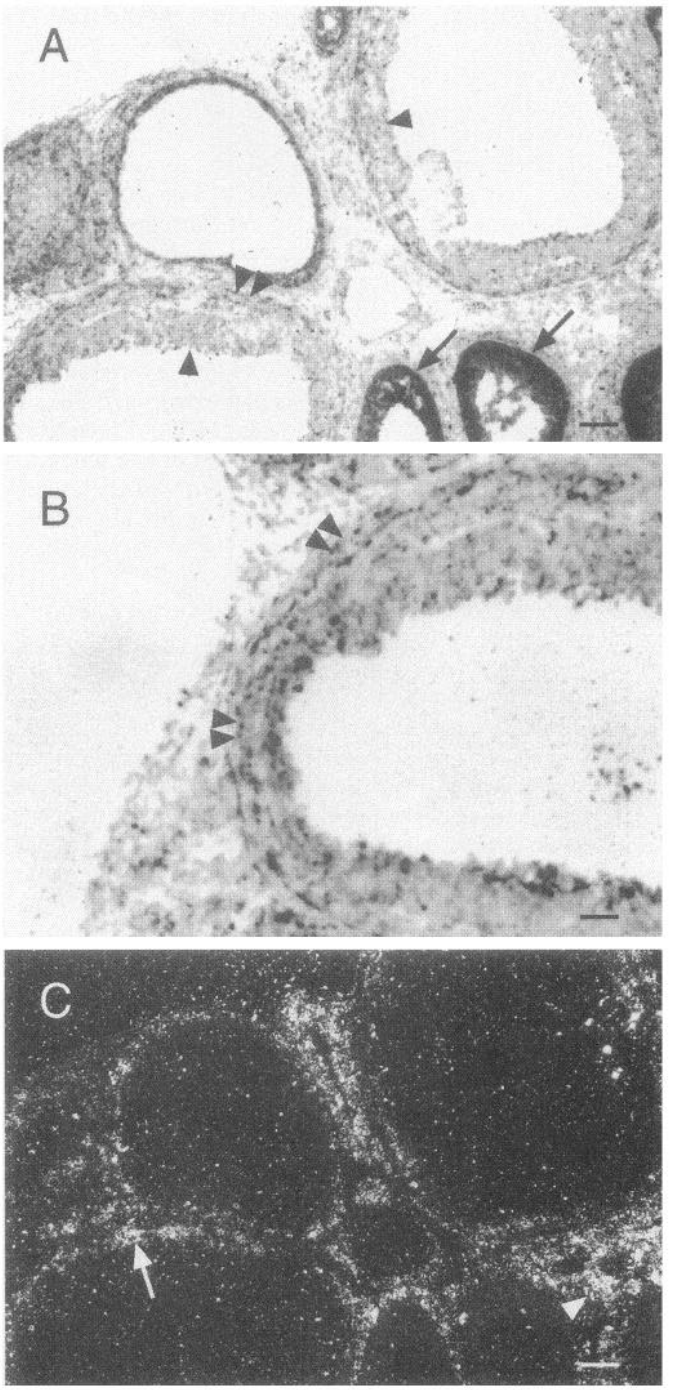

FIG. 1. A, Detection of Cx43 mRNA in follicles of preovulatory ovaries by hybridization histochemistry, using a digoxygenin UTP-labeled Cx43 antisense RNA probe. Notice the strong hybridization signal in granulosa cells of medium and small antral follicles (*arrows*), the loss of Cx43 mRNA in large periovulatory follicles (*arrowheads*), and the less prominent, but distinct, presence of Cx43 mRNA in the thecal compartment (*double arrowheads*). *Bar* = 100 μm. B, Higher magnification view of the thecal compartment of preovulatory follicles showing the presence of Cx43 mRNA in thecal cells of a large antral follicle (*double arrowheads*). *Bar* = 50 μm. C, Darkfield illumination of the same section shown in A, demonstrating the presence of *trk*A mRNA in interstitial cells (*arrowhead*) and thecal (*arrow*), but not granulosa, cells of preovulatory ovaries. *Bar* = 100 μm. The results represent *in situ* hybridization experiments performed on ovaries from three animals.

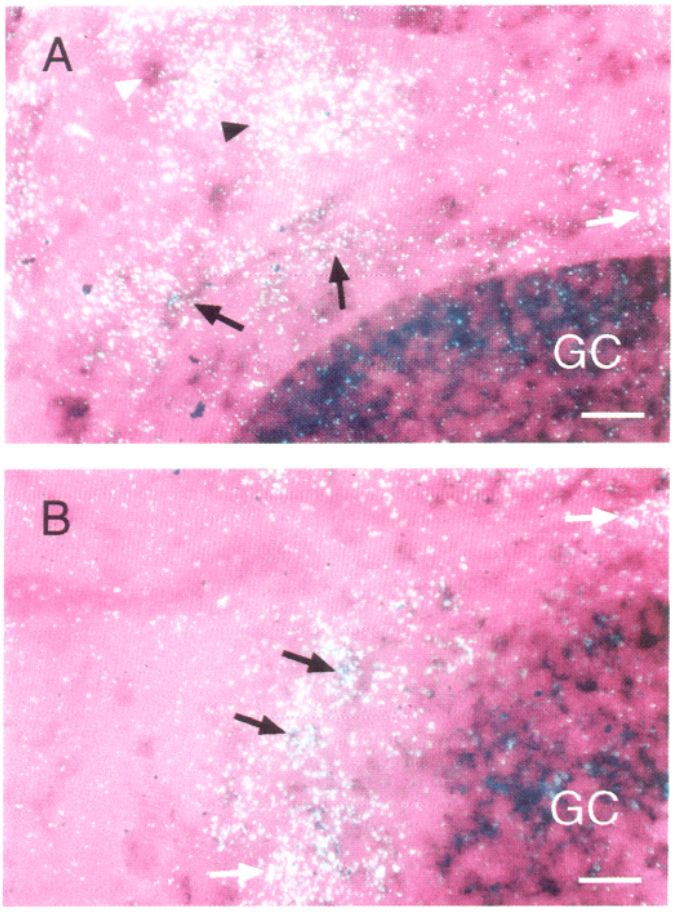

FIG. 2. Coexpression of *trk*A mRNA and Cx43 mRNA in cells of the follicular wall of periovulatory follicles, as determined by double hybridization histochemistry. Cx43 mRNA was detected with a digoxigenin UTP-labeled cRNA probe; *trk*A mRNA was detected using a [^{35}S]UTP-labeled rat *trk*A cRNA probe. *Black arrows* indicate cells containing both Cx43 and *trk*A mRNAs; *white arrows* show cells expressing *trk*A mRNA, but not cx43 mRNA; the *black arrowhead* indicates interstitial cells containing only *trk*A mRNA; the *white arrowhead* shows interstitial cells containing only Cx43 mRNA. GC, Granulosa cells. *Bars* = 20 μm. The examples shown represent results obtained using the ovaries from three animals collected at 2100 h on the day of the expected preovulatory LH surge induced by administration of PMSG to immature rats.

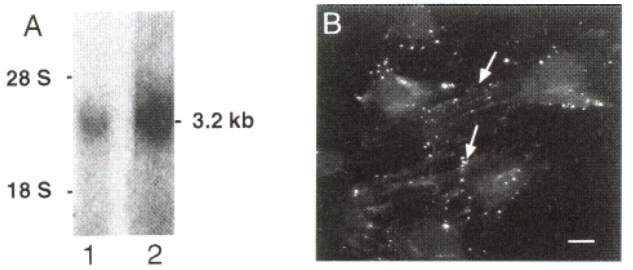

FIG. 3. A, Detection of Cx43 mRNA by Northern blot analysis in bovine thecal cells. Each lane contains 15 μg total RNA extracted from thecal cells of large follicles (lane 1) or from cultured thecal cells (lane 2). B, Immunohistochemical detection of Cx43 in cultured bovine thecal cells. The Cx43 immunoreactivity is punctate and is primarily detected at touching borders of adjacent cells (*arrows*). *Bar* = 12.5 μm.

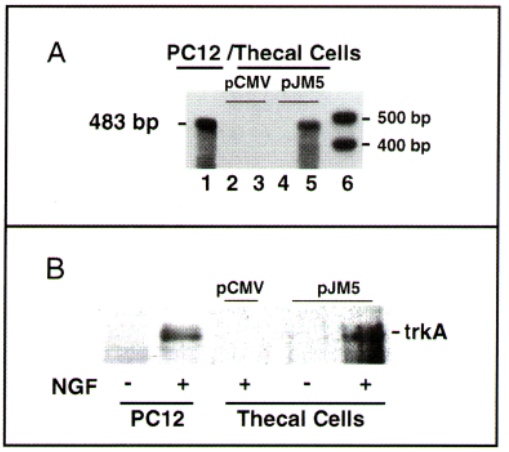

FIG. 4. A, Detection of *trk*A mRNA in bovine thecal cells transfected with a rat *trk*A expression construct (pJM5) or vector alone (pCMV), as assessed by RNase protection assay. The RNA was isolated 48 h after transfection. Before the assay, contaminant plasmid DNA extracted along with the cellular RNA, was digested with deoxyribonuclease (DNase). The specificity of the mRNA:cRNA protection was further verified by treating some samples with RNase before incubation with the cRNA probe. Lane 1, Total RNA from the *trk*A-expressing cell line PC12 (predigested with DNase); lane 2, RNA from pCMV-transfected cells (predigested with RNase and DNase); lane 3, RNA from pCMV-transfected cells (predigested with DNase only); lane 4, RNA from rat *trk*(pJM5)-transfected cells (predigested with RNase and DNase); lane 5, RNA from rat *trk*A(pJM5)-transfected cells (predigested with DNase only); lane 6, RNA size markers. The autoradiograms represent the results from two experiments. B, Tyrosine phosphorylation of *trk*A in response to NGF. PC12 cells or thecal cells transfected with plasmid alone (pCMV) or with rat *trk*A expression vector (pJM5) were treated with NGF (100 ng/ml; 5 min), lysed in RIPA buffer, and immunoreacted with rat *trk*A antibody 203, followed by precipitation with protein A-Sepharose. The immunoprecipitates separated by SDS-PAGE and blotted onto nitrocellulose were reacted with antibody 4G10 to phosphotyrosine before visualization using enhanced chemiluminescence. The autoradiograms depicted represent the results of three experiments.

Our previous studies in the rat demonstrated that the *trk*A gene becomes expressed in thecal-interstitial cells only at the time of the preovulatory surge of LH (16). To simulate this condition *in vitro*, in the absence of other gonadotropin-dependent events, we increased *trk*A expression in purified bovine thecal cells by transient transfection of an expression construct (pJM5) containing a rat *trk*A complementary DNA (cDNA) under the control of the CMV immediate early promoter (32). Forty-eight hours later, we assessed the response of the *trk*A-overexpressing cells to NGF. Other cells were transfected with the pCMV vector alone. Cells transfected with the *trk*A cDNA synthesized rat *trk*A mRNA, as determined by RNase protection assay (Fig. 4A), and rapidly responded to NGF with tyrosine phosphorylation of a ~140-kDa protein species immunoprecipitated with *trk* antibodies (43) (Fig. 4B). The phosphorylated protein was similar in size to authentic *trk*A phosphorylated by NGF in the NGF-responsive pheochromocytoma cell line PC12. A similar species was not detected either in cells transfected with pCMV only and challenged with NGF (Fig. 4B) or in *trk*A cDNA-transfected cells treated with NT-3, brain-derived neurotrophic factor, or NT-4 (not shown), demonstrating the specificity of the *trk*A construct.

NGF induces phosphorylation of thecal Cx43 at serine residues

Addition of NGF (100 ng/ml) to *trk*A-transfected cells induced rapid phosphorylation of membrane-associated Cx43 (approximate M_r of 46,000) at serine residues as early as 10 min after treatment (Fig. 5A). In all three experiments performed, serine phosphorylation was robustly present after 30 min and persisted for at least 60 min. Exposure to EGF (100 ng/ml), a growth factor known to induce Cx43 serine phosphorylation in epithelial cells (44), resulted in some serine phosphorylation, which was variably detected at 10 or 30 min in *trk*A and pCMV-only transfected cells (Fig. 5). This effect of EGF is probably mediated by interaction with its receptor, which is present in normal thecal cells (45). No NGF- or EGF-induced phosphorylation of Cx43 at tyrosine residues was detected (Fig. 5B). NGF induced some Cx43 serine phosphorylation at 30 min in control thecal cells (Fig. 5A), suggesting the presence of endogenous *trk*A in these cells.

NGF, but not other NTs, disrupts thecal cell-cell communication

To determine whether the effect of NGF on Cx43 phosphorylation is associated with a reduction in cell-cell communication, we performed fluorescent dye transfer studies using plasmid- and *trk*A-transfected bovine thecal cells preloaded with the fluorescent dyes DilC18 and calcein/AM (42). During loading, DilC18, a lipophilic dye, binds to cell membranes, whereas calcein/AM freely enters the cell, where it is transformed into calcein by cellular esterases. Calcein cannot diffuse though the cell membrane, but passes through gap junctions. Exposure of *trk*A-transfected thecal cells to NGF significantly reduced calcein dye transfer between DilC18-calcein/AM-preloaded and nonloaded cells. Figure 6 depicts the results of a representative experiment. The *upper panels* show that in the absence of NGF, calcein was transferred from *trk*A-transfected preloaded cells to six naive cells. The *lower panels* demonstrate that upon exposure of the cells to NGF (100 ng/ml), gap junction-mediated dye transfer is reduced. Figure 7 summarizes these findings and presents the statistical evaluation of the results. The effect of NGF was ligand and receptor specific, as it was not seen in either cells transfected with the pCMV vector alone (Fig. 7A) or in *trk*A-transfected cells exposed to NT-3, NT-4, or EGF (Fig. 7B). Treatment of the cells with heptanol, a potent inhibitor of gap junctional communication (46), eliminated all detectable calcein transfer (not shown), thus verifying the specificity of the method (42). Although the magnitude of the changes observed was seemingly small (~30% decrease), this figure does not take into account the actual fraction of cells that was effectively transfected. Lipofectamine-mediated transfection of the fibroblastic 3T3 cell line has been shown to result in transfection of 40–80% of the cells (47, 48), suggesting that the transfectability of thecal cells in primary culture may be, at best, near the lower part of this range.

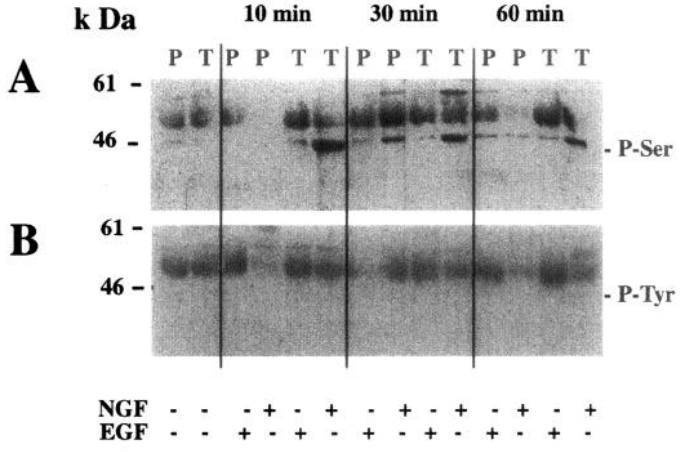

FIG. 5. Phosphorylation of Cx43 induced by NGF or EGF on bovine thecal cells transfected with the *trk*A expression construct pJM5. The cells were treated with the growth factors 48 h after transfection. Thecal cells transfected with the rat *trk*A cDNA (T) or plasmid only (P) were treated with NGF (100 ng/ml) or EGF (100 ng/ml) for 10, 30, or 60 min. After immunoprecipitation with a specific Cx43 antiserum, SDS-PAGE size separation, and transfer to nitrocellulose membranes, the Cx43-phosphorylated species were identified with a monoclonal antibody to phosphoserine (A) and a monoclonal antibody (4G10) to phosphotyrosine (B). Three individual experiments yielded similar results.

Discussion

The preovulatory surge of gonadotropins initiates a cascade of events within both the granulosa and thecal cell compartments of the Graafian follicle that ultimately results in ovulatory rupture and differentiation of both thecal and granulosa cells into luteal cells. Although it is not always easy to establish a clear distinction between events leading to luteinization and those involved in follicular rupture, it appears clear that follicular rupture is not required for luteinization to occur (30). Consequently, the dissociation and rupture of the follicular wall must be determined by events not coupled to the differentiation of follicular cells into their luteal counterparts.

Morphological and biochemical examination of the follicular wall have indicated that disruption of cell-cell communication between thecal cells and weakening of the collagenous matrix within the follicular wall are critical events in the process leading to follicular rupture (reviewed in Ref. 30). Dissociation of thecal cells in the theca externa begins within a few hours of the gonadotropin surge and is markedly accelerated near the actual time of ovulation (49). The cellular dissociation of the theca externa is accompanied by increased collagenolytic and protease enzyme activity, and edema and dissociation of the theca interna (49–52). Although there is little doubt that these complex events are critical for ovulation to take place, the factors that set in motion the cellular dissociation of the follicular theca have not been identified.

During the course of experiments aimed at characterizing the presence of neurotrophic genes of the NGF family in the developing rat ovary, we unexpectedly found that expression of *trk*A, the NGF tyrosine kinase receptor, increases massively during the evening of the first proestrus (16). Importantly, this change was transient and limited to the thecal-interstitial compartment of the ovary, suggesting that activation of *trk*A receptors may be one of the mechanisms

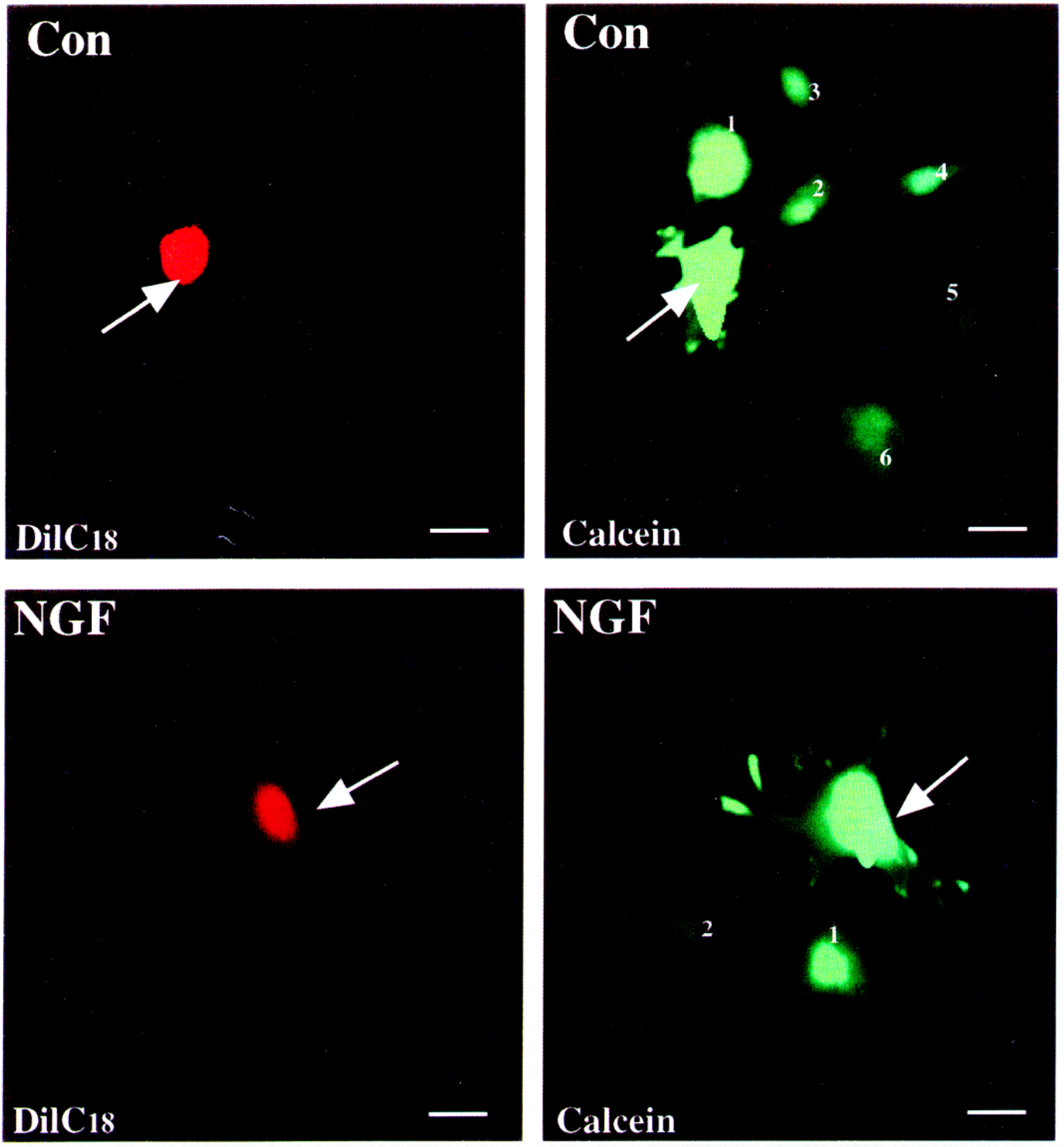

FIG. 6. Inhibitory effect of NGF on calcein dye transfer between thecal cells expressing the rat *trk*A receptor. The method employed (42) is based on the fact that after loading cells with calcein AM and DilC18, calcein can only be transferred to neighboring cells via gap junctions, whereas DilC18 remains in the originally loaded cells attached to cell membranes. For additional details, see *Materials and Methods*. The examples depicted are taken from a culture of *trk*A-transfected thecal cells showing calcein transfer from a donor cell to six neighboring cells in the absence of NGF (*upper panels*; *arrows* indicate the donor cell) and from an NGF-treated culture in which calcein transfer to only two adjacent cells was detected (*lower panels*; *arrows* indicate the donor cell). *Bars* = 10 μm.

underlying the changes in follicular wall homeostasis associated with the ovulatory process.

Ectopic expression of *trk* receptors in nonneuronal cells of mesenchymal origin, such as fibroblasts, endows these cells with the ability to proliferate in response to NTs (28, 53, 54). In the presence of a potentiating p75 receptor form, *trk*A- and

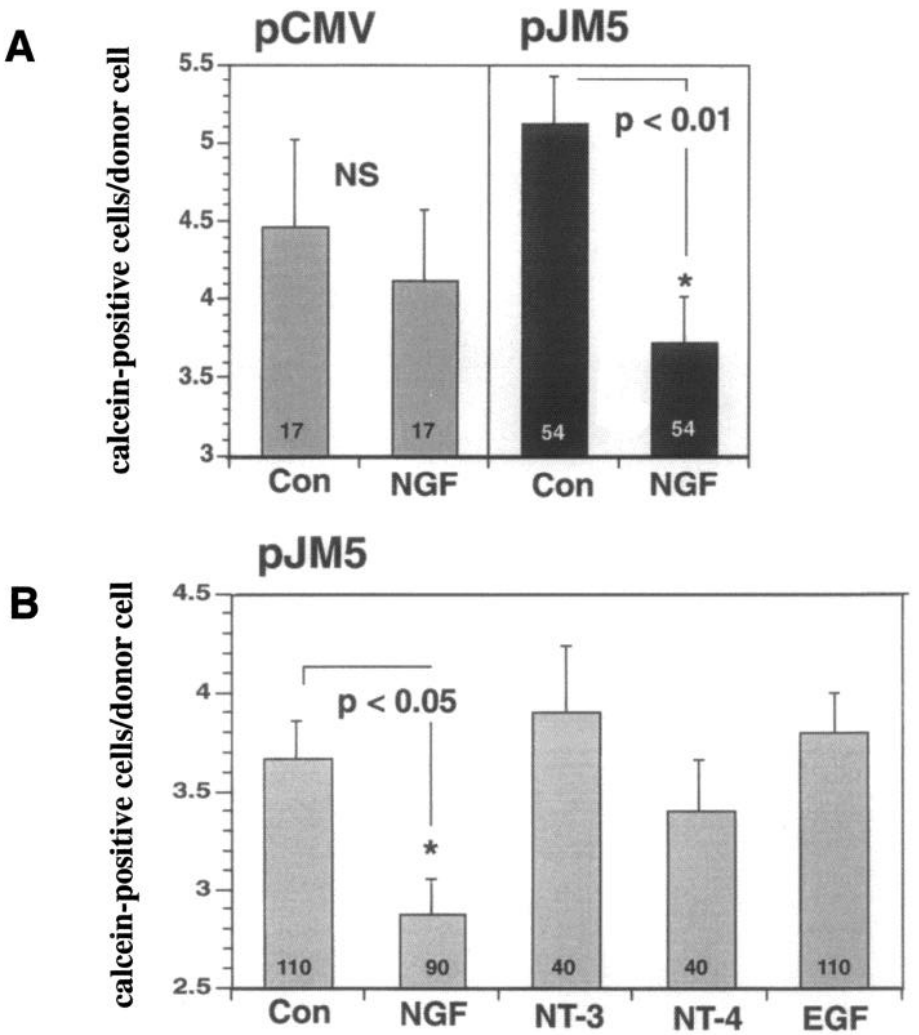

FIG. 7. Analysis of the results obtained from calcein dye-transfer experiments. A, NGF (100 ng/ml) did not significantly reduce calcein transfer in plasmid only (pCMV)-transfected cells, but decreased calcein transfer in *trk*A (pJM5)-transfected cells ($P < 0.01$). B, Failure of other NTs (NT-3 and NT-4) or EGF to reproduce the inhibitory effect of NGF on dye transfer between thecal cells transfected with a *trk*A cDNA. *Bars* represent the mean ± SEM. *Numbers inside bars* are the number of donor cells examined. The results represent pooled values from four different experiments. For methodological details, see Fig. 3 and *Materials and Methods*.

*trk*B-expressing fibroblasts also show loss of contact inhibition in response to ligand stimulation (28). Although cells of mesenchymal origin do not normally express *trk*A receptors, the activation of *trk*A gene expression detected in thecal-interstitial cells of the ovary during the hour preceding ovulation (16) indicates that these highly specialized cells may represent a unique example of a nonneuronal target for NT action.

The results of the present study suggest that NGF-dependent activation of *trk*A receptors in thecal cells may contribute to initiating the preovulatory loss of cell-cell contact within the follicular wall. Exposure of thecal cells bearing *trk*A receptors to NGF resulted in the rapid serine phosphorylation of Cx43 and, as shown by dye transfer experiments, the subsequent reduction of cell-cell contacts via attenuation of gap junctional communication. We do not know whether, under the experimental conditions used, NGF affects cell coupling by preventing the formation of new gap junctions, disrupting those already existing, or both. Cell-cell communication via gap junctional coupling is established within seconds or minutes after the cells come into contact with one another (55). As NGF was added 30 min after seeding the cells, and at this time dye transfer was already detected, it would appear that at least part of the NGF effect is exerted on newly formed gap junctions. A potential inhibitory effect of NGF on *in vitro* gap junction formation remains, however, a distinct possibility. Regardless of the cellular mechanism used by NGF to affect thecal cell communication, the present results indicate that the net outcome of its actions is a reduction in the number of viable gap junctional cell-cell contacts.

Although our study does not demonstrate a cause-effect relationship between serine phosphorylation of Cx43 and a reduction in gap junction viability, prior reports have implicated serine phosphorylation of Cx molecules as one of the initial steps in the sequence of events leading to gap junctional disruption in several cell types (19, 44, 56), including ovarian cells (26). The study of Lau *et al.* (44) is particularly enlightening in this regard. These researchers found that EGF-induced disruption of gap junctions coincided with increased serine phosphorylation of Cx43 and, more importantly, that okadaic acid, a serine/threonine phosphatase inhibitor, prevented both the subsequent dephosphorylation of Cx43, and the restoration of gap junctional communication. Nevertheless, the ability of cells to sustain a basal level of Cx43 phosphorylation may also be important for the es-

tablishment of functional gap junctions (57). As neither the presence of Cx43 nor the detection of Cx43 in its phosphorylated stage can be taken as proof that the cells are or are not actively communicating (57), the functional competence of gap functions must always be evaluated. The dye transfer experiments we have conducted provide the required physiological assessment of the functional status of gap junctions in thecal cells exposed to NGF.

The ability of NGF to serine phosphorylate Cx43 is consistent with the intracellular signaling pathways activated by binding of the growth factor to its *trk*A receptor. NGF activates, among other signaling elements, mitogen-activated protein kinases (3, 40), which have been recently shown to phosphorylate Cx43 on consensus serine phosphorylation sites in the carboxyl-terminal tail of the protein (58). In contrast to NGF, only a small increase in the serine phosphorylation of Cx43 was observed after a 10- to 30-min exposure to EGF, and this change was not accompanied by a reduction in dye transfer. Although it is entirely possible that the dye transfer method employed is unable to detect small changes in gap junctional communication, the lack of effect of EGF was unexpected, because in epithelial cells, EGF strongly induces Cx43 phosphorylation and disrupts gap junctional communication (44). At present, we have no clear explanation for this difference.

Thecal cells in culture may not represent an ideal model to study preovulatory events, because they undergo luteinization *in vitro*. Nevertheless, they provide the best available system to analyze the actions of putative regulators of thecal cell function, as potential confounding influences generated by other ovarian cell types (particularly granulosa cells) are avoided. Transfection of thecal cells with a *trk*A expression vector allowed us to mimic the physiological acquisition of *trk*A receptors that occurs after the preovulatory surge of gonadotropins without having to expose the cells to LH. Thus, the effect of *trk*A activation could be examined in the absence of additional gonadotropin-dependent events that may have modified the results. Thecal cells in culture retain their cell-cell contacts via Cx43-containing gap junctions and respond to NGF with a reduction in the number of viable junctions, indicating that despite their *in vitro* luteinization, isolated thecal cells can respond to a putative regulatory factor transiently expressed before they become luteinized during the normal process of ovulation. Importantly, overexpression of the *trk*A receptor did not affect the overall signal transduction machinery of the cells, as evidenced by the inability of other NTs acting via tyrosine kinase receptors (such as NT-3 and NT-4) to induce Cx43 phosphorylation and affect gap junctional communication in cells transiently expressing *trk*A receptors. Thus, the experimental conditions used allowed us to examine, under controlled *in vitro* conditions, the specific manifestations of NGF-dependent, *trk*A-mediated events.

Our experiments do not allow us, however, to define the type of thecal cells most prominently affected by NGF, because the preparation we used contains cells derived from both theca interna and theca externa. The finding that *trk*A mRNA is mostly expressed in theca externa of rat preovulatory follicles implicates theca externa as the main target for NGF action within the follicular wall. In bovine follicles, however, *trk*A mRNA can be detected by RNase protection assay in both theca interna and externa of antral follicles (Dissen, G. A., J. A. Parrott, M. K. Skinner, and S. R. Ojeda, unpublished data). Recently, Espey advanced the view (30) that the principal events leading to the degradation of the follicular wall, and ultimately to follicular rupture, may take place in thecal fibroblasts of the theca externa, which during the hour preceding ovulation "switch from a quiescent condition into a motile proliferating state." Our results suggest that activation of *trk*A receptors may be one of the molecular events implicated in this process, as thecal cells bearing *trk*A receptors not only lose gap junctional communication (this paper), but also proliferate in response to NGF (Dissen, G. A., D. F. Hill, J. A. Parrott, M. K. Skinner, and S. R. Ojeda, in preparation). Thus, neurotrophic genes may not only be important for the development of the nervous system, but they may also play important roles in the regulation of specific differentiation processes affecting the endocrine system. Participation of the NGF-*trk*A regulatory complex in the ovulatory process appears to provide a unique example of this type of neuroendocrine integration.

Acknowledgments

We thank Dr. Richard Simerly (Division of Neuroscience, Oregon Regional Primate Research Center) and the members of his lab for advice and help in using the equipment for the dye transfer studies, Dr. E. Beyer (Washington University, St. Louis, MO) for the Cx43 cDNA, Dr. Luis Parada (Southwestern Medical Center, Dallas, TX) for the pJM5 *trk*A expression vector, and Dr. David Kaplan (NCI-Fredrick Cancer Research and Development Center, Montreal Neurological Institute, Montreal, Canada) for his generous supply of phosphotyrosine antibodies.

References

1. **Levi-Montalcini R** 1987 The nerve growth factor 35 years later. Science 237:1154–1162
2. **Snider WD** 1994 Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77:627–638
3. **Raffioni S, Bradshaw RA, Buxser SE** 1993 The receptors for nerve growth factor and other neurotrophins. Annu Rev Biochem 62:823–850
4. **Thoenen H** 1991 The changing scene of neurotrophic factors. Trends Neurosci 14:165–170
5. **Otten U, Ehrhard P, Peck R** 1989 Nerve growth factor induces growth and differentiation of human B lymphocytes. Proc Natl Acad Sci USA 86:10059–10063
6. **Matsuda H, Coughlin MD, Bienenstock J, Denburg JA** 1988 Nerve growth factor promotes human hemopoietic colony growth and differentiation. Proc Natl Acad Sci USA 85:6508–6512
7. **Ehrhard PB, Erb P, Graumann U, Otten U** 1993 Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. Proc Natl Acad Sci USA 90:10984–10988
8. **Horigome K, Pryor JC, Bullock ED, Johnson Jr EM** 1993 Mediator release from mast cells by nerve growth factor. J Biol Chem 268:14881–14887
9. **Missale C, Boroni F, Frassine M, Caruso A, Spano P** 1995 Nerve growth factor promotes the differentiation of pituitary mammotroph cells *in vitro*. Endocrinology 136:1205–1213
10. **Polak M, Scharfmann R, Seilheimer B, Eisenbarth G, Dressler D, Verma IM, Potter H** 1993 Nerve growth factor induces neuron-like differentiation of an insulin secreting pancreatic beta cell line. Proc Natl Acad Sci USA 90:5781–5785
11. **Kanaka-Gantenbein C, Tazi A, Czernichow P, Scharfmann R** 1995 *In vivo* presence of the high affinity nerve growth factor receptor *trk*-A in the rat pancreas: differential localization during pancreatic development. Endocrinology 136:761–769
12. **Missale C, Boroni F, Sigala S, Zanellato A, Dal Toso R, Balsari A, Spano P** 1994 Nerve growth factor directs differentiation of the bipotential cell line HG-3 into the mammotroph phenotype. Endocrinology 135:290–298
13. **Scharfmann R, Tazi A, Polak M, Kanaka C, Czernichow P** 1993 Expression of functional nerve growth factor receptors in pancreatic beta cell lines and fetal rat islets in primary culture. Diabetes 42:1829–1836
14. **Djakiew D, Pflug B, Dionne C, Onoda M** 1994 Postnatal expression of nerve growth factor receptors in the rat testis. Biol Reprod 51:214–221
15. **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
16. **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
17. **Dekel N** 1988 Regulation of oocyte maturation by cell to cell communication. In: Piva F, Bardin CW, Forti G, Motta M (eds) Cell to Cell Communication in Endocrinology. Raven Press, New York, pp 181–194
18. **Dermitzel R, Spray DC** 1993 Gap junctions in the brain: where, what type, how many and why? Trends Neurosci 16:186–192
19. **Bennett MVL, Barrio LC, Bargiello TA, Spray DC, Hertzberg E, Sáez JC** 1991 Gap junctions: new tools, new answers, new questions. Neuron 6:305–320
20. **Amsterdam A, Rotmensch S** 1987 Structure-function, relationships during granulosa cell differentiation. Endocr Rev 8:309–337
21. **Erickson GF, Magoffin DA, Dyer CA, Hofeditz C** 1985 The ovarian androgen producing cells: a review of structure/function relationships. Endocr Rev 6:371–399
22. **Mayerhofer A, Garfield RE** 1995 Immunocytochemical analysis of the expression of gap junction protein connexin 43 in the rat ovary. Mol Reprod Dev 41:331–338
23. **Granot I, Dekel N** 1994 Phosphorylation and expression of connexin-43 ovarian gap junction protein are regulated by luteinizing hormone. J Biol Chem 269:30502–30509
24. **Risek B, Guthrie S, Kumar N, Gilula NB** 1990 Modulation of gap junction transcript and protein expression during pregnancy in the rat. J Cell Biol 110:269–282
25. **Wiesen JF, Midgley Jr AR** 1994 Expression of connexin 43 gap junction messenger ribonucleic acid and protein during follicular atresia. Biol Reprod 50:336–348
26. **Wiesen JF, Midgley Jr AR** 1993 Changes in expression of connexin 43 gap junction messenger ribonucleic acid and protein during ovarian follicular growth. Endocrinology 133:741–746
27. **Dissen GA, Hill DF, Costa ME, Ma YJ, Ojeda SR** 1991 Nerve growth factor receptors in the peripubertal rat ovary. Mol Endocrinol 5:1642–1650
28. **Hantzopoulos PA, Suri C, Glass DJ, Goldfard MP, Yancopoulos GD** 1994 The low affinity NGF receptor, p75, can collaborate with each of the trks to potentiate functional responses to the neurotrophins. Neuron 13:187–201
29. **Hirshfield AN** 1991 Development of follicles in the mammalian ovary. Int Rev Cytol 124:43–101
30. **Espey LL, Lipner H** 1994 Ovulation. In: Knobil E, Neill JD (eds) Physiology of Reproduction, ed 2. Raven Press, New York, pp 725–780
31. **Roberts AJ, Skinner MK** 1990 Hormonal regulation of thecal cell function during antral follicle development in bovine ovaries. Endocrinology 127:2907–2917
32. **Loeb DM, Maragos J, Martin-Zanca D, Chao MV, Parada LF, Greene LA** 1991 The *trk* proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. Cell 66:961–966
33. **Berg-von der Emde K, Dees WL, Hiney JK, Hill DF, Dissen GA, Costa ME, Moholt-Siebert M, Ojeda SR** 1995 Neurotrophins and the neuroendocrine brain: different neurotrophins sustain anatomically and functionally segregated subsets of hypothalamic dopaminergic neurons. J Neurosci 15:4223–4237
34. **Meakin SO, Suter U, Drinkwater CC, Welcher AA, Shooter EM** 1992 The rat *trk* protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc Natl Acad Sci USA 89:2374–2378
35. **Beyer EC, Paul DL, Goodenough DA** 1987 Connexin 43: a protein from rat heart homologous to gap junction protein from liver. J Cell Biol 105:2621–2629
36. **Sakai N, Tabb T, Garfield RE** 1992 Studies of connexin 43 and cell-to-cell coupling in cultured human uterine smooth muscle. Am J Obstet Gynecol 167:1267–1277
37. **Hill DF, Dissen GA, Ma YJ, Ojeda SR** 1992 Detection of nerve growth factor and one of its receptors. In: Conn PM (ed) Methods in Neurosciences: Gene Expression in Neural Tissues. Academic Press, New York, vol 9:179–196
38. **Lara HE, Hill DF, Katz KH, Ojeda SR** 1990 The gene encoding nerve growth factor is expressed in the immature rat ovary: effect of denervation and hormonal treatment. Endocrinology 126:357–363
39. **Kaplan DR, Morrison DK, Wong G, McCormick F, Williams LT** 1990 PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell 61:125–133
40. **Ping X, Greene LA, Kaplan DR, Stephens RM** 1995 Deletion of a conserved juxtamembrane sequence in Trk abolishes NGF-promoted neuritogenesis. Neuron 15:395–406
41. **Ma YJ, Hill DF, Junier M, Costa ME, Felder SE, Ojeda SR** 1994 Expression of epidermal growth factor receptor changes in the hypothalamus during the onset of female puberty. Mol Cell Neurosci 5:246–262
42. **Goldberg GS, Bechberger JF, Naus CCG** 1995 A pre-loading method of evaluating gap junctional communication by fluorescent dye transfer. BioTechniques 18:490–497
43. **Hempstead BL, Rabin SJ, Kaplan L, Reid S, Parada LF, Kaplan DR** 1992 Overexpression of the *trk* tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. Neuron 9:883–896
44. **Lau AF, Kanemitsu MY, Kurata WE, Danesh S, Boynton AL** 1992 Epidermal growth factor disrupts gap-junctional communication and induces phosphorylation of connexin43 on serine. Mol Biol Cell 3:865–874
45. **Mulheron GW, Schomberg DW** 1993 The intraovarian transforming growth factor system. In: Adashi EY, Leung PCK (eds) The Ovary. Raven Press, New York, pp 337–361
46. **Nedergaard M** 1994 Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. Science 263:1768–1770
47. **Macdonald AS, Schifferli K, Anderson D, Jessee J, Ciccarone V** 1996 A simple method for cationic lipid reagent selection for transfection. Focus 18:6–9
48. **Hawley-Nelson P, Ciccarone V, Gebeychu G, Jesse J, Felgner PI** 1993 Lipofectamine reagent: a new, higher efficiency polycationic liposome transfection reagent. Focus 15:73–78
49. **Bjersing L, Cajander S** 1974 Ovulation and the mechanism of follicle rupture. Cell Tissue Res 153:15–30
50. **Reich R, Daphna-Iken D, Chun SY, Popliker M, Slager R, Adelman-Grill BC, Tsafriri A** 1991 Preovulatory changes in ovarian expression of collagenases and tissue metalloproteinase inhibitor messenger ribonucleic acid: role of eicosanoids. Endocrinology 129:1869–1875
51. **Curry Jr TE, Mann JS, Estes RS, Jones PBC** 1990 α_2-Macroglobulin and tissue inhibitor of metalloproteinases: collagenase inhibitors in human preovulatory ovaries. Endocrinology 127:63–68
52. **Curry Jr TE, Dean DD, Sanders SL, Pedigo NG, Jones PBC** 1989 The role of ovarian proteases and their inhibitors in ovulation. Steroids 54:501–521
53. **Glass DJ, Nye SH, Hantzopoulos P, Macchi MJ, Squinto SP, Goldfarb M, Yancopoulos GD** 1991 TrkB mediates BDNF/NT-3-dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. Cell 66:405–413
54. **Cordon-Cardo C, Tapley P, Jing S, Nanduri V, O'Rourke E, Lamballe F, Kovary K, Jones K, Reichardt LF, Barbacid M** 1991 The *trk* tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66:173–183
55. **Kumar NM, Gilula NB** 1996 The gap junction communication channel. Cell 84:381–388
56. **Goldberg GS, Lau AF** 1993 Dynamics of connexin 43 phosphorylation in pp60$^{v\text{-}src}$-transformed cells. Biochem J 295:735–742
57. **Musil LS, Cunningham BA, Edelman GM, Goodenough DA** 1990 Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. J Cell Biol 111:2077–2088
58. **Warn-Cramer BJ, Lampe PD, Kurata WE, Kanemitsu MY, Loo LWM, Eckhart W, Lau AF** 1996 Characterization of the mitogen-activated protein kinase phosphorylation sites on the connexin-43 gap junction protein. J Biol Chem 271:3779–3786