Korean J. Zool. 37:304-310, 1994

Neuroanatomical Localization of Cells Containing Gonadotropin Releasing Hormone mRNA in the Brain of Frog, *Rana dybowskii*, by *in situ* Hybridization

Wan Sung Choi[1], Jeong-Woo Kim, Sang Ho Baik[2], Myeong Ok Kim[1], Kyungjin Kim[3], Hyuk Bang Kwon and Wook-Bin Im

Department of Biology, College of Natural Sciences, Chonnam National University, Kwangju, 500-757, [1]Department of Anatomy, College of Medicine, Gyeongsnag National University, Chinju, 660-280; [2]Department of Anatomy, College of Medicine, Seoul National University 110-744; and [3]Department of Molecular Biology, College of Natural Sciences, Seoul National University, Seoul, 151-742

Using *in situ* hybridization, we have mapped the anatomical localization of perikarya containing mRNA that codes for gonadotropin releasing hormone (GnRH) in the brains of female frogs, *R. dybowskii*. DNA oligomers, with sequences complementary to the GnRH portion of pro-GnRH mRNA sequence, were synthesized and hybridized to paraformaldehyde-fixed, sagittal sections of the whole brain stem. The distribution of the GnRH mRNA containing cell bodies was similar to that described for GnRH peptide by immunohistochemistry. That is, cells containing GnRH mRNA were observed in the medial septal area, anterior preoptic area, ventromedial hypothalamus and infundibular regions. However, another cell groups which contains GnRH mRNAs were also detected by *in situ* hybridization in the bed nucleus of hippocampal commissure, preoptic area, nucleus infundibularis dorsalis, mesencephalic nuclei and intermediolateral cell column of spinal cord areas. These results demonstrate the feasibility of using *in situ* hybridization as a strategy to study the distribution of GnRH neurons and the detection of GnRH gene expression in the vertebrates.

KEY WORDS: GnRH mRNA, Frog, *in situ* hybridization

Gonadotropin-releasing hormone (GnRH), originally isolated from porcine (Matsuo *et al.*, 1971) and ovine (Amoss *et al.*, 1971) hypothalamus, is the physiological regulator of luteinining hormone (LH) and follicle-stimulating hormone (FSH) release from the anterior pituitary gland (Kalra, 1986). It was thought that GnRH was a unique molecular form until variant forms were demonstrated in the brains of nonmammalian vertebrates (King and Millar, 1979). Several structural variants have been sequenced from rat, chicken, and so on. These members of the GnRH family are decapeptides with gonadotropin-releasing activities and significant structural homologies (mammalian GnRH, salmon GnRH, chicken GnRHI, chicken GnRHII, and lamprey GnRH) (King and Millar, 1987; Sherwood, 1987). Therefore, GnRH neuron system in vertebrates has been more complicated.

In amphibian species, mammalian GnRH together with chicken GnRHII and salmon GnRH

This work was supported by Genetic Engineering Research Grant (1991) from the Ministry of Education.

have been identified and the neuroanatomical locations of these peptides have been investigated by immunocytochemistry (ICC, Sherwood *et al.*, 1986). However, interpretation of peptide content data by ICC or by analysis of neuropeptide content has been controversial. Specially, the presence of GnRH neurons in the mesencephalic nucleus and spinal cord is conflicted according to the researchers. To observe the more direct distribution of peptide-secreting neurons, it is necessary to search the expression of GnRH mRNA. *In situ* hybridization makes it possible to detect and measure the specific cellular mRNAs within the neuroanatomically intact nervous system (Shivers *et al.*, 1986; Standish *et al.*, 1987).

On the basis of the sequence of human placental GnRH cDNA (Seeburg and Adelman, 1984), we have synthesized radiolabeled oligonucleotide complementary to the GnRH mRNA nucleoptide sequence. Using the *in situ* hybridization technique with these oligonucleotide probe, we now report the anatomic localization of cells containing GnRH mRNA and compare the distribution of GnRH mRNA with immunoreactive GnRH in the brain of the female frog, *R. dybowskii*.

Material and Methods

Five adult female frogs, *R. dybowskii*, weighing 45-50g were deeply anesthetized with sodium pentobarbital and perfused intracardially with physiological saline, followed by 4% para-formaldehyde in phosphate buffer and 4% paraformaldehyde. The brain of each animals was quickely removed, and a block of tissue containing the hypothalamus and basal forebrain was frozen with dry ice and subsequently sectioned at 10 micron. Each sagittal section was mounted on acid cleaned, gelatin-coated, microscopic slide and stored at −70°C until *in situ* hybridization.

DNA Oligonucleotide Probe Synthesis and Labeling

Oligomer dCCG/GTC/GTA/ACC/AGG/ATA/CCC/AAC/GCG/GGA (3'-5') was synthesized by the phosphate/phosphoester method on automated DNA synthesizer (Genetic Engineering Center, KAIST, Seoul, Korea). GnRH oligomer (29 mer) is complementary to the sequence of the rat GnRH mRNA coding for amino acids -1 to 9 of decapeptide (Adelman *et al.*, 1986). This oligomer was labeled according to the 5'- end labeling method (Davis *et al.*, 1986) in order to use a hybridization probe. Briefly, 50 pmole of 29 mer was incubated with 150 μCi of ^{35}S-dATP (S.A. 3000 Ci/mmole), 70 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM DTT, 20 unit T4 polynucleotide kinase at 37°C for 30 min. Radiolabeled oligomer was then separated from free forms by sephadex G-50 superfine chromatography (1 × 20 cm column) eluted triethanolamine bicarbonate buffer (pH 7.50). ^{35}S-labeled oligomer fractions were then pooled, lypohilized, resuspended in autoclaved distilled water and stored at −20°C prior to use. The final specific activity was 0.7 - 1.2 × 10^8 cpm/50pmole.

Treatments for *in situ* Hybridization

In situ hybridization was carried out essentially as described by Cox *et al.* (1984). All pretreatments of tissue were carried out under RNase free conditions. Tissue sections were dried at 37°C for 2 hrs, rehydrated through 70% and 50% alcohol and twice water washes, and incubated in 1 g/ml proteinase K in 0.1 M Tris-HCl, pH 8.0, 50 mM EDTA at 37°C for 30 min. Slides were next rinsed in distilled water and then rinsed in 2× SSC (1× SSC = 0.15 NaCl, 0.015 M sodium citrate). Samples were prehybridized for 2 hr at room temperature in 100 μl of a solution consisting of 2× SSC, 0.02% ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA, 1 mM/ml yeast tRNA. ^{35}S- end labeled GnRH probe were then diluted in hybridization buffer (the same components as prehybridization buffer), and applied to the tissue section in 50 μl. The sections were then incubated in humidified boxes at 45°C for 2 days. The slides were washed twice at room temperature for 10 min with 2× SSC, 1 mM sodium phosphate. The slides were further washed in 0.5× SSC at 45°C for 1 hr and again at room

temperature for 1 hr. The sections were then dehydrated in 70%, 90% and 100% ethanol, and air-dried. The sections were dipped in Kodak NTB2 emulsion (diluted to 1 : 1 with water). Slides were dipped twice in the emulsion, blotted on paper towels, and air dried for 30 min. Slides were stored in light-tight boxes at 4°C for 2 weeks. Slides were developed at 20°C for 6 min in D-19 developer diluted 1 : 1 with H_2O, rinsed in water, fixed for 10 min in Kodak rapid fixer, washed for 20 min in distilled water, and washed extensively in cold tap water.

Slides were then dehydrated through distilled H_2O, 70%, 95% and 100% alcohol, delipided through two changes of xylene, and rehydrated through the alcohol series and distilled H_2O. The tissue was then counterstained with cresyl violet for 10 min. The slides were rinsed briefly in distilled H_2O, destained for 1 min or less in acidified alcohol (2% acetic acid in 70% ethanol), dehydrated through 70%, 95% and 100% ethanol, then passed through two changes of xylene and coverslipped with permount.

Twenty tissue sections from each frog were searched systematically with darkfield optics for the presence of clusters of autoradiographic grains. The presence of such clusters indicated a high concentration of radiolabeled probe-GnRH mRNA hybridization. Such clusters were defined as GnRH cells, that is, cells containing mRNA for the coding of GnRH synthesis.

Both light and dark field microscopy (at 400×) were used to analyze quantitatively auto-radiographic tissue sections. A cluster of grains seen in dark field was considered to indicate the presence of a GnRH cell only if light field optics revealed the presence of an identifiable cell lying below the cluster of grains.

In each tissue section, we determine the number of cells containg clear GnRH message, as well as the anatomic localization of cells containing GnRH mRNA and the concentration of signal in each cell within each anatomic region.

Results

In situ hybridization histochemistry for GnRH mRNA demonstrated the presence of hybridizing cells in several regions of the forebrain and brain stem. The distribution pattern of GnRH mRNA containing cells in the brain of the frog is summarized in figures 1 and 2. A cluster of autoradiographic grains in the perikarya represents the GnRH mRNA. Pretreatment with RNase before hybridization resulted in complete loss of cluster of sliver grains (data not shown here). GnRH mRNA containing cells were observed consistently in the following anatomic regions: the medial septal nuclei (MSN), the preoptic area (PA), anterior preoptic area (APOA), and ventromedial hypothalamus-infundibular region (VHI) (Figs. 1, 2-I, 2-II and 2-III). Distribution of GnRH mRNA containing cells was similar to that of GnRH peptide containing cells by immunohistochemistry (Kim *et al.*, 1994). That is, cells containing GnRH-mRNA and GnRH peptides were observed in almost same areas.

However, particulary in the bed nucleus of hippocampal commissure (BH) (Figs. 1 and 2-III), preoptic area (PA) (Figs. 1 and 2-II), nucleus infundibularis dorsalis (NID) (Figs. 1 and 2-III) mecencephalic nucleus (MN) (Figs. 1 and 2-IV) and spinal cord area, GnRH mRNA containing cell groups were indentified in a larger number of cells

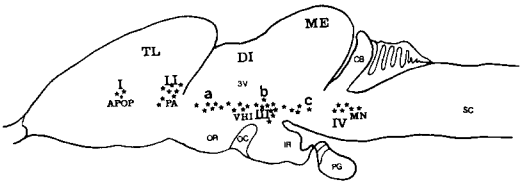

Fig. 1. Schematic illustration of the distribution of GnRH mRNA containing perikarya in sagittal section of the female frog brain. GnRH mRNA containing neurons were identified by *in situ* hybridization of GnRH mRNA with cDNA oligomer which is complementary to the rat hypothalamic GnRH mRNA sequence. The (*) represents GnRH mRNA containing perikarya. The density of this symbol means the density of hybridized GnRH mRNA.
abbreviations: APOA; anterior preoptic area, PA; preoptic area,ON; optic nerve, OC; optic chiasma, PG; pituitary gland, TL; telencephalon, DI; diencephalon, ME; mecencephalon, VHI;ventromedial hypothalamus infundibular CB;cerebellum, SC; spinal cord, 3V; third ventricle, IR; infundibulum, MN;mecencephalic necleus and ME; median eminence

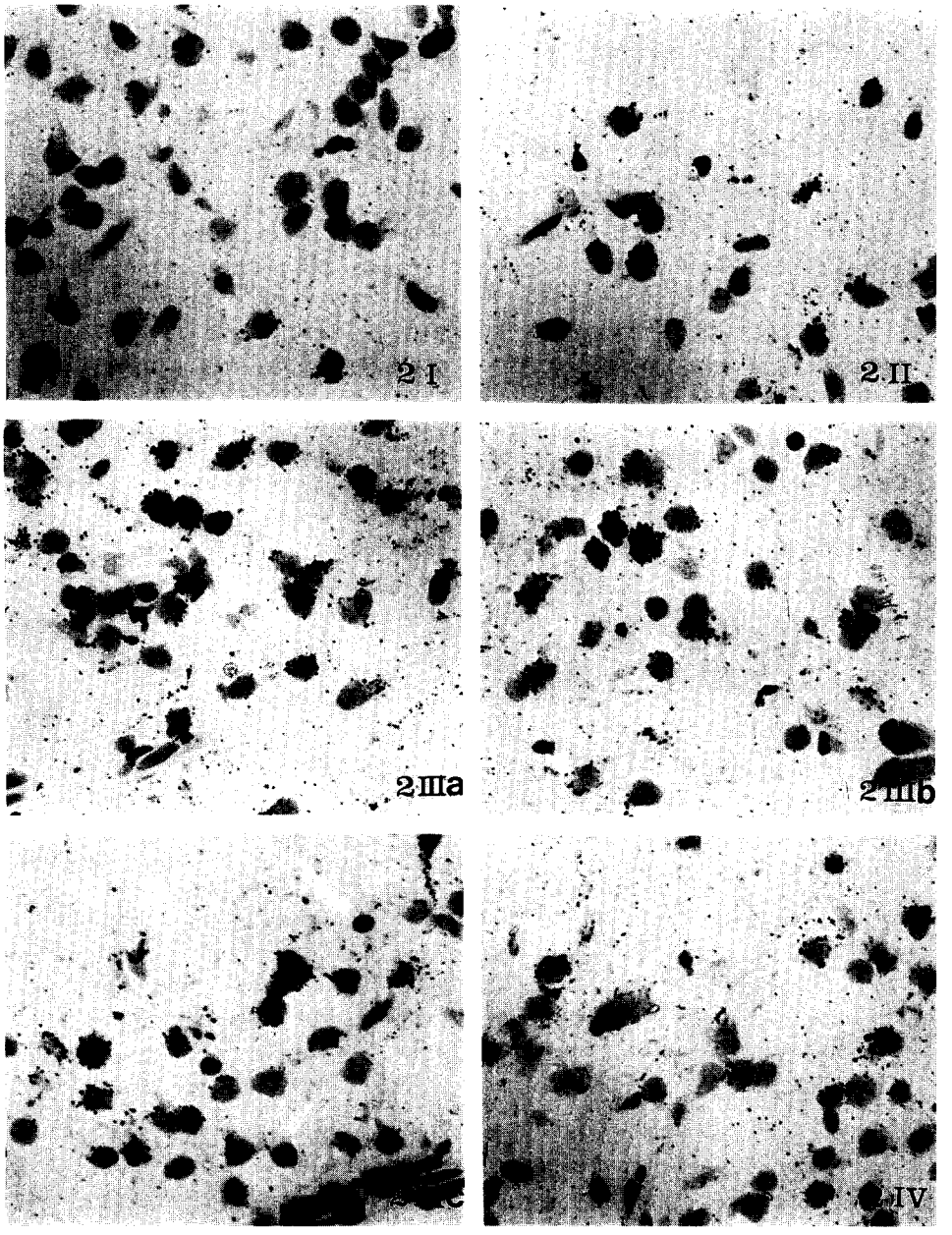

Fig. 2. Photomicrographs illustrating GnRH mRNA containing neurons at various brain areas of the female frogs, *Rana dybowskii*. I, II, IIIa, IIIb, IIIc and IV are the cross sections through corresponding points indicated on the diagram of the sagittal brain section in Fig. 1. GnRH mRNA was stained in black dots and cells were counterstained with cresyl violet. (400×)

which is not coincided with the GnRH distribution data by ICC. The number of GnRH mRNA containing cells was larger than that of GnRH peptide containing cells. Differences in number of GnRH mRNA-containing cells were evident across the regions with the higher number of such neurons in the PA, BH, APOA, SN, MN, NID and VHI regions. Averages across five independent

experiments with five female frog indicated that BH and PA regions, and lateral to 3rd ventricle contained greatest number of GnRH mRNA-containing neurons. Finally, GnRH neurons were also localized in the intermediolateral cell column of spinal cord.

Discussion

In situ hybridization histochemistry is a powerful technique for localizing specific mRNAs in neurons in discrete regions of the central nervous system. (Coughlan *et al.*, 1985). In this study, we used this method to identify the anatomical sites of GnRH gene expression in the frog brain which was demonstrated as silver grains. *In situ* hybridization using GnRH oligonucleotide probe demonstrated the presence of neurons in anterior preoptic area (APOA), medial septal nucleus (MSN), ventromedial hypothalamus (VH), infundibular region and tegmentum area.

Good agreement exists between the localization of GnRH mRNA containing cell by *in situ* hybridization immunohistochemistry in the present study and localization of GnRH peptide containing cells as determined by ICC studies and these results have verified the specificity of GnRH immunostaining in most regions reported previously (Rastogi *et al.*, 1990; Kim *et al.*, 1994).

However, another GnRH mRNA containing cell groups were identified by *in situ* hybridization which were not detected by ICC. That is, GnRH mRNA containing cell bodies were localized in the bed nucleus of hippocampal commissure, preoptic area, mesencephalic nucleus of medullary tegmentum and intermediolateral cell column of spinal cord in the frog brain. GnRH neurons in the bed nucleus of hippocampal commissure and tegumental area were also detecetd by ICC (Rastogi *et al.*, 1990) but cell mumber of GnRH neurons was very few. The reasons for this new existence of GnRH neurons in the frog are not clear. Since it appears more than three forms GnRH analogs exist in the frog brain and GnRH is synthesized from pro-GnRH and processing to active form to transport to the axon terminal, it could not be detected by ICC with GnRH antibody directed to a certain form of GnRH. The presence of a large collection of GnRH mRNA containing midbrain cells in the frog is surprising since many researchers failed to detect GnRH immunoractivity in this area (Nozaki and Kobayash; 1979; Nozaki *et al.*, 1984). However, mesencephalic GnRH immunoractive neurons were first identified in the dorsal tegmentum in teleost (Münz *et al.*, 1981; Kah *et al.*, 1986) and also detected in elasmobranchs (Wright and Demski, 1990). In amphibian, mesencephalic GnRH immunoreactive cells extending as a longitudinal band of immunoreactive cells beneath the ventricle was also identified by Branton *et al.* (1986). More detailed descriptions of the GnRH mRNA containing cell populations are needed to make valid comparison. However, based on antaomical location, cell morphology and number, midbrain GnRH systems reported here in frog appear to be similar to those observed in tetrapods and elasmobranches. This would suggest that mesencephalic GnRH neurons could be a relatively common feature in vertebrates.

Branton *et al.* (1986) also found another groups of GnRH neurons in the intermediolateral columns of the spinal cord. Our results clearly show the exsitence of GnRH neurons in the midbrain and the intermediolateral columns of the spinal cord. Whether or not midbrain GnRH systems in frogs are homologus to those in species in other vertebrate classes remains to be determined.

We also detected GnRH mRNA containing cells in the ventral thalamic area and brain stem. A few GnRH neurons was appeared in these area by ICC. However, *in situ* hybridization exhibited a large number of GnRH mRNA containing cell in these area. Recently, GnRH neurons were localized in the sympathetic ganglia of frog and functioned as a neurotransmitter (Crim and Vigna, 1983; Peter, 1983). GnRH neurons located in the brain stem of frog might have a similar function to the GnRH in the sympathetic ganglia.

Our *in situ* hybridization data showed the large number of GnRH mRNA containing cells than the GnRH peptide containing cells. As reported by Ronnekleiv *et al.* (1989), it is well concide with

the hypothesis that it is more difficult to detect GnRH by ICC because GnRH is posttranslationally changed to translocate directly to the axon terminals. The presence of GnRH mRNA in neurons not previously recognized to contain GnRH strengthens the possibility that GnRH peptides contained within the precusor molecule which could not detected by ICC or varient forms of GnRH are expressed in these cells. *In situ* hybridization used in this study demonstrates GnRH mRNA containing cells within several neuroanatomic regions previosly shown to include immunoreactive GnRH perikarya and establishes the feasibility of using *in situ* hybridization as a quantitative tool to study the development and the regulation of GnRH gene expression in the frog brain.

References

Adelman, J.P., A.J. Mason, J.S. Hayflick, and D.H. Seeburg. 1986. Isolation of the gene and hypothalamic cDNA for the common presursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. *Proc. Natl, Acad. Sci.* **83:** 179-213

Amoss, M., R. Burgus, R. Blackwell, V. Vale, S. Fellows, and R. Guillemin. 1971. Purification, amino-acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. *Biochem. Biophys. Res. Comm.* **44:** 205-210

Branton, W.D., H.S. Phillips, and Y.N. Jan. 1986. The LHRH family of peptide messengers in the frog nervous system. *Prog. Brain Res.* **68:** 205-215

Coughlan, J.P., P. Aldred, J. Haraiambidis, H.D. Niall, J.D. Penschow, and G.W. Tregear, 1985. Hybridization histochemistry. *Anal. Biochem.* **149:** 1-7

Crim, J.W., and S.R. Vigna. 1983. Brain, gut and skin peptide hormone in lower vertebrates. *Amer. Zool.* **23:** 621-638

Cox, K.H., D.V. Deleon, L.M. Angerer, and R.C. Angerer. 1984. Detection of mRNAs in sea urchin embryos by *in situ* hybridization. *Dev. Biol.* **101:** 485-502

Davis, L.G., M.D. Dibner, and J.F. Battey, 1986. "Methods in Molecular Biology" New York: Elsevier, pp139-142

Kah, O., B. Breton, J.G. Dulka, J. Nunez-Rodrigues, R.E. Peter, A. Corrigan, J.E. Rivier, and W. Vale, 1986. A reinvestigation of the GnRH (gonadotropin-releasing hormone) systems in the goldfish brain using antibodies to salmon GnRH. *Cell Tissue Res.* **244:** 327-337

Kalra, S.P. 1986. Neural circulatary involved in the control of LHRH secretion: a model for preovulatory LH release. In:Ganong, WF, Martini, L. (eds) Frontiers in Neuroendocrinology. Raven Press, New York, pp 31-75

Kim, J.-W., W.S. Choi, M.O. Kim, H.B. Kwon, C.H. Ra and W.-B. Im, 1994. Localization of gonadotropin releasing hormone (mGnRH, sGnRH and cGnRH II) in the brain of three species of frog, *R. nigromaculata, R. dybowskii and R. rugosa. Kor. J. Zool.* **37:** 161-173.

King, J.A., and R.P. Miller. 1979. Hypothalamic luteinizing hormone-releasing hormone content in relation to the seasonal reproductive cycle of *Xenopus laevis. Gen. Comp. Endocrinol.* **39:** 309-312

King, J.A., and R.P. Millar. 1987. Phylogenetic diversity of LHRH. In B.H. Vickery and J.J. Nestor (Eds.), LHRH and Its Analogus. Contraceptive and Therapeutic Applications, Book 2, MRP Press, Lancaster, pp 53-74

Matsuo, J., Y. Bada, R.M.G. Nair, A. Arimura, and A.V. Schally. 1971. Structure of the porcine LH and FSH-releasing hormone I. The proposed amino acid sequence. *Biochem. Biophys. Res. Commun.* **43:** 1334-1339

Münz, H., W.E. Stumpf, and L. Lennes. 1981. LHRH systems in the brain of platyfish. *Brain Res.* **221:** 1-13

Nozaki, M., and H. Kobayashi. 1979. Distribution of LHRH-like substance in the vertebrate brain as revealed by immunohistochemistry *Arch. Histol. Jpn.* **42:** 201-219

Nozaki, M., T. Tsukahara, and H. Kobayashi. 1984. An immunocytological study on the distribution of neuropeptides in the brain of certain species of fish. *Biomed. Res.* [Suppl.] **4:** 135-145

Peter, R.E. 1983. Evolution of neruohormonal regulation of reproduction in lower vertebrates. *Amer. Zool.* **23:** 685-695

Rastogi, R.K., M.D. Meglio, and L. Iela. 1990. Immunoreactive luteinizing hormone in the frog (*Rana esculenta*) brain: Distribution pattern in the adult, seasonal changes, castration effects, and developmental aspects. *Gen. Comp. Endocrinol.* **78:** 444-458

Ronnekleiv, O.K., B.R. Naulor, C.T. Bond, and J.P. Adelman. 1989. Combined immunohistochemistry for gonadotropin-releasing hormone (GnRH) and pro-

GnRH and *in situ* hybridiztion for GnRH messenger ribonucleic acid in rat brain. *Molecular Endocrinology,* **3:** 363-371

Seeburg, P.H., and J.P. Adelman. 1984. Characterization of cDNA for precursor of human luteinizing hormone-releasing hormone. *Nature* **311:** 666-6

Sherwood, N. 1987. The GnRH family of peptides. *Trends Neurosci.* **10:** 129-132

Sherwood, N., R.T. Zoeller, and F.L. Moore. 1986. Multiple forms of gonadotropin-relesaing hormone in amphibian brains. *Gen. Comp. Endocrinol.* **61:** 313-322

Shivers, B.P., R.E. Harlan, J.F. Hejtmancik, P.H. Conn, and D.W. Pfaff. 1986. Localization of cells containing LHRH-like mRNA in rat forebrain using *in situ* hybridization. *Endocrinology* **188:** 883-885

Standish, L.J., L.A. Adams, L. Vician, K.D. Clifton, and R.A. Steiner. 1987. Neuroanatomical localization of cells containing gonadotropin-hormone messenger ribonucleic acid in the primate brain by *in situ* hybridization histochemistry. *Molecular Endocrinology* **1:** 371-376

Wright, D.E., and L.S. Demski. 1990. Gonadotropin hormone-releasing hormone (GnRH) immunoreactivity in the mesencephalon of sharks and rays. *J. Comp. Neurology* **307:** 49-56

In situ hybridization법에 의한 북방산개구리 뇌에서 GnRH mRNA를 함유한 세포의 분포 연구.

최완성[1] · 김정우 · 백상호[2] · 김명옥[1] · 김경진[3] · 권혁방 · 임욱빈(전남대학교 자연과학대학 생물학과, [1]경상대학교 의과대학 해부학실, [2]서울대학교 의과대학 해부학실, [3]서울대학교 자연과학대학 분자생물학과)

In situ hybridization법으로 생식소 자극 호르몬 분비 호르몬(GnRH)의 mRNA를 함유한 신경세포체의 분포를 암컷 북방산개구리 뇌에서 조사하였다. GnRH mRNA에 상보적인 염기의 DNA oligomer를 합성하여 고정된 뇌의 시상단면 절편에 처리하였다. GnRH mRNA 함유 세포의 분포는 면역조직화학법으로 밝혀진 GnRH의 분포와 비슷하였다. 즉, GnRH mRNA 함유 세포는 medial septal area, anterior preoptic area, ventromedial hypothalamus 그리고 infundibular region에 분포하였다. 또한 the bed nucleus of hippocampal commissure, preoptic area, nucleus infundibularis dorsalis, mesencephalic nuclei, intermediolateral cell column of spinal cord area 등에서도 발견되었다.

이러한 결과는 척추동물에서 GnRH 유전자의 발현 조사와 GnRH 뉴론의 분포 연구에 *in situ* hybridization 법을 이용할 수 있음을 보여준다.