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Expression and Cellular Localization of Gonadotropin-Releasing Hormone (GnRH)-like Messenger Ribonucleic Acid in the Rat Gonad

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Gonadotropin releasing hormone (GnRH) is known to be extrahypothalamically localized with a broad range including gonad. It remains, however, unknown whether GnRH is locally synthesized in the gonad. The present study aims to identify expression and cellular localization of GnRH-like mRNA and immunoreactive GnRH in the rat gonad. GnRH radioimmunoassay and chromatographic extracts on G-50 sephadex column showed that rat gonadal extracts contained a substantial amount of immunoreactive GnRH similar to the hypothalamic and synthetic GnRH. Although a wide distribution of immunostainable GnRH-like molecule with different cell types in the rat ovary was observed, the major cell population hybridized with GnRH probe appears to be granulosa, theca cells and corpus luteum. Immunoreactive GnRH-like peptides were distributed in various regions of testis, including spermatogenic cells, Sertoli cells and Leydig cells. In situ hybridization revealed that positive signals of GnRH-like mRNA were predominantly present in Sertoli cells within some seminiferous tubules, but absent in the outside of seminiferous tubules in the testis. This study clearly demonstrated that GnRH-like molecule present in the rat gonad may be resulted from the local synthetic machinery of GnRH, supporting the notion that this peptide may act as autocrine and/or paracrine role in intra-gonadal communication.

KEY WORDS: Gonadotropin releasing hormone (GnRH), Gene expression, Gonad, *In situ* hybridization, Immunohistochemistry.

Gonadotropin releasing hormone (GnRH) is a key brain regulator of the control of gonadotropin in the mammalian reproduction. Considerable evidence showed that GnRH or GnRH-like peptide was extrahypothalamically localized with a broad range including gonad (Eiden and Brownstein, 1981).

Depending on experimental conditions and the status of gonadal maturation, GnRH and its analogs may exert inhibitory and/or stimulatory effects on a variety of gonadal functions, such as

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gonadal development, steroidogenesis, ovulation, and implantation (Sharpe, 1982; Hsueh and Johnes, 1981). GnRH receptor similar to that found in the rat pituitary was also present in the ovarian and testicular membranes (Bourne et al., 1980; Clayton et al., 1980). Moreover, it is generally believed that GnRH synthesized in the hypothalamus is unable to reach to the gonad in an effective concentration because of its high rate of turnover and dilution in the general circulation (Hsueh and Johnes, 1981), indicating that GnRH or GnRH-like peptide may be synthesized locally within gonad. It remains, however, to be resolved on the origin or the source of biologically active GnRH-like peptide in the gonad.

The present study exmaines the local expression of GnRH-like mRNA in the rat gonad and the precise localization of specific cell types containing immunoreative GnRH-like peptide using *in situ* hybridization and immunohistochemistry.

Materials and Methods

Animals

Immature (70-90 g) or adult (200-250 g) Sprague-Dawley rats were obtained from Seoul National University Animal Breeding Center (Seoul, Korea), and maintained under a temperature controlled light-dark cycle (lights on 0600-200 hr). Food and water available *ad libitum*.

Sephadex G-50 Gel Chromatography and GnRH Radioimmnuoassay (RIA)

Tissue samples of ovary, testis, hypothalamus and cortex were obatined from rats. Ovaries of mature rats were collected regardless of the estrous cycle and pooled. Ovaries and testes were homogenized in 0.02 N acetic acid and centrifuged at 3,000 rpm. The supernatant was mixed with dry charcoal (10% vol/vol) and rotated overnight at 4 °C for removing the steroid hormones (Bhasin *et al.*, 1983). The mixture was centrifuged at 3,000 rpm, and followed by a second centrifugation at 36,000 × g in a ultracentrifuge at 4 °C for 1 hr. The supernatant was defatted with diethyl ether and freeze-dried. The defatted and steroid-free gonadal extracts were dissolved in GnRH RIA buffer, and used for GnRH RIA and gel chromatography as described below. Hypothalami and cortex were homogenized in 0.02 N acetic acid, neutralized and centrifuged. The supernatant was used for GnRH RIA parallelism and gel chromatography, respectively. The recovery of GnRH in gonad, and hypothalamus was ranged from 70-80%.

Five to 100 ul of gonadal extracts were used to determine the GnRH RIA parallellism of suspected GnRH-like molecule with hypothalmic and synthetic GnRH. In other hands, 350 ul of extract was applied to the glass column (1 × 50 cm) to show similarity of chromatographic profile. This column was packed with Sephadex G-50 fine and preequilibrated in 0.02 N acetic acid at 4 °C. The column was eluted with 0.02 N acetic acid at a flow rate of 5 ml/hr. Fractions (1 ml) were collected, neutralized and then assayed for GnRH.

The GnRH concentration was measured in duplicate by a GnRH RIA procedure using Chen-Ramirez GnRH antiserum (CRR-11-B-72) at a final dilution of 1:200,000 as previously described (Kim *et al.*, 1989; Park *et al.*, 1988). Synthetic GnRH was used radioiodination and served as the reference standard. The sensitivity at 80% binding was about 0.5 pg/tube.

Immunohistochemistry

Immunohistochemical localization of GnRH in the rat ovary was performed by the avidine-biotine complex (ABC) method (Hsu *et al.*, 1981). Briefly, the sections were dried, post-fixed for 30 min with 4% para-formaldehyde and rinsed in PBS for 10 min. Then, 50 ul of normal goat serum was applied on the sections for 30 min to reduce the non-specific binding. Rabbit anti-GnRH antiserum was diluted at 1:2000 in tris--phosphate buffer and incubated overnight at 4 °C in Nalgen utility box. Then, the sections were washed in PBS and reacted sequentially at room temperature with biotinylated goat anti-rabbit *r*-globulin fractions at 1:500 for 60 min and ABC conjugated to HRP for 60 min. The sections were reacted in 3,3'-DAB-Ni solution, and the reaction was stopped in water. The slides were dehydrated with alcohol and poly-mounted with balsam. Reaction products were observed underlight microscope.

In situ Hybridization Histochemistry

Two types of oligomers were synthesized by the phosphate/phosphoester method on automated DNA synthesizer (Genetic Engineering Center, KAIST, Seoul, Korea). Probe 1 and 2 consist of 29 and 34 deoxyribonucleotides (29-and 34-mer) complementary to the sequence of the rat pre-proGnRH cDNA coding for amino acids −1 to 9 and 10 to 20 of preprohormone of GnRH (Adelman et al., 1986; 1987), respectively. These probes are named GnRH and GAP probes for a convinience according to its coding region of the preprohormone of GnRH. These oligomers were labeled according to the 5'-end labeling method (Davis *et al.*, 1986). Briefly, 50 pmole of 29-mer and 34-mer were incubated with a 150 uCi of [$r-^{32}$P]ATP (S. A. 3,000 Ci/mmole, NEN) or [$r-^{35}$S]ATP (S.A. 800 Ci/mmole, Amersham) in a kinase buffer (70 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$ and 5 mM DTT) and 20 unit T_4 polynucleotide kinase (BRL) for 30 min at 37 °C. Radiolabeled oligomers were then separated from free by Sephadax G-50 superfine chromatography eluted TE buffer (pH 8.0). The final specific activity was $1-2\times10^8$ cpm/50 pmole for ^{32}P-labeled oligomer and $5\text{-}6\times10^6$ cpm/50 pmole for ^{35}S-labeled oligomer, respectively.

Protocol of *in situ* hybridization histochemistry was based on the method reported previously (Shivers *et al.*, 1986). Pentobarbital anesthetized rats were perfused with 0.1 M phosphate-buffered (pH 7.4) paraformaldehyde (4%). Then, the gonad and the hypothalamus were removed, postfixed in 0.1 M phosphate-buffered paraformaldehyde (4%) for 4 hr and were immediatly frozen at −70 °C. The frozen were cut in a 10 um-thick section with a cryostat (American Optics) at −70 °C until hybridization.

The sections were warmed at room temperature, and digested in 1 ug/ml proteinase K (Behringer Menheim Biochem.) at 37 °C for 30 min. Slides were prehybridized for 2 hr at room temperature in a 50 ul of prehybridization buffer consisting of 1 × Denhardt's solution, 1 mM phosphate, pH 7.4, 100 ug/ml sonicated, heat denatured salmon sperm DNA and 1 mg/ml yeast tRNA. Hybridization was carried out a 50 ul of prehyridization solution supplemented ^{35}S-labeled oligomer for 48 hr at room temperature. The control section were pretreated with RNase A (Sigma) or a unlabeled GnRH probe before hybridization step. After hybridization, the slides were washed twice with 2 × SSC, dehydrated and air-dried. The slides dipped in Kodak NTB_2 emulsion (diluted 1:1 with water), exposed at 4 °C for 2 weeks, developed with the Kodak D-19 developer and the Kodak rapid fixer. And silver grains as positive signals were observed under light microscope.

Results

Identification and Cellular Localization of Immunoreactive GnRH Molecule in the Rat Gonad

A serial dilution (5−100 ul) of gonadal tissue extracts readily allowed the measurement of immunoreactive GnRH. Fig. 1 showed that there is a fairly good parallelism of immunoreactive GnRH in ovarian, testicular, hypothalamic extracts and synthetic GnRH standards. When the gonadal extracts were chromatographed on Sephadex G-50 gel filtration, the majority of immunoreactive GnRH molecule as a single peak was coeluted with those of synthetic GnRH and hypothalamic GnRH, indicating that the molecular size of gonadal GnRH appears similar to those of hypothalamic and synthetic decapeptide. Notice, however, that chromatographic profiles of gonadal extracts showed minor peaks emerged earlier than that of authentic GnRH (Fig. 2). The higher molecular size indicates the presence of more than one molecular form of GnRH molecule in the rat gonad.

Immunohistochemical study was performed to determine the precise cellular localization of immunostainable GnRH peptide in the rat gonad (Plate I). Hypothalamic sections served as the positive control contained immunostainable GnRH or GnRH-like peptide. No immunostainable GnRH peptide was found when primary antiserum was replaced with normal rabbit serum or with buffer constituents. A serial dilution of antiserum resulted in reduction of the intensity of staining reaction. These controls suggest that immunostaining was specific under the condition used in this study.

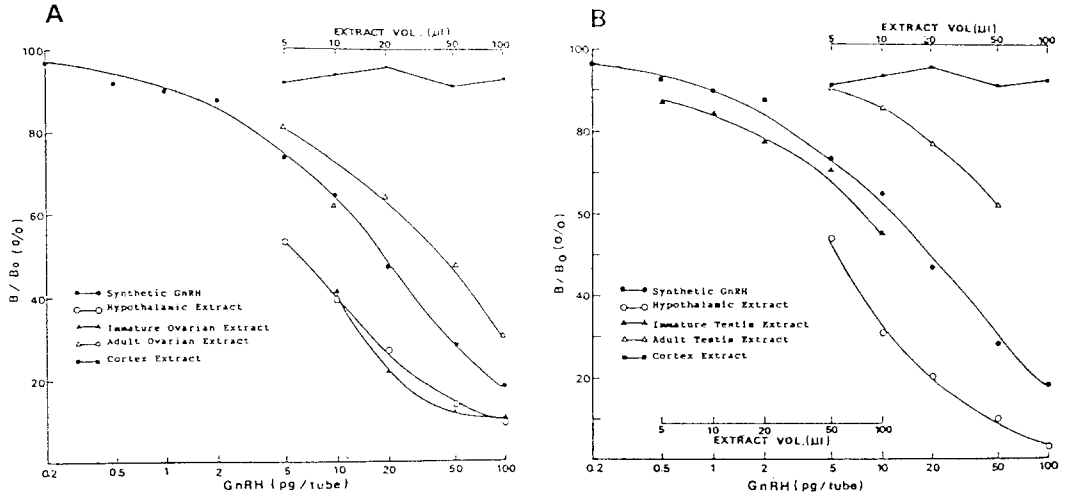

Fig. 1. RIA parallelism of immunoreactive GnRH in the ovarian (A), testicular (B) extracts and synthetic GnRH. B/Bo, bound to free ratio.

In immature and mature rat ovaries, a strongly positive staining was shown in granulosa, theca, cumulus and interstitial cells (Plate I, 3). The immunoreactive GnRH or GnRH-like molecule was widely distributed in the cytoplasm of the Leydig cells, and in the nuclei of spermatogonia within some seminiferous tubules, but not all (Plate I, 4). The nuclei of the Leydig cells were negative. Some spermatogenic cells and most of cytoplasm of Sertoli cells were slightly positive. No reaction product was identified within the interstitial blood vessels or connective tissue element.

Expression of GnRH mRNA in the Rat Gonad

The cellular localization of GnRH-like mRNA in the rat gonad was determined by *in situ* hybridization. To ensure the specificity of GnRH mRNA *in situ*, control groups were employed using the neighboring tissue sections (Plate II). As a positive control, GnRH neurons in the hypothalamus showed GnRH-like mRNA signal (Plate II, 5). The nonspecific binding of GnRH-like mRNA on tissue sections were greatly reduced, and no photographic clusters were practically observed, indicating that silver grains determined seems to be specific (Plate II, 6). In addition, to confirm the localization of GnRH-like mRNA in the gonad, two kinds of oligomer probes coding for GnRH and GAP were used.

As shown in Plate III, the density of hybridization signal with GnRH probe (29-mer) was more dense than that with GAP probe (34-mer). However, both hybridization signals were shown in the same cell types, confirming that GnRH mRNA signal is specific, and GnRH and GAP are expressed simultaneously. Ovarian GnRH-like mRNA predominantly existed in the granulosa/theca cells, although GnRH mRNA appeared in interstitial cells as well (Plate III, 9 and 10). The photomicrographs of *in situ* hybridization histochemistry showed that the signals of testicular GnRH mRNA were mostly found in some discrete but not all seminiferous tubules, but not observed in the interstitial tissue including Leydig cells. There is a strong signal in the Sertoli cells of the seminiferous tubules containing early spermatogonial cells, whereas there is no signals in those containing mature sperms (Plate III, 11 and 12).

Discussion

In the present study, we have clearly demonstrate that GnRH-like mRNA was expressed in the rat gonad, indicating that this gonadal peptide is locally synthesized in the rat gonad.

GnRH-like substance, but chemically different from hypothalamic GnRH was recently found in

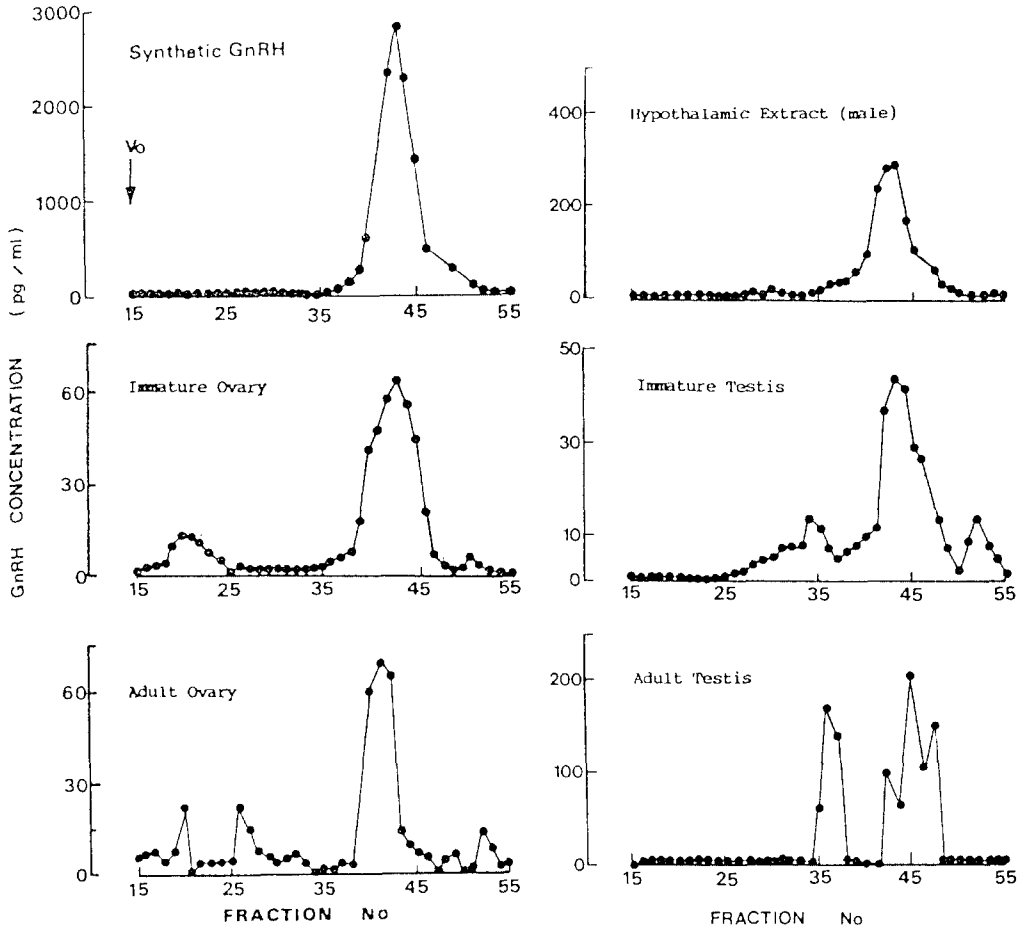

Fig. 2. Sephadex G50 chromatography of GnRH immunoreactivity in ovarian, testicular and hypothalamic extracts and synthetic GnRH. Arrow indicates void volume (V_o).

the rat and human follicular fluid (Ying *et al.*, 1981; Li *et al.*, 1987). Aten *et al.* (1986) recently claimed that the rat ovary contained a protein which is GnRH-like, but distinctly different from hypothalamic and authentic GnRH. For instance, the heat instability and chromatographic behavior during reverse phase high performance liquid chromatography was clearly unlike hypothalamic GnRH. In addition, they found that ovarian GnRH-like substance exhibited the lack of a cross-reactivity with anti-GnRH antiserum (A/S 311) different from our own. However, in our study using anti GnRH antiserum (CRR-11-B-72), there was a fairly good parallelism between immunoreactivities in ovarian, hypothalamic and synthetic GnRH. It was also evident that when the gonadal extracts were chromatographed on Sephadex G-50 gel filtration, the majority of immunoreactive GnRH molecule as a single peak was coeluted with those of synthetic and hypothalamic GnRH molecules. The chromatographic profile from gonadal extracts was more complex than that from hypothalamic extracts, indicating the presence of more than one molecular form of GnRH-like molecule in the rat gonad as previously described (Dultow and Millar, 1981; Gauton *et al.*, 1981). It is noteworthy that GnRH immunoreactivity in the rat testis cross-reacted with antiserum raised against GnRH agonists, but not with anti -GnRH antiserum (Paull *et al.*, 1981;

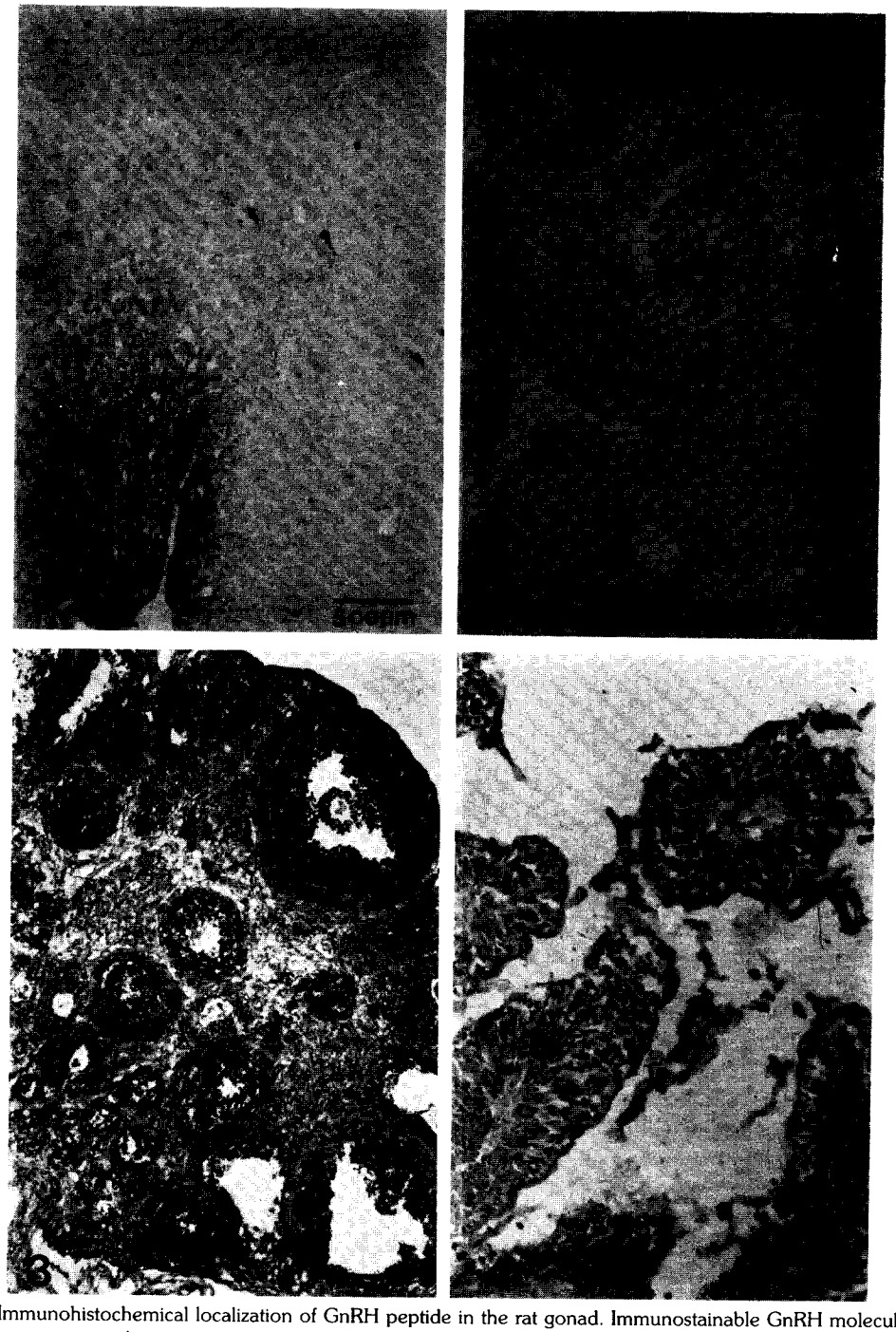

Plate I. Immunohistochemical localization of GnRH peptide in the rat gonad. Immunostainable GnRH molecules are shown in neurons and axons in the preoptic area of hypothalamus as a positive control (1). No immunoreactive GnRH in the ovary when anti-GnRH serum is replaced with normal rabbit serum (NRB) (2). Immunoreactive GnRH is localized in the folliclular cells, interstitial, and luteal cells in the ovary (3)and Leydig and spermatogonial cell in the testis (4).

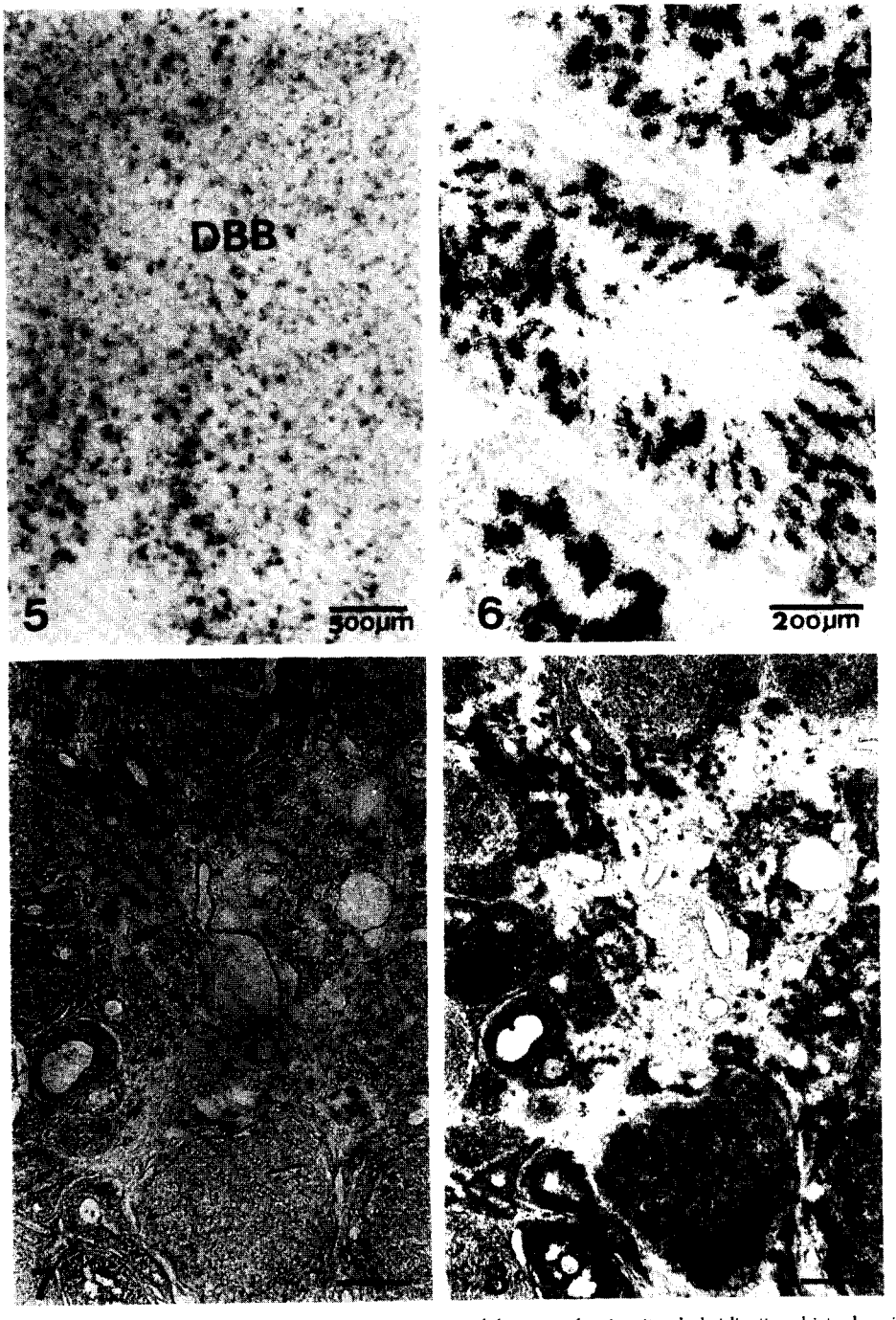

Plate II. Localization of GnRH-like mRNA in the rat gonadal tissues by *in situ.* hybridization histochemistry with S^{35}-end labeled GnRH oligomer. The tissue sections of testis hybridized with ^{35}S-end labeled GnRH ligomer and autoradiographed with NTB_2 solution (see Materials and Methods for details). GnRH-like mRNA is shown in the Diagonal band of Broca (DBB) as a positive control (5). No hybridization signal is shown in the ovarian tissues pretreated with RNase served as a negative control (6). GnRH-like mRNA are localized in the follicular, interstitial and luteal cells in the ovary (7) and in certain seminferous of the testis (8).

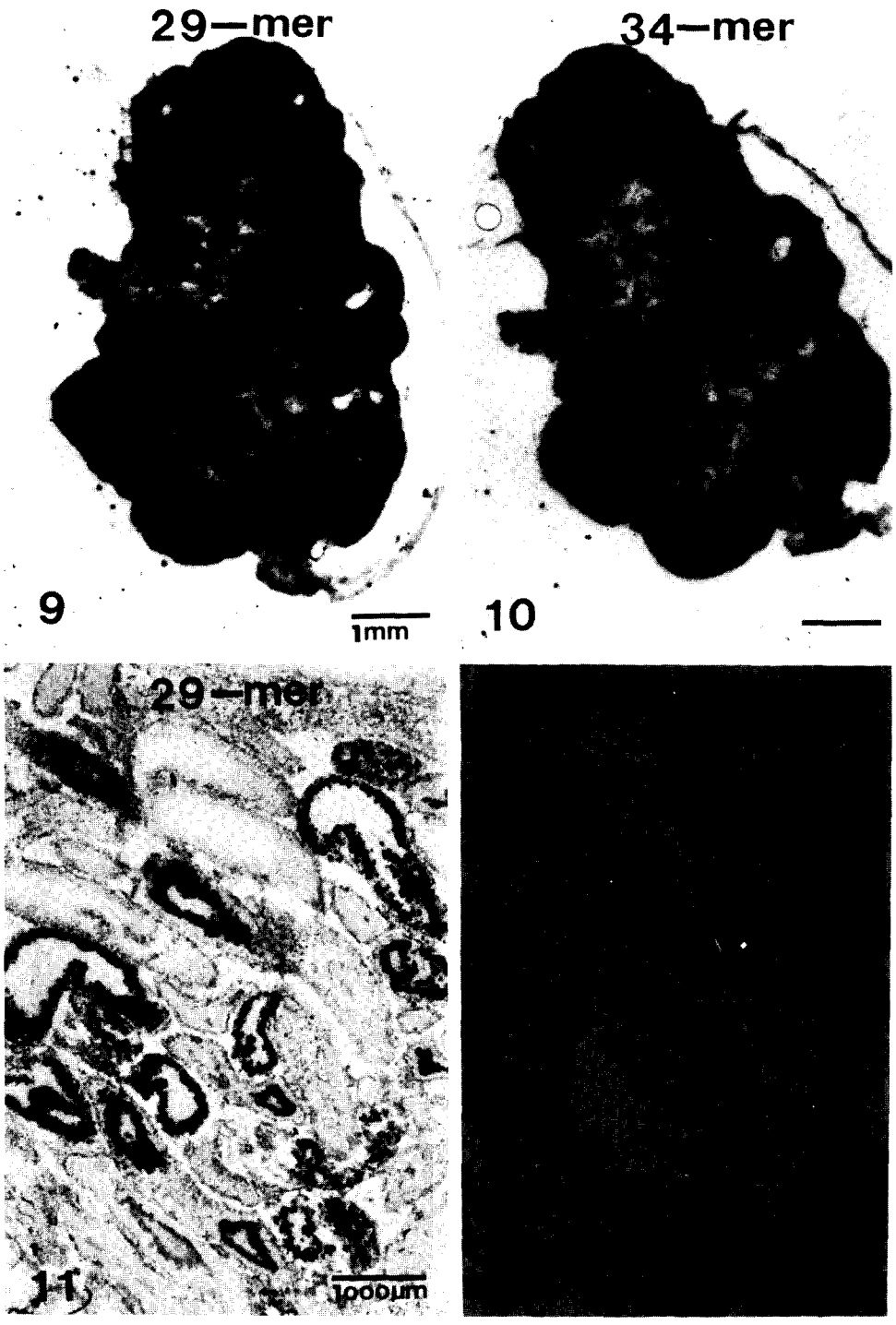

Plate III. Hybridization signals of GnRH and GAP mRNA in the rat gonadal tissues by *in situ* hybridization histochemistry. GnRH-like and GAP mRNA signals are colocalized in the same types of the cells in the rat ovary (9 and 11) and testis (10 and 12).

Sharpe *et al.*, 1981; Swerdloff *et al.*, 1984). At this moment, we have no detailed information on the antigenic determinants of anti-GnRH antisera used in this study. Therefore, we cannot exclude the possibility that immunoreactive GnRH found in the rat gonad is probably not the decapeptide GnRH, since any compound within the ovary having a similar amino acid residue would be detected by anti-GnRH antisera. Alternatively, there may be the heterogeneity of processing of GnRH precusor. Processing of GnRH and GAP from precusor in the rat ovary may differ from those in hypothalamus. Indeed, it has been recently demonstrated that the GnRH precursor contained GnRH and GAP in the rat hypothalamus, and that the multiple immunoreactive forms of GnRH prohormone existed in the hypothalamus, in particular, preoptic hypothalamus. Moreover, the post-translational processing of GnRH in the rat gonad may differ from that in hypothalamus (Wetsel *et al.*, 1988). A further study is needed to delineate the biosynthetic processing of GnRH in the ovary.

It has been well known that GnRH or GnRH analogs exert direct inhibitory and/or stimulatory effects on gonad. The effects of GnRH appeared to be mediated through binding to high-affinity GnRH receptor. In fact, GnRH receptor similar in the binding characteristics to that in the anterior pituitary, was present in granulosa cell (Pieper *et al.*, 1981), corpus luteum (Harwood *et al.*, 1988) and oocyte (Dekel *et al.*, 1988). Recent studies also showed that GnRH action in the ovary is mediated probably through stimulation of phosphatidyl-inositol metabolism and calcium mobilization, and activation of protein kinase C (Leung, 1985). In the present study, immunostainable GnRH peptides were widely distributed in the rat ovary with several cell populations including granulosa/theca, cumulus, interstitial cell, and corpus luteum. *In situ* hybridization data reinforced that the expression of GnRH-like mRNA appears predominant in the granulosa/theca cells and corpus luteum. It is, therefore, reasonable to conclude that these cells are responsible for the synthesis of GnRH peptide. Together with the previous findings described above, the present study strongly suggests that ovarian GnRH-like peptides probably synthesized in granulosa/theca cells and corpus luteum as well are able to diffuse within the ovary, and then acts as autocrine and/or paracrine role in regulating granulosa cell growth, follicular differentiation and steroidogenesis as previously suggested (Hsueh and Jones, 1981). Since GnRH immunoreativities were observed in the cumulus cells, it is also plausible to assume that GnRH peptides in the cumulus cells may directly act on the oocyte membrane receptor and may participate the induction of oocyte maturation (Hillensjo and LeMaire, 1980).

Although the source and nature of the testicular GnRH is still unclear, the expression of GnRH-like mRNA appears to be restricted the Sertoli cells into the some seminiferous tubules and immature spermatogonia. These result confirms the previous reports which the testicular GnRH is secreted from the Sertoli cells and then acts exclusively on the Leydig cells (Sharpe, 1984). It is of interest to note that there is a 3-fold increase of GnRH-like mRNA in the adult testis when compared with that in the immature ovary. It seems then that gene expression of testicular GnRH may be regulated during the process of testis maturation. Finally, it is worth pointing out that the hybridization signals of GnRH mRNA are shown in heterogenous population of seminiferous tubules, indicating that testicular GnRH may function differentially depending on the stages of cycles of seminiferous epithelium. GnRH mRNA signals in the early stage of seminiferous tubules are much higher than those shown in the late stage of seminiferous tubules. It appears that GnRH produced from seminiferous tubules may control the spermatogenesis directly (autocrine) or indirectly (paracrine) throuth the regulation of function of Leydig cells, i. e. androgen biosythesis. Further study is, however, needed to prove the possibility.

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흰쥐 생식소에서 GnRH-like mRNA의 발현과 세포내 분포

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시상하부에서 합성, 분비되는 gonadotropin-releasing hormone (GnRH)의 면역반응성이 생식소를 비롯한 여러 부위에서도 검출됨이 알려졌으나, 이 펩타이드가 과연 생식소에서 국부적으로 합성되는 지에 관해서는 아직 밝혀지지 않았다. 본 연구에서는 흰쥐 생식소에서 GnRH 유전자발현을 연구하기 위하여 GnRH-like mRNA와 GnRH펩타이드의 발현과 세포내 분포양상을 조사하였다. GnRH 방사면역측정법과 GnRH를 크로마토그라피 방법으로 분리한 결과, 시상하부에서 합성되는 GnRH와 유사한 GnRH 면역반응성이 흰쥐 생식소 추출물에서 상당량 검출되었다. GnRH-면역반응성이 흰쥐 난소의 다양한 세포군에서 나타남에 반하여, GnRH-like mRNA는 granulosa, theca 그리고 luteal 세포에서만 주로 발현되었다. 또한 흰쥐 정소에서 GnRH면역반응성은 원시정세포, Sertoli, Leydig 세포에서 검출된 반면에, GnRH-like mRNA는 성세관내의 Sertoli세포에서만 발현되었다. 따라서 이 연구는 생식소에 존재하는 GnRH는 생식소 내에서 국부적으로 합성, 발현되는 결과라고 사료되며, 생식소 내에서 생성된 GnRH는 생식소 내 세포군간의 정보교환의 매개자로서 역할을 수행하고 있다고 추정된다.