Neurotrophic Control of Ovarian Development

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ABSTRACT Substantial evidence now exists indicating that the neurotrophins, a family of growth factors required for the survival, development, and differentiation of various neuronal populations of the nervous system, are also important for the development of nonneuronal tissues. Such a function was first suggested by studies showing the presence of high-affinity neurotrophin receptors in a variety of nonneuronal tissues including those of the cardiovascular, endocrine, immune, and reproductive systems. Within the latter, the gonads appear to be a preferential site of neurotrophin action as suggested by the presence in the mammalian ovary of at least four of the five known neurotrophins and all of the neurotrophin receptors thus far identified. While the various functions that the neurotrophins may have in the ovary are still being elucidated, it is now clear that in addition to recruiting the ovarian innervation, they play a direct role in the regulation of two different maturational periods that are critical for the acquisition of female reproductive function: early follicular development and ovulation. Neurotrophins facilitate the development of newly formed follicles by promoting the initial differentiation and the subsequent growth of primordial follicles. These actions appear to be related to the ability of neurotrophins to sustain the proliferation of both mesenchymal and granulosa cells, and to induce the synthesis of follicle stimulating hormone (FSH) receptors. At the time of the first ovulation, neurotrophins contribute to the ovulatory cascade by increasing prostaglandin E_2 release, reducing gap junction communication, and inducing cell proliferation within the thecal compartment of preovulatory follicles.

INTRODUCTION

In recent years, it has become apparent that growth factors involved in regulating the development and differentiation of neural cells also contribute to the control of these processes in nonneuronal cells. A conspicuous example of such a dual function can be found in the epidermal growth factor family of polypeptide growth factors. Members of this family are required not only for the development of neuronal populations in the brain and peripheral nervous system, but also for formation of the heart (Lemke, 1996).

Evidence is now accumulating that another family of growth factors, known as the neurotrophins (NTs), also display such a dual activity and contribute to the development of a variety of nonneural tissues, inducing the pancreas, thymus, heart, and adenohypophysis (Tessarollo, 1998). As will be discussed below, ovarian development is also supported by NTs (Ojeda et al., 1994). This function was first inferred by earlier observations showing that when the ovary is transplanted into an ectopic site, reinnervation occurs promptly (Lara et al., 1991), implying that the gland produces substances able to promote and facilitate the ingrowth of nerve fibers from extrinsic sources. Such a plasticity is also evident during normal postnatal development as shown by the twofold increase in the density of the innervation that occurs during prepubertal development of the rhesus monkey ovary (Schultea et al., 1992). That the neurons projecting to the ovary are supported by neurotrophic molecules produced in the gland was demonstrated by the almost complete loss of sympathetic innervation observed in the ovaries of prepubertal rats treated during the first days of postnatal life with antibodies able to neutralize the biological actions of nerve growth factor (NGF) (Lara et al., 1990b), the most prominent member of the NT family. These animals also exhibited stunted follicular development, reduced estrogen output, and disrupted estrous cyclicity. An active retrograde transport of NGF by the innervating fibers was suggested by the accumulation of the peptide, in the gland, following transection of the ovarian nerves (Lara et al., 1990a).

NGF appears to promote ovarian development by at least two separate, but functionally related, mechanisms. On the one hand, it supports the ovarian innervation; on the other, it acts directly on ovarian cells via specific membrane-bound recognition molecules. By supporting the ovarian innervation, NGF facilitates antral follicular growth as the extrinsic sympathetic innervation of the ovary has been shown to exert a

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facilitatory influence on this process (for reviews see Burden, 1985; Dissen et al., 1993). In addition to this effect, the extrinsic nerves of the ovary appear to play a role in early follicular development by promoting the acquisition of gonadotropin receptors and responsiveness to gonadotropins (Hirshfield, 1991; Richards et al., 1987) by the developing follicles. This effect seems to be a function of neurotransmitters acting via activation of the cyclic AMP generating system, such as NE and VIP, which are present in the ovary before the formation of follicles. In vitro exposure of neonatal ovaries to either a β-adrenergic agonist that mimics NE actions in the ovary, or to VIP, resulted in increased FSH receptor gene expression, and in the formation of biologically active FSH receptors (Mayerhofer et al., 1997). Thus NGF, by supporting the ovarian innervation, contributes to both the initial molecular differentiation of newly formed follicles into gonadotropin-responsive structures, and to their subsequent maturation towards ovulatory competence.

In this review, we will discuss evidence suggesting that neurotrophins contribute to two key phases of ovarian maturation: the initial stages of follicular development, and follicular rupture at the time of the first ovulation. By influencing these critical stages of gonadal function, NTs appear to be critical components of the molecular interface used by the nervous and endocrine systems to facilitate the acquisition of ovarian reproductive competence.

NEUROTROPHINS AND THEIR RECEPTORS IN THE OVARY

Neurotrophins belong to a family of target-derived trophic factors required for the survival and differentiation of neuronal populations in both the central and peripheral nervous systems. To date, five NTs have been identified, including NGF (Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Leibrock et al., 1989), neurotrophin-3 (NT-3) (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990), neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991; Ip et al., 1992), and NT-6 (Götz et al., 1994). They initiate their biological actions by binding to high-affinity transmembrane tyrosine kinase receptors encoded by members of the trk proto-oncogene family (Barbacid et al., 1991; Raffioni et al., 1993; Yancopoulos et al., 1990). There are three members of the trk receptor family: trkA that binds NGF, trkB that binds BDNF and NT-4/5, and trkC that binds NT-3 (Raffioni et al., 1993; Yancopoulos et al., 1990). In addition, all NTs (and perhaps also NT-6) are recognized with similar low affinity by a more abundantly expressed receptor, a member of the tumor necrosis receptor family (Dechant and Barde, 1997), known as the low-affinity neurotrophin (NTR) receptor or p75 NTR (Bothwell, 1991; Chao et al., 1986). The actions of the p75 NTR are complex; while NGF binding results in the death of glial cells and some neurons that express p75 NTR, but not trkA (Barrett, 2000), the presence of p75 NTR in sensory neurons and PC12 cells (which also contain trkA receptors) causes cell death even in the absence of NGF (Barrett, 2000). Ectopic expression of p75 NTR in NIH-3T3 fibroblasts demonstrated that NGF uses the receptor to activate sphingomyelin hydrolysis, resulting in the production of ceramide, which is thought to initiate an apoptotic signaling cascade (Barrett, 2000; Dobrowsky et al., 1995). Co-expression of trkA with the p75 NTR in these cells blocked the hydrolysis of sphingomyelin, suggesting that this is a mechanism by which trkA receptors may counteract the death signal conveyed by the binding of NGF to p75 NTR (Barrett, 2000). In addition to the direct signaling mediated by p75 NTR, NGF binding to p75 NTR leads to either amplification (Hantzopoulos et al., 1994) or inhibition (Kohn et al., 1999) of trkA-mediated biological responses. These interactions are especially evident in the case of NGF and BDNF, which have been shown to exert antagonistic effects on both the growth of sympathetic neurons and the ability of these neurons to innervate their target tissues, via alternative binding to p75 NTR (Kohn et al., 1999).

The ovary not only contains four of the known NTs (NGF, BDNF, NT-3, and NT-4/5) (Berkemeier et al., 1991; Dissen et al., 1995, 1996; Ernfors et al., 1990; Hallböök et al., 1991; Lara et al., 1990a, but also expresses the receptors for each of them (p75 NTR, trkA, trkB, and trkC) (Dissen et al., 1991, 1995; Klein et al., 1989; Lamballe et al., 1991). Studies in rats have shown that the genes encoding all of these NTs and their respective receptors are present in the ovary before the initiation of folliculogenesis (Dissen et al., 1995), which in rodents takes place during the first few days after birth (Eppig and O'Brien, 1996; Malamed et al., 1992; Rajah et al., 1992).

EXPRESSION OF NEUROTROPHINS DURING OVARIAN DEVELOPMENT

Time of Folliculogenesis

The NTs and their respective receptors can be detected in the feto-neonatal rat ovary before formation of the first primordial follicles (Dissen et al., 1995). It appears that at least some of the NTs exhibit a developmental pattern of expression related to the phase of definitive ovarian histogenesis and the completion of folliculogenesis (Dissen et al., 1995). Cellular localization of p75 NTR in perinatal rat ovaries using a specific monoclonal antibody demonstrated that the receptor is predominantly expressed in mesenchymal cells (Dissen et al., 1995). By gestational day 18, these cells begin to infiltrate the adjacent epithelium forming "pocket"-like structures, which as gestation approaches term, separate the epithelial, presumptive pre-granulosa cells into groups surrounding individual oocytes. This enclosure continues postnatally resulting in the organization of the three cell types (oocytes/epithelial cells/mesenchymal cells) into primordial follicles between 48h and 72 hours after birth. As predicted by these immunohistochemical studies, the content of p75 NTR mRNA, measured by RNase protection assay, increases after birth to become maximally elevated at the time of follicular assembly. In contrast to this increase in p75 NTR, the ovarian content of both NGF mRNA and trkA mRNA, also measured by RNase protection assay, appears to decrease at the time of folliculogenesis (Dissen et al., 1995). The levels of both NT-4/5 mRNA (detected by semi-quantitative PCR) and those of the mRNA encoding trkB, the NT-4/5 high-affinity receptor (detected by RNase protection assay), increased at this time. In situ hybridization showed that the main increase in NT-4/5 mRNA expression occurred in a sub-

population of oocytes between 24–48 hours after birth, and that the trkB gene was predominantly expressed at this time in epithelial, pre-granulosa cells (Dissen et al., 1995). No major changes in either NT-3 mRNA or trkC mRNA, which encodes the high-affinity receptor for NT-3, were detected. Noteworthy, both of these mRNAs were unambiguously expressed in the ovary by 18 days of fetal life, the earliest fetal age studied. Additional studies are required to define the potential role that NT-3 and its trkC receptor may play in early ovarian development.

These results led to the suggestion that NGF and the NT-4/5-BDNF complex may play different but complementary roles in ovarian histogenesis: the former, facilitating predifferentiation, proliferative processes; the latter, promoting either the organization of germ and somatic cells into follicular structures, or gonadotropin-independent follicular growth. Recent studies examining these issues have demonstrated the validity of these initial concepts (see below).

Time of First Ovulation

Following the completion of follicle formation, the bulk of newly formed follicles remains in a state of quiescence. Selected cohorts are, however, recruited into waves of gonadotropin-dependent proliferative pools from which one or more follicles are selected for ovulation. These gonadotropin-responsive follicles undergo many biochemical and morphological changes during and after the preovulatory surge of gonadotropins (for a review see Richards et al., 1998; Robker et al., 2000). Both *trk*A and NGF gene expression increase following the gonadotropin surge and preceding the ovulatory rupture of the follicle (Dissen et al., 1996). The fugacious nature and magnitude of the NGF/*trk*A gene activation suggests that NGF-initiated *trk*A-mediated responses are integral components of the ovulatory process; whereas 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, more than a 100-fold increase in mRNA levels occurs shortly after the LH surge. Immunohistochemistry and hybridization histochemistry showed that both NGF and *trk*A are produced by thecal cells of large antral follicles and interstitial cells (Dissen et al., 1996). The activation of the *trk*A gene in nonneural cells of the ovarian follicle at a time when the follicle is becoming biochemically and cytologically differentiated into a new structure, the corpus luteum, suggests that ligand-mediated activation of *trk*A receptors contributes to these acute differentiating events.

FUNCTIONS OF NEUROTROPHINS IN THE DEVELOPING OVARY

Functions Around the Time of Folliculogenesis

NGF-trkA Signaling Module. As previously indicated, the receptor for NGF—known as trkA—is a membrane-spanning tyrosine kinase protein that binds NGF with high affinity (Raffioni et al., 1993). NGF and trkA mRNAs, detected by RNase protection assay, are already present in the rat ovary during late fetal development, and their content decrease postnatally at the time of folliculogenesis, i.e., between 24–48 hours after birth (Dissen et al., 1995). Thereafter, trkA mRNA levels remain at very low levels until the time of puberty, but ovarian NGF mRNA content increases during prepubertal days.

The decline of both NGF and trkA mRNA expression that occurs in the neonatal ovary about the time of follicular assembly suggests that this signaling complex may be influencing processes other than cellular differentiation. One of these processes appears to be the proliferation of mesenchymal cells, which is prominent in the fetal ovary but is markedly reduced around the time of folliculogenesis (Hirshfield, 1991). An involvement of NGF in promoting the proliferation of mesenchymal cells was suggested by the ability of the peptide to induce cell proliferation in fibroblastic cell lines ectopically expressing the trkA receptor (Cordon-Cardo et al., 1991; Hantzopoulos et al., 1994). In a recent study using mice lacking the NGF gene (Crowley et al., 1994), we demonstrated that NGF is required for early follicular development (Dissen et al., 2001). The ovaries from NGF knock out (KO) mice analyzed at the time of birth show a dramatic decrease in the number of mesenchymal cells labeled with antibodies to PCNA (proliferating cell nuclear antigen) (Dissen et al., 2001), a nuclear protein associated with the cell cycle (Liu et al., 1989; Xiong et al., 1992). PCNA accumulates during the transition between the G_1 and S phases of the cell cycle, reaches a plateau during the G_2 phase and decreases to much lower levels during the M phase, to disappear during G_0. Thus, its presence in a cell can be used as an index of proliferation. Because PCNA expression may not always be associated with cell proliferation (Hall et al., 1990), we performed additional experiments in which the ovaries of newborn mice were exposed in vitro to bromodeoxyuridine (BrdU) and the ovaries were collected 24 hours later for immunohistochemical analysis of the cells that incorporated the nucleotide analog. The results confirmed the findings with PCNA, by showing that the rate of proliferation of mesenchymal cells in the ovaries from NGF KO animals was about half of that detected in wildtype littermates (Dissen et al., 2001). In addition to this proliferative deficiency detected before the formation of primordial follicles (i.e., follicles having one single layer of flattened epithelial cells surrounding an oocyte). NGF KO mice sacrificed at the end of the first week of postnatal life exhibited a marked delay in early follicular development, characterized by a decrease in the number of primary (one single layer of cuboidal epithelial cells) and secondary (two or more layers of granulosa cells) follicles per ovary (Dissen et al., 2001). This deficiency does not appear to be caused by a gonadotropin deficiency, because serum FSH and LH levels are similar in wild type animals and those carrying either one disrupted NGF allele or the homozygotic mutation. Interestingly, NGF KO mice showed only a marginal decrease in the number of primordial follicles, suggesting that the absence of NGF does not impair follicular formation, but instead disrupts the subsequent growth of primordial follicles.

The mechanisms underlying the supportive effect of NGF on follicular growth includes not only a proliferative signal on mesenchymal cells but also the induction of FSH receptors (FSHR). This effect is manifested after a relatively short time (8 hours) and appears to be mediated by intracellular pathways independent of

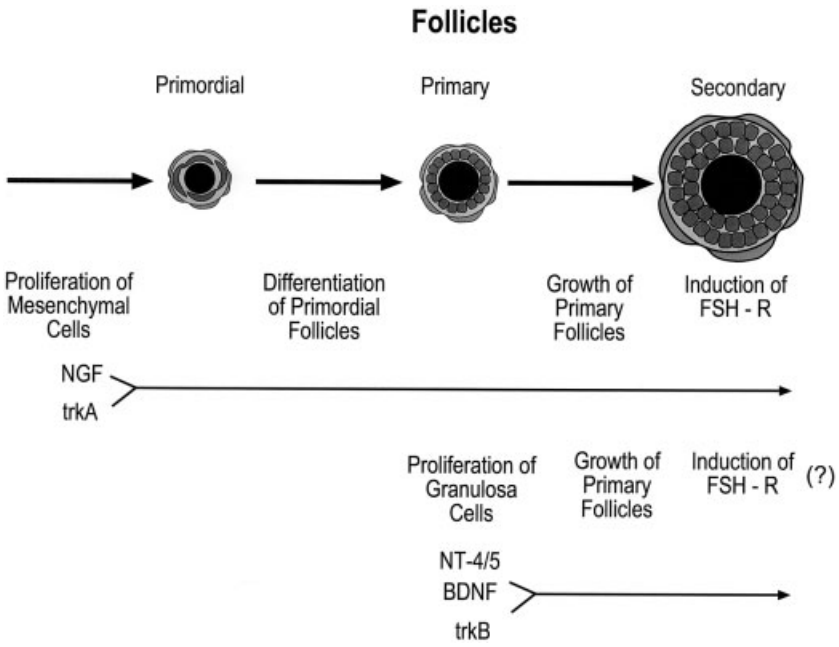

Fig. 1. Postulated roles of the neurotrophins (NGF, NT-4/5, and BDNF) and their respective receptors (trkA and trkB) in early follicular development.

cAMP. Previous studies in neuronal systems have shown that although NGF on its own does not induce cAMP accumulation, it effectively facilitates the effect of adenylate cyclase-stimulating agents on cellular responses (Berg et al., 1995; Heidemann et al., 1985), suggesting that a cAMP-mediated pathway and NGF may act in collaboration to induce cellular differentiation (Heidemann et al., 1985). Consistent with this concept, our results show that, in the neonatal ovary, NGF potentiates the effect of cAMP accumulation on FSHR mRNA levels without affecting cAMP response to activation of adenylate cyclase by other activators (Romero et al., 2001). That NGF is, indeed, required for formation of FSHR during early ovarian development in demonstrated by the reduced content of FSHR mRNA seen in mice lacking the NGF gene when compared to wild type controls (Romero et al., 2001). The finding that NGF facilitates the initiation of follicular growth (Dissen et al., 2001) and is involved in the initial biochemical differentiation of growing follicles into gonadotropin-responsive structures (Romero et al., 2001) defines the developing ovary as one of the nonneuronal, endocrine targets of NGFs actions (Fig 1).

NT-4/5-BDNF-trkB Signaling Module. As indicated above, there was an increase in NT-4/5 mRNA levels at the time of follicular assembly, coinciding with the abrupt appearance of trkB mRNA (the receptor for NT-4/5 and BDNF) (Dissen et al., 1995). The developmental pattern and cellular site of NT-4/5 expression in the neonatal ovary suggested the possibility that the neurotrophin may be a signaling molecule utilized by the oocyte to communicate with pregranulosa cells at the time of follicular formation. According to this concept, NT-4/5 would contribute to the organization of primordial follicles, so that in its absence a deficit in follicular formation would occur. Contrary to this expectation, the ovaries from mice carrying a null mutation of the trkB gene (which eliminates the expression of both full-length and truncated trkB receptors) have no defects in follicular formation (Romero et al., unpublished data). The ovaries, analyzed after completion of ovarian histogenesis, exhibited a normal number of primordial follicles, but a gonadotropin-independent deficiency in early follicular growth, demonstrated by a selective reduction in the formation of secondary follicles. Mitogenic activity of follicular granulosa cells, which is required for the growth of primary follicles, is markedly reduced in trkB KO mice. These results indicate that NT-4/5 and BDNF contribute to regulating mammalian ovarian development by providing a proliferative signal transduced by trkB receptors to granulosa cells of growing follicles. Because NGF-deficient mice also show a defect in ovarian cell proliferation during early postnatal life, it would appear that facilitation of cell proliferation may represent a general mechanism used by neurotrophins to regulate nonneural cell function during development (Fig. 1).

Overall, these results support the emerging concept that NTs play important roles in the development of nonneuronal systems (Donovan et al., 1996). The factors involved in regulating the expression of NTs and their receptors in the ovary remain to be elucidated, but they might be similar (or related) to the Wnt factors recently shown to regulate neurotrophin expression in a nonneuronal system in which ectodermal/mesenchymal interactions are prominent (Patapoutain et al., 1999).

Potential Functions at the Time of Ovulation

NGF-trkA Signaling Module. Ovulation is another major cytodifferentiation phase of ovarian development in which NGF appears to play a role. Mammalian ovulation resembles an inflammatory process, which, instead of being initiated by injury, is set in motion by hormonal stimulation. The inflammatory-like changes that occur in the preovulatory follicle as a

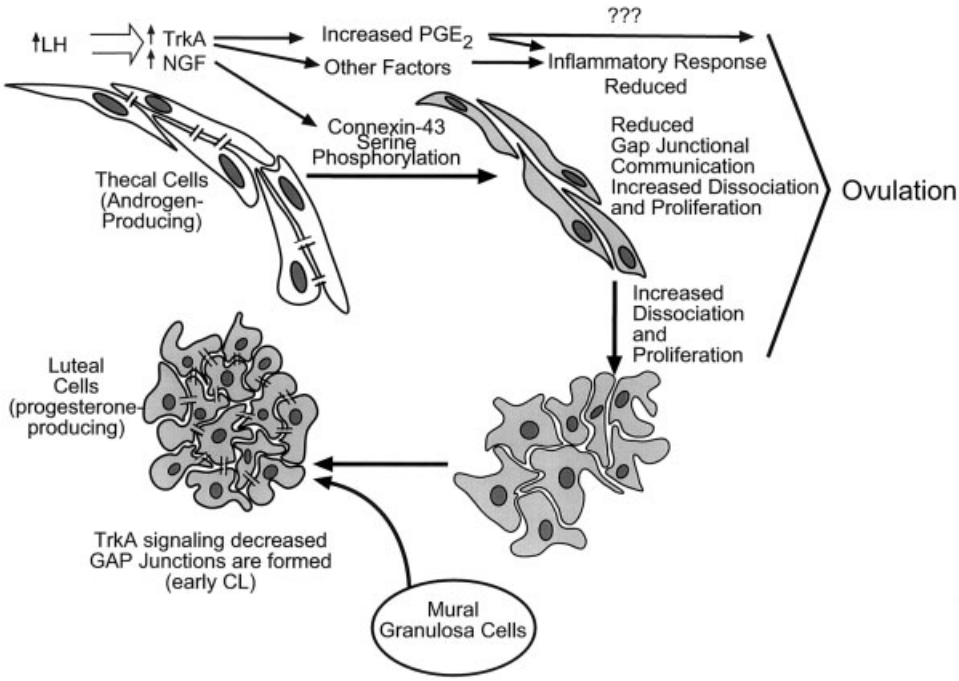

Fig. 2. Schematic representation of postulated actions of NGF as mediated by the high affinity trkA receptor in ovulation. Reproduced from Dissen et al. (2000a) with permission of the publisher.

consequence of LH stimulation result in the dissolution of the follicular wall and ovulatory rupture. A number of substances involved in inflammation, such as interleukins, prostaglandins, and vasoactive factors, have been found in periovulatory follicles (Espey, 1994). Injury of the peripheral nervous system results in rapid activation of NGF synthesis and NGF-dependent processes (see, for instance, Lindholm et al., 1987). The ovary behaves similarity, as shown by the dramatic increase in ovarian trkA gene expression, and the simultaneous elevation in NGF mRNA levels that accompanies and follows the first preovulatory surge of gonadotropins (Dissen et al., 1996). The increase in trkA mRNA content is striking (> 100-fold), it lasts for at least 8 hours, is mainly observed in cells of the follicular wall and interstitial gland, and is accompanied by a corresponding increase in immunoreactive trkA protein. In vitro and in vivo experiments demonstrated that this preovulatory increase in trkA expression is an LH-dependent phenomenon.

The proestrous LH surge stimulates ovarian synthesis of the cytokinin interleukin-1β (IL-1β) (Hurwitz et al., 1991), which appears to play a role in the preovulatory increase of prostaglandin release (Kokia et al., 1992). Our studies showed that IL-1β enhances both trkA and NGF gene expression in ovarian cells, and that this effect was prevented by the natural IL-1β receptor antagonist, Il-1ra (Dissen et al., 1996). The increase in prostaglandin E_2 elicited by IL-1β was reduced by both immunoneutralization of NGF actions and by the pharmacological blockade of trk receptors with the tyrosine kinase inhibitor K-252a (Dissen et al., 1996). NGF stimulated PGE_2 release from ovarian cells in culture, and NGF antibodies administered in vivo reduced the preovulatory increase in ovarian PGE_2 synthesis further suggesting that part of the preovulatory increase in ovarian PGE_2 release is, at least in part, an NGF-dependent event. That activation of the ovarian NGF-trkA ligand/receptor complex is a required component of the ovulatory cascade was suggested by experiments in which PMSG-induced ovulation was inhibited by the intrabursal administration of NGF antibodies or a blocker of trk tyrosine kinase activity (Dissen et al., 1996).

Hints as to the mechanism by which NGF may affect the ovulatory process were first provided by the cellular distribution of NGF and its receptor. The localization of both NGF and its trkA high-affinity receptor in thecal cells of periovulatory follicles suggested that the neurotrophic factor may play a role in follicular rupture, instead of the intrafollicular processes governing granulosa cell and/or oocyte physiology at the time of ovulation. As indicated before, activation of trkA receptors ectopically expressed in fibroblasts results in proliferative responses (Cordon-Cardo et al., 1991; Hantzopoulos et al., 1994). This would suggest that acquisition of neurotrophin receptors by mesenchymal cells engaged in specialized functions, such as thecal cells, may lead to a similar response. In fact, evidence exists that during the hours preceding ovulation, fibroblast-like thecal cells switch from a quiescent to an active, proliferative condition (Espey and Lipner, 1994). The marked increase in trkA and NGF gene expression detected in the follicular wall at this time suggests that an NGF-dependent activation of trkA receptors may contribute to the preovulatory proliferation of thecal cells. In recent studies, we have observed that purified bovine thecal cells engineered to transiently express trkA receptors in culture do proliferate in response to NGF stimulation (Dissen et al., 2000b). In another study (Mayerhofer et al., 1996), we used a similar preparation of purified bovine thecal cells transfected with a trkA expression vector to gain insight into some of the cytodifferentiation processes affected by NGF in

the follicular wall during the preovulatory period. The results showed that activation of trkA receptors by NGF results in serine phosphorylation of connexin-43, the main protein constituent of gap junctions in thecal cells of preovulatory follicles. The phosphorylating effect of NGF is rapid (10–30 min) and is followed by a disruption in cell-cell communication, as indicated by a reduction in the ability of thecal cells exposed to NGF to transfer fluorescent dye via gap junctions. Thus, NGF-dependent activation of trkA receptors in periovulatory thecal cells appears to represent a signal for the loss of cell adhesion that occurs in the follicular wall before ovulation (Fig. 2).

CONCLUSIONS

Mammalian ovarian maturation is influenced by NTs acting at different, but critical developmental windows. While NGF, NT-4/5, and/or BDNF appear to have complementary functions in the regulation of early follicular development, NGF may represent a facilitatory signal for follicular rupture at ovulation. In both cases, the NTs appear to act via regulation of fundamental cellular processes related to both proliferation and cytodifferentiation of ovarian somatic cells.

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