Involvement of Nerve Growth Factor in the Ovulatory Cascade: \textit{trkA} Receptor Activation Inhibits Gap Junctional Communication Between Thecal Cells* \\


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ABSTRACT \\

Activation of \textit{trkA}, 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 \textit{trkA} and NGF gene expression occurs in thecal-interstitial cells of the ovary. Immunoneutralization of NGF actions or pharmacological blockade of \textit{trkA} transducing activity inhibits ovulation, suggesting that activation of the NGF-\textit{trkA} 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 \textit{trkA} receptors in isolated ovarian thecal cells disrupts cell to cell communication by affecting the functional integrity of gap junctions. Bovine thecal cells expressing \textit{trkA} 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 neurophin 3, neurophin 4, and brain-derived neurotrophic factor. Thus, cell-specific activation of \textit{trkA} 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)
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 trkA abundance triggered by the preovulatory surge of gonadotropins in this ovarian compartment suggest that activation of trkA receptors may contribute to the LH-dependent changes in thecal cell homeostasis that precede ovulation. Fibroblasts ectopically expressing trkA 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 trkA 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 trkA receptors caused by LH in vivo via transfer of the trkA gene into purified cultures of unstimulated bovine 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 trkA 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 trkA 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 [35S]UTP-labeled rat trkA complementary RNA (cRNA) probe complementary to nucleotides 226–752 in trkA mRNA (34) and a digoxigenin-UTP-labeled rat Cx43 cRNA probe complementary to nucleotides 226–561 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 antisera (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 [32P]UTP. Detection of trkA mRNA by RNase protection assay also employed the same cRNA used for in situ hybridization, but labeled with [32P]UTP.

**Protein phosphorylation**

To determine whether transfection of thecal cells with the trkA expression vector resulted in expression of biologically active receptors, thecal cells that had been transfected with either plasmid alone or with the trkA expression vector were treated with NGF (100 ng/ml; 5 min), lysed in RIPA buffer (20 mm Tris, pH 7.4, 137 mm NaCl, 0.2% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, 1 mm sodium orthovanadate) (39), and immunoprecipitated for 4 h at 4°C with trkA 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 × 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 DiIC18 (1,1'-diocadecyl-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 “native” trkA-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 contact.
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 DilCl8), interfaced with a 486DX/66 IBM-compatible personal computer (Comtrate, 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 DilCl8 and calcein) to neighboring cells (positive for calcein, but not for DilCl8) was evaluated between 90-120 min after seeding by counting the number of calcein-positive cells that surround one calcein/DilCl8-positive donor cell. This evaluation was performed blind, i.e. without knowledge of the treatment.

Statistics
The differences in dye transfer between trkA-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), trkA 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 trkA 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 trkA mRNA and Cx43 mRNA appeared to be localized in the theca externa (black arrows), but not all trkA 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 trkA 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 immunohistochemistry, respectively (Fig. 3, A and B).
Our previous studies in the rat demonstrated that the trkA 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-independent events, we increased trkA expression in purified bovine thecal cells by transient transfection of an expression construct (pJM5) containing a rat trkA complementary DNA (cDNA) under the control of the CMV immediate early promoter (32). Forty-eight hours later, we assessed the response of the trkA-overexpressing cells to NGF. Other cells were transfected with the pCMV vector alone. Cells transfected with the trkA cDNA synthesized rat trkA 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 trkA phosphorylated by NGF in the NGF-responsive pheochromocytoma cell line PC12. A similar specificity was not detected either in cells transfected with pCMV only and challenged with NGF (Fig. 4B) or in trkA cDNA-transfected cells treated with NT-3, brain-derived neurotrophic factor, or NT-4 (not shown), demonstrating the specificity of the trkA construct.
NGF induces phosphorylation of thecal Cx43 at serine residues

Addition of NGF (100 ng/ml) to trkA-transfected cells induced rapid phosphorylation of membrane-associated Cx43 (approximate Mr 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 trkA 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 trkA 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 trkA-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 through the cell membrane, but passes through gap junctions. Exposure of trkA-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 trkA-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 trkA-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 transection 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.

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 disassociation 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 trkA, 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 trkA receptors may be one of the mechanisms...
Fig. 6. Inhibitory effect of NGF on calcein dye transfer between thecal cells expressing the rat trkA 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 trkA-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, trkA- and
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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 trkB (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 trkA 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.

trkB-expressing fibroblasts also show loss of contact inhibition in response to ligand stimulation (28). Although cells of mesenchymal origin do not normally express trkA receptors, the activation of trkA 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 trkB receptors in thecal cells may contribute to initiating the preovulatory loss of cell-cell contact within the follicular wall. Exposure of thecal cells bearing trkB 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-
establishment 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 trkA 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 trkA expression vector allowed us to mimic the physiological acquisition of trkA receptors that occurs after the preovulatory surge of gonadotropins without having to expose the cells to LH. Thus, the effect of trkA 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 trkA 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 trkA receptors. Thus, the experimental conditions used allowed us to examine, under controlled in vitro conditions, the specific manifestations of NGF-dependent, trkA-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 trkA 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, trkA mRNA can be detected by RNase protection assay in both theca interna and externa of antural 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 trkA receptors may be one of the molecular events implicated in this process, as thecal cells bearing trkA 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-trkA regulatory complex in the ovulatory process appears to provide a unique example of this type of neuroendocrine integration.

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