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Ovarian Development is Influenced by a Neuroendocrinotrophic Regulatory Complex

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Abstract: Evidence is presented in support of the concept that mammalian ovarian development is under the regulatory influence of a "neuroendocrinotrophic" complex. We postulate that this complex is formed by three components: the extrinsic innervation to the gland, an intragonadal source of catecholamines, and growth factors of the neurotrophin family. Although these components likely interact throughout the life span of the ovary, each of them may exert its greatest impact on ovarian function during different developmental windows. Thus, an important role of the extrinsic innervation may be to facilitate the initial, pituitary-independent growth of small follicles. The intragonadal source of catecholamines may contribute to regulating subsequent follicular growth and selection by, at least in part, controlling oocyte growth. Finally, neurotrophins may affect more predominantly the phases of follicular assembly and ovulatory rupture. Evidence exists that one of the neurotrophins, nerve growth factor, may contribute to the ovulatory process by disrupting cell-cell communication between thecal cells and facilitating the cytodifferentiation of these cells into their luteal counterparts.

Key Words: Ovarian nerves, neurotrophic factors, follicular development, neurotransmitters, first ovulation

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A. The Extrinsic Innervation of the Ovary

It is well established that the mammalian ovary is a terminal field for both sympathetic and sensory neurons. The projecting neurons innervate the different structural components of the gland, such as the vasculature, interstitial tissue and the follicular wall, with different degrees of complexity.

The sympathetic innervation of the ovary is represented by noradrenergic (NE)- and neuropeptide Y (NPY)-containing fibers; the sensory innervation by substance P (SP)- and calcitonin-gene related peptide (CGRP)-containing nerves [reviewed in 1]. Vasoactive intestinal peptide (VIP)-containing fibers of both sympathetic and sensory nature also innervate the gland [2]. Work emanating from several laboratories, including ours, has demonstrated a stimulatory involvement of catecholamines and VIP in the control of follicular steroidogenesis [for reviews see 2-4], and has led to the concept that this neural control operates independently from, but in harmony with, the well-established hormonal regulation exerted by adenohypophyseal hormones [2,4]. The extrinsic innervation reaches the ovary via two different routes [5]: the ovarian plexus nerve, which travels along the ovarian artery and carries most of the sensory fibers, NPY-ergic nerves, and the bulk of the NE fibers innervating the ovarian vasculature [5-7]; and the superior ovarian nerve (SON), which is associated with the suspensory ligament and carries most of the NE fibers innervating the follicular wall, and all of the VIP innervation to the gland [5,6]. The importance of the SON in the acute control of steroidogenesis is demonstrated by the drop in steroid secretion that follows SON transection at the time of maximal gonadotropin stimulation, in the afternoon of proestrus [8].

The ovarian nerves appear to be endowed with a high degree of plasticity as they

promptly reinnervate the gland after transplantation to an ectopic site [9]. The density of the ovarian innervation -- at least in primates -- increases more than two-fold as the animal approaches puberty [10]. These and other observations have led to the suggestion that, in addition to a stimulatory effect on ovarian steroidogenesis, the extrinsic innervation of the ovary may exert a facilitatory influence on follicular growth [for reviews see 1,11]. Strong support for this view comes from experiments in which permanent loss of the sympathetic innervation, caused by either passive or active neonatal immunosympathectomy, resulted in delayed follicular development, reduced steroidal response to gonadotropins, and marked irregularities of the estrous cycle [12,13].

In addition to facilitating follicular growth, the extrinsic nerves of the ovary may play a role in early follicular development by initiating the molecular differentiation of newly formed follicles into gonadotropin-responsive structures. Once primordial follicles are formed, they begin a differentiation process that results in the acquisition of gonadotropin receptors and responsiveness to gonadotropins [14,15]. While it appears clear that the initiation of this process is gonadotropin-independent [14,15], we recently found that stimulation of adenylate cyclase with forskolin or via VIP- or isoproterenol-mediated receptor activation results in upregulation of both the genes encoding cytochrome P-450 aromatase and the FSH receptor in primary follicles (Mayerhofer A et al., submitted). Since the innervation of the ovary develops before the formation of follicles [16], and the ovarian content of VIP and NE is already high before the gland becomes responsive to gonadotropins [17,18], it would appear that these adenylate cyclase-activating neurotransmitters may represent a candidate signal for the initiation of granulosa cell differentiation at the onset of follicular growth. Support for this notion stems from the

finding that neonatal ovaries in which FSH receptor gene expression was activated by exposure to VIP or isoproterenol, responded to FSH with cAMP formation and follicular growth (Mayerhofer A et al., submitted). Thus, neurotransmitters contained in ovarian nerves are capable of inducing the formation of biologically active FSH receptors at the onset of follicular development.

B. An Intragonadal Source of Catecholamines

In addition to its extrinsic innervation, the monkey ovary contains a network of neuron-like cells, some of which are catecholaminergic [19]. The perikarya of these cells are located in the interstitial tissue, with their processes occasionally targeting thecal cells and innervating blood vessels. To investigate the molecular basis of these findings, we used RT-PCR and oligodeoxynucleotides complementary to highly conserved sequences in exons 1 and 3 of the human and macaque tyrosine hydroxylase (TH) genes, to isolate from the rhesus monkey ovary a cDNA species, which proved to be homologous, but not identical, to the corresponding regions of both the human and rhesus monkey TH genes [20]. The TH cDNA isolated from the ovary encodes amino acid substitutions, the most notable being the addition of an extra serine residue within exon 3. Using this cDNA as a template for the synthesis of antisense RNA, we prepared a cRNA probe for in situ hybridization and detected in preliminary experiments the presence of TH mRNA-containing cells (presumably catecholaminergic) in the ovary of peripubertal monkeys.

The function of an intrinsic network of cells expressing a neuronal phenotype in the primate ovary is not yet known; however, its presence in the gland implies that these cells may play a role in the control of primate ovarian physiology. The fact that some of them are catecholaminergic suggests that they may contribute to stimulating ovarian

steroidogenesis. Catecholamines acting through β_2-adrenergic receptors stimulate ovarian steroid secretion [21-25]. Since catecholamines are able to amplify the stimulatory effect of gonadotropins on ovarian steroidogenesis [23,24,26], it has been hypothesized that this is one of the mechanisms by which catecholaminergic extrinsic nerves affect ovarian steroidogenesis [1,27]. The presence of catecholaminergic cells in the monkey ovary raises the possibility that, in primates, the effect of gonadotropins on ovarian steroidogenesis is also amplified by regulatory inputs provided by neuron-like cells intrinsic to the gland. It appears that these ovarian neuron-like cells are tightly interconnected; while this indicates that their functions are synchronized, it also suggests that they may work in conjunction with the extrinsic innervation to regulate primate ovarian function. A neuronal regulatory system intrinsic to the ovary may not only participate in the control of normal ovarian function in higher primates, but it may also have relevance to the etiology of gonadotropin-independent ovarian pathologies such as psychogenic amenorrhea and stress-related anovulatory syndromes.

Very recent experiments indicate the existence of an additional source of catecholamines in the primate ovary [28]. Reverse transcription-PCR of ovarian RNA, using oligodeoxynucleotides complementary to highly conserved sequences of the human and rat dopamine-beta-hydroxylase (DBH) genes, yielded a cDNA encoding a mRNA species identical to adrenal DBH mRNA. Surprisingly, in situ hybridization identified oocytes as a major site of DBH mRNA expression, a localization verified by RT-PCR cloning and sequencing of a DBH cDNA from cumulus-free oocytes. Immunohistochemical localization of DBH protein by conventional and confocal laser microscopy showed that oocyte DBH mRNA is translated into its protein product. Moreover, oocytes were found

to be devoid of TH mRNA and protein, but did express a dopamine (DA) transporter gene, similar to the one found in human brain. Incubation of oocytes with DA led to the formation of a product which induced cAMP formation in granulosa cells. Since this effect was blocked by propranolol, a β-adrenergic receptor blocker (Mayerhofer A. et al, in preparation), it would appear that oocytes can metabolize DA to NE. Thus, these findings suggest that follicular development in the primate ovary may be affected by a novel cell-cell regulatory loop involving a) DA released by nerve terminals and TH-positive neuron-like cells, b) uptake of DA by oocytes, and c) conversion of DA to NE, which may then feed back on cumulus/granulosa cells to affect their function, presumably via β-adrenergic receptors.

C. Neurotrophins and Ovarian Function

It is now evident that some growth factors controlling the differentiation and development of neural cells also participate in regulating the differentiation and fate of nonneuronal cells.

Recent reports have shown that the neurotrophic family of target-derived trophic factors displays such an activity. To date, five neurotrophins have been identified, the best known of which is nerve growth factor (NGF) [29]. The other members of the family are brain-derived neurotrophic factor (BDNF) [30], neurotrophin-3 (NT-3) [31-33], neurotrophin-4/5 (NT-4/5) [34,35], and the recently identified NT-6 [36]. All of them are required for the survival of different and overlapping neuronal populations in both the central and peripheral nervous system. The biological actions of neurotrophins are mediated by transmembrane tyrosine kinase receptors encoded by members of the trk proto-oncogene family [37-39]. In addition, all neurotrophins (perhaps also NT-6) are recognized by another unrelated,

more abundantly expressed receptor, known as the low-affinity NGF receptor or p75 NGFR [40,41]. The p75 NGFR is not only expressed in nervous tissue, but also in a variety of nonneuronal cells, including the ovary [42-44]. Binding of either NGF or BDNF to p75 NGFR leads to amplification of trk-mediated biological responses [45].

The ovary contains four of the known neurotrophins (NGF, BDNF, NT-3 and NT-4/5) [34,46-50], and the receptors for each of them (p75 NGFR, trkA, trkB and trkC) [51,52]. Expression of these genes is already detectable in the feto-neonatal rat ovary [50].

Recently, we performed studies to determine whether neurotrophins and their receptors are expressed at the time of definitive ovarian histogenesis, and whether any of them exhibit a developmental pattern of expression related to the completion of folliculogenesis [50]. Immunohistochemical identification of p75 NGFR in rat embryonic ovaries using a specific monoclonal antibody revealed that the receptor is predominantly expressed in mesenchymal cells. By gestational day 18, these cells begin to form pockets, which as gestation proceeds, separate the presumptive pre-granulosa cells into discrete groups surrounding individual oocytes. This enclosure continues postnatally resulting in the abrupt formation of primordial follicles between 24 and 48h after birth. Consistent with these observations, p75 NGFR mRNA content, measured by RNase protection assay, increased after birth and was still elevated at the time of follicular assembly. Although the low cellular abundance of NGF and trkA mRNA made it difficult to identify by hybridization histochemistry the cells in which they are produced, RNase protection assay revealed that the ovarian content of both mRNAs varied in parallel during feto-neonatal development, decreasing at the time of folliculogenesis. In contrast to the drop in NGF and trkA mRNA expression, NT-4/5, but not BDNF, mRNA levels increased at the time of follicular

assembly, coinciding with the abrupt appearance of trkB mRNA (the receptor for NT-4/5 and BDNF). In situ hybridization showed that the increase in NT-4/5 mRNA expression occurred in a subpopulation of oocytes between 24-48h after birth; the trkB gene was predominantly expressed at this time in epithelial pre-granulosa cells. No major changes in either NT-3 mRNA or trkC mRNA, which encodes the high-affinity receptor for NT-3, were detected. Noteworthy, both mRNAs were unambiguously expressed in the ovary by 18 days of fetal life, the earliest fetal age studied. These results suggest that NGF and NT-4 may play different but complementary roles in ovarian histogenesis: the former facilitating predifferentiation processes, the latter perhaps contributing to the organization of germ and somatic cells into follicular structures. Alternatively, NT-4 may be regulating some early event of follicular growth such as oocyte viability or the transition of oocytes from a resting to a growing condition.

In other experiments we demonstrated that the gene encoding trkA, the NGF receptor tyrosine kinase, becomes transiently expressed in periovulatory follicles during the hours preceding the first ovulation at puberty [49]. The increase in trkA mRNA content is dramatic (> 100-fold), it lasts for at least 8 h, is mainly localized to cells of the follicular wall, and is accompanied by an increase in immunoprecipitable trkA protein. Both in vivo and in vitro experiments demonstrated that the pubertal increase in ovarian trkA expression is caused by the preovulatory discharge of luteinizing hormone (LH). The changes in trkA mRNA were closely followed by similar changes in NGF gene expression; in both cases peak values were detected about 4-6h before ovulation.

The proestrous LH surge stimulates ovarian synthesis of the cytokinin interleukin-1β (IL-1β) [53], which in turn causes prostaglandin release. IL-1β enhances both trkA and

NGF gene expression in ovarian cells, an effect prevented by the natural IL-1β receptor antagonist, Il-1ra [49]. The increase in prostaglandin E_2 elicited by IL-1β was reduced by both NGF antibodies and a trk receptor blocker. NGF antibodies administered in vivo also reduced the preovulatory increase in ovarian PGE_2 synthesis suggesting that part of the preovulatory increase in PGE_2 release seen in the ovary is NGF-dependent. More importantly, immunoneutralization of NGF actions or pharmacological blockade of trkA receptors targeted to one ovary resulted in the ipsilateral inhibition of ovulation, indicating the importance of an NGF-trkA interaction for the ovulatory process. The striking, but transient, increase in trkA gene expression at the time of the first preovulatory surge of gonadotropins and the accompanying elevation in NGF mRNA content suggests that NGF, acting as a neuroendocrinotrophic signal during a very narrow developmental window affects the acute cytodifferentiation process that leads to the first ovulation.

Activation of trkA receptors ectopically expressed in fibroblasts results in proliferative responses [45,54], suggesting that acquisition of neurotrophin receptors by fibroblasts engaged in specialized functions may lead to a similar response. During the hours preceding ovulation, fibroblast-like thecal cells switch from a quiescent to an active, proliferative condition [55]. The marked increase in trkA and NGF gene expression detected in the follicular wall at this time suggests that the preovulatory proliferation of thecal cells may be, at least in part, an NGF-dependent phenomenon. Support for this concept comes from recent studies showing that purified bovine thecal cells in culture do proliferate in response to NGF stimulation (Dissen GA, Mayerhofer A, Skinner M, Hill DF and Ojeda SR, unpublished data). In another recent study (Mayerhofer A et al., submitted), we used 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 demonstrated that activation of trkA receptors by NGF disrupts cell-to-cell communication via serine phosphorylation of connexin-43, the main protein constituent of gap junctions in thecal cells of preovulatory follicles. The effect of NGF on connexin-43 phosphorylation is rapid (10-30 min) and is followed by a reduction in the ability of thecal cells to transfer fluorescent dye via gap junctions. These results indicate that NGF-dependent activation of trkA receptors in periovulatory thecal cells is a signal for the cellular dissociation of the follicular wall that precedes ovulation.

Taken altogether, the results described in this article suggest that ovarian development is influenced by a "neuroendocrinotrophic" regulatory complex that includes three basic interactive components: the extrinsic innervation of the gland, an intrinsic source of catecholamines, and growth factors of the neurotrophin family.

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