Korean J. Zool. 37:311-317, 1994

Reproductive Cycle-related Changes in GnRH Immunoreactivity in the Brains of Three Congeneric Species of Frog.

Wook-Bin Im, Jeong-Woo Kim, Wan Sung Choi*, Dong Gyu Bai, Mi-Seung Kim, and Hyuk Bang Kwon

*Department of Biology, College of Natural Sciences, Chonnam National University, Kwangju, 500-757, Korea; *Department of Anatomy, College of Medicine, Gyeongsang National University, Chinju, 660-280, Korea*

Seasonal variations of GnRH were investigated by immunohistochemical technique in three species of frog, *Rana nigromaculata*, *R. dybowskii* and *R. rugosa* with different ovulation period in order to examine the relationship between GnRH expression and reproductive function. In all three species of frog, the intensity of GnRH immunoreactivity and the number of GnRH neurons in the brain were relatively high in frogs at the pre-ovulation period and markedly increased at the ovulation period. Those were then decreased after ovulation and further lowered during early hibernation period.

These results indicate that GnRH experssion is closely related to specific phases of annual reproductive cycle in frog.

KEY WORDS: Gonadotropin-releasing hormone, GnRH, Reproductive cycle, Seasonal change, Immunohistochemistry, Frog.

Reproductive cycle is closely related to the seasonal climatic changes in amphibians. It has been reported that environmental factors such as temperature and light play an important role in the circannual endogenous testicular functions in the frog, *Rana esculanta* (Rastogi *et al.*, 1981). However, the sexual cycle in amphibians may be regulated basically through the hypothalamus-pituitary-gonad axis like in mammals. The seasonal changes in gonadotropins and sex steroids were studied in amphibians with to the involvement of these hormones in the regulation of sexual cycle (Licht *et al.*, 1983; Pierantoni *et al.*, 1984). It appeared that seasonal changes in the concentrations of gonadotropins and sex steroids are closely related to the reproductive condition. It also appeared that amphibian reproduction is controlled at least in part by the hypothalamus (Dierickx, 1964, 1966). Gonadotropin-releasing hormone GnRH may mediate the environmental signals to the brain endocrine system.

GnRH was identified in the brain of amphibians and immunohistochemical studies demonstrate that GnRH neurons were localized in nucleus medialis septi NMS, nucleus diagonal band of Broca NDB and median eminace (Nozaki and Kobayashi, 1979; Kubo *et al.*, 1979; Jokura and Urano, 1985), indicating GnRH is invoved in the control of gonatropin secretion from pituitary. Admnistration of GnRH to amphibians stimulated secretion of gonadotropins, (McCreery *et al.*, 1982), and stimulated sexual behavior (Kelley, 1982; Moore *et al.*, 1982)

This work was supported by Genetic Engineering Research Grant (1992) from the Ministry of Education.

Since gonadotropins and sex steroids change seasonally and GnRH regulates the secretion of gonadotropins, the seasonal changes in reproduction are probably controlled by seasnoal changes in GnRH. We investigated the changes in GnRH immunoreactivity in brain areas of frogs collected throughout the reproductive cycle and compared among different congeneric species of frogs with different breeding seasons.

GnRH immunoreactive neurons were changed according to the reproductive condition, not to the climatic season in all three species. These results indicate that GnRH is the primary regulator in the control of reproductive cycle in frogs.

Materials and Methods

Frogs of *R. dybowskii*, *R. rugosa* and *R. nigromaculata* were collected throughout the reproductive cycle in Kwangju province in South Korea. Female frogs of *R. dybowskii* (40-50g), *R. rugosa* (18-23g) and *R. nigromaculata* (40-45g) were used. Breeding seasons are as follows: May-July in *R. rugosa*, February-March in *R. dybowskii* and May-June in *R. nigromaculata*.

Immunohistochemistry

Frogs were perfused with 50ml of 4% neutral buffered paraformaldehyde fixative solution. Brains were carefully removed and post-fixed with same fixative. Tissues were rinsed twice in 0.1 M phosphate buffer for 4 hr and transferred to the same buffer containing 30% sucrose and 0.1% sodium azide for storage. Serial cross-, sagittal- and horizontal sections of the entire brains were cut on a cryostat (AO, Cryo-cut II) at 10 μm and thaw-mounted onto 1% gelatine-coated slides.

Serial sections of approximately every 10th or 15th section were mounted on the same slide to reach a maximum of 6 sections per slide.

The immunohistochemical staining procedure was basically same to that of Hsu *et al.* (1981) and the antibody-antigen complex was visualized using avidin-biotin-peroxidase technique and described in details (Kim *et al.*, 1994). Briefly, sections were rinsed with phosphate buffered saline (PBS, pH 7.4). Sections were then sequentially treated with dilute normal goat serum, primary GnRH antisera (diluted 1:500 - 1:2,000), biotinylated secondary goat anti-rabbit IgG (1:200 - 1:500) and a preformed avidin-biotinylated horseradish peroxidase complex (ABC Kit, Vectastain; Vector Labs, CA). Sections were washed twice for 10 min with PBS between steps. All antisera solutions were diluted as indicated in PBS, pH 7.4.

After washing the slides twice sequentially with PBS and 0.05 M Tris buffer (pH 7.6), slides were dipped for 10 min in PBS solution containing 0.05% diaminobenzidine (DAB, Sigma), 0.04% NiCl, 0.003% hydrogen peroxide. Immunoreactive GnRH perikarya and fibers turned to black color.

For immunohistochemical staining two different primary antisera including rabbit derived anti-sGnRH (A6 1668, a gift of J. King, South Africa), and anti-mGnRH antibody (Incstar, Lot 8932025, Puchased from Immunonuclear Corp.) were used depending on the frog species used. It has been shown that these antibodies does not cross-react with heterogeneous antigens (King and Millar, 1987). (for further details of preparation and immunological charateristics see King and Millar, 1987).

The controls for a specific immunoreactive staining were: (a) omission of the primary antisera or secondary antisera, (b) substitution of primary antisera with non-immune rabbit serum, (c) substitution of anti-rabbit secondary antisera with anti-mouse antisera. These control tests resulted in the complete loss of immunoreactive staining.

Results

Three congeneric species of frogs, with different seasonal ovulation periods, *R. dybowskii*, *R. nigromaculata* and *R. rugosa* were used in order to elucidate the relationship between GnRH expression and reproductive functions.

Since sGnRH was dominant in *R. dybowskii* and mGnRH was predominant in *R. rugosa* and *R. nigromaculata*, and there were no brain area-specific variation in the molecular forms of GnRH (Kim *et al.*, 1994), different antisera against species-specific dominant variants of GnRH were

used. Specifically, antisera raised to mGnRH were used in *R. rugosa*, *R. nigromaculata* and antisera against sGnRH were used in *R. dybowskii*.

There were no seasonal changes in the distribution of GnRH immunoreactive (GnRH-ir) perikarya and fibers in consistent with the previous results (Kim *et al.*, 1994).

Fig 1. is photomicrographs of sagittal sections through anterior preopticarea (APOA) of the brain in frogs, *R. nigromaculata* whose breeding season is from the end of April to the end of May. GnRH were visualized by immunohistochemical techniques using anti-mGnRH antisera.

As shown in Fig. 1, the intensity of immunoreactivity in NMS and NDB was relatively high in frogs, *R. nigromaculata* collected in March, the hibernation and pre-ovulation period and was much stronger in frogs collected at the beginning of May, the ovulation period, throughout the circannual reproductive cycle. The abundunce of GnRH-ir perikarya and fibers are also increased in line with the increase in GnRH immunostaining intensity.

The GnRH neurons clearly decreased in frogs collected in June, just after ovulation. The intensity and abundance further decreased in July and October. Since August is fast oocytes growing period (Kwon *et al.*, 1991), a relatively lower appearance of GnRH neurons might be related to the growth of oocytes.

This result suggests that GnRH expression may be involved in the regulation of reproductive cycle. In order to clarify the possibility, we studied the changes in GnRH immunoreactivity in other species of frogs with different breeding seasons.

Fig 2. is mGnRH-immunohistochemical photomirographs of sagittal sections through APOA and optic area of the brain in frogs, *R. rugosa*, whose breeding season starts from the end of May and lasts to the end of June.

As shown in Fig. 2, the intensity and abundance of immunoractivity in NMS and NDB were changed throughout the year as in *R. nigromaculata*. However, it should be noted the intensity and abundance of GnRH was much higher in frogs, *R. rugosa* collected at the end of May, the ovulaltion period.

The number of GnRH-ir neurons in NMS and NDB was relatively high in *R. rugosa* collected in April, the hibernation and pre-ovulation period and was much stronger in frogs collected in May, during the ovulation period throughout the year.

The immunoreactive GnRH neurons clearly decreased in frogs collected in August, just after ovulation. The intensity and abundance slightly decreased in August and lasted until the end of September, though the intensity was lower than those in pre-ovulation and ovulation period. Since August and September is vitellogenesis period, the decrease in GnRH intensisty might be related to the growth of oocytes. Thereafter, the number decreased from the period, before entering hibernation to deep hibernation period, January.

In *R. dybowskii*, the GnRH expression was only compared between ovulation period and hibernation period, because of the difficulty in collecting the frogs. The breeding season of *R. dybowskii*, was from early February to March, which had the earliest ovulation period among three species of frog. As shown in Fig. 3, the GnRH immunoreactivity and GnRH neurons were markedly increased in frog brain collected in February, the breeding season, in consistant with those results in two other species of frogs with different breeding seasons.

These results indicate that GnRH is closely implicated in the regulaltion of annual reproductive cycle in frogs.

Discussion

The present result clearly revealed that GnRH expression is implicated in the regulation of reproductive functions in frogs, with seasonal breeding period.

In this study, we examined and compared the annual changes in GnRH immunoreactivity and the number of GnRH neurons in the brain of three congeneric species of frogs, *R. nigromaculata*, *R. dybowskii* and *R. rugosa*. Consistently, in all three species, the intensities of GnRH immunoreactivity and the number of GnRH neurons conincided with the reproductive cycle. The GnRH concentrations and the number of GnRH perikarya or fibers were highest during ovulation period in all three species.

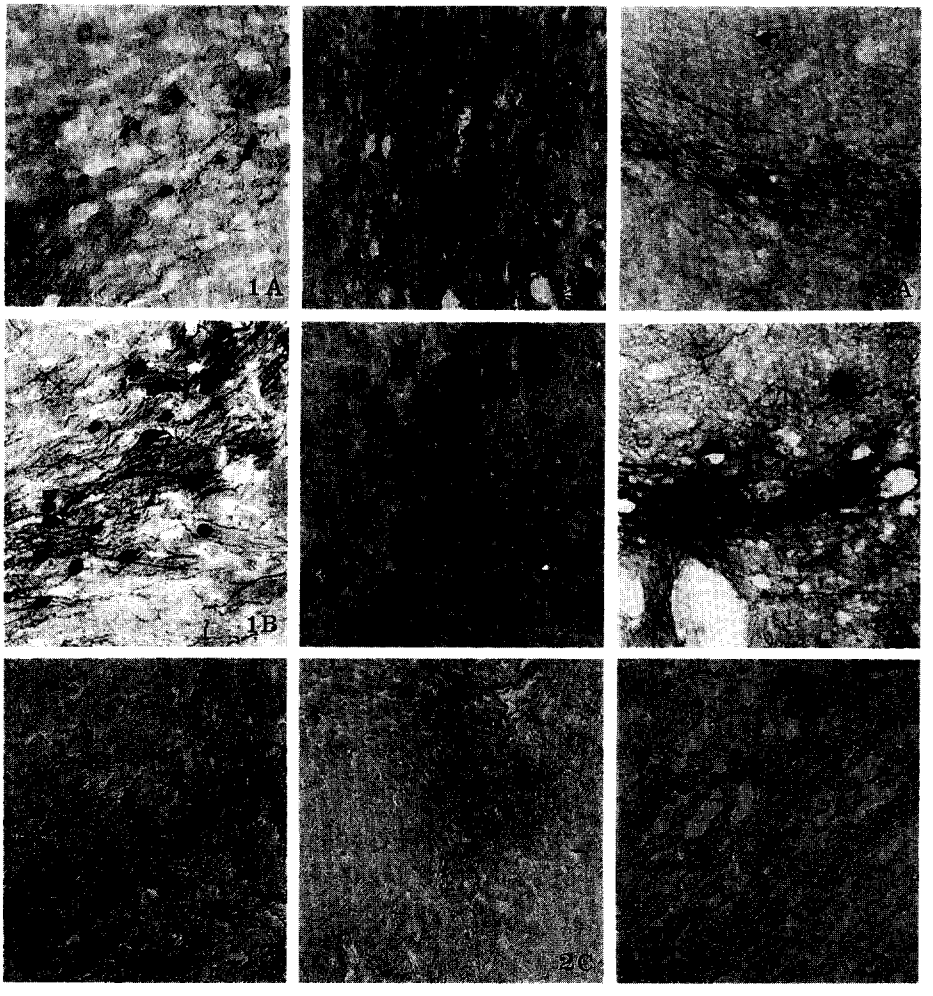

Fig. 1. Seasonal changes in GnRH immnoreactivity in the brain of frog, *R. nigromaculata*. Photomicrographs of mGnRH neurons in NMS-NDB region at pre-ovulatory (A), ovulatorly (B) and post-ovulatory period (C). GnRH were identified by immunohistochemical staining using anti-sGnRH antisera. GnRH immunoreactive cells were located dorsal and ventral area near the midline of the mesencephalon. The dense immunostaining of GnRH perikarya and fibers in (B) was charateristics in the brain sections of all frogs at the breeding season. In contrast, few or sometimes no immunoreactive neurons were detected in corresponding brain areas at the pre- or post-ovulatory periods. (250×)

Fig. 2. Seasonal differences in GnRH concentrations in the NMS-NDB region of the brain of frog, *R. rugosa* collected at pre-ovulatory (A): (250×), ovulatory (B): (250×) and post-ovulatory period (C): (125×). GnRH were identified by immunohistochemical staining using anti-mGnRH antisera.

Fig. 3. Reproductive cycle-related changes in GnRH concentrations in the brain of frog, *R. dybowskii*. Photomicrographs of sGnRH neurons in NMS-NDB region at pre-ovulatory (A), ovulatory (B) and post-ovulatory period (C). GnRH were visualized by immunohistochemical staining using anti-sGnRH antisera. (250×)

In addition, those were relatively higher during the vitellogenesis periods. More convincing evidence came from the fact that these species are different one another in their ovulation period, as follows; February-March in *R. dybowskii*, May-June in *R. nigromaculata* and May-July in *R. rugosa*.

GnRH-ir neurons were changed according to the reproductive condition, not to the climatic season in all three species. These results strongly support that GnRH regulates the reproductive

cycle in frogs.

Although it has long been recognized that there is close reltionship between reproductive cycles and seasonal climatic changes in amphibians as well as in most of vertebrates, it is postulated that the reproductive functions in amphibians is also regulated basically by gonadotropic hormones and sex steroids as in mammals. Thus, the seasonal changes in gonadotropins and sex steroids were studied in amphibians focusing on the relation of these hormones to the regulation of sexual cycle (Licht *et al.*, 1983; Pierantoni *et al.*, 1984; Kwon *et al.*, 1991). It appeared that seasonal changes in the concentrations of gonadotropins and sex steroids are closely related to the reproductive condition.

Although reproductive function is under the control of endogeneous gonadotropins and sex hormones in amphibians, it was also reported that the annual endogenous testicular functions were influenced severely by several environmental factors such as temperature and light in the frog, *Rana esculanta* (lela *et al.*, 1980; Rastogi *et al.*, 1981). In addition, it also reported that amphibian reproduction is controlled al least in part by the hypothalmus (Dierickx, 1964, 1966).

Hypothalamo-hypophyseal portal vessels and neurohumoral molecules which stimulates the anterior pituitary to secrete gonadotropins make it possible to explain the means how environmental stimuli, such as temperature and light regulate the reproductive functions. Hypothalamus and gonadotropin-releasing hormone may mediate the environmental signals to the brain endocrine system.

GnRH, a neurohumoral peptide which stimulate gonadotropin secretion from pituitary, was identified and determined its 10 amino acids sequence in the brain of amphibians. Subsequent immunohistochemical studies demonstrate that GnRH neurons were localized in NMS, NDB and median eminence (Nozaki and Kobayashi, 1979; Kubo *et al.*, 1979; Crim, 1985), indicating GnRH is invoved in the control of gonadotropin secretion from pituitary. Application of purified GnRH or synthetic GnRH to amphibians induced secretion of gonadotropins, and gonadal steroid hormones (Daniels and Licht, 1980; Moore *et al.*, 1982; McCreery *et al.*, 1982), induced vitellogenesis and ovulation (Thornton and Geschwind, 1974), and stimulated sexual behavior (Kelley, 1982; Moore *et al.*, 1982), indicating these functions are GnRH dependent.

It is postulated that GnRH concentration in the brain may change seasonally associated with the reproductive cycle, since frogs ovulate during a definite breeding period. The result in frog, *R. esculenta*, that the intensity of immunoreactive LHRH in the median eminence and medial septal and APOA was higher in animals collected in May, the breeding season than those from other monthes (Rastogi *et al.*, 1990) is consistent with the present data that brain GnRH amount change concomitantly with the reproductive cycle in three species of frogs, *R. nigromaculata, R. dybowskii* and *R. rugosa*.

Seasonal changes in GnRH secretion may also be a primary regulator of the neuroendocrine control of seasonal reproduction in other species. The seasonal changes in the brain LHRH were described in several species, including frogs, newts and mares, focusing on the relation to the reproductive stage. Seasonal change in brain LHRH concentration showed two peaks, one was in February and the other in June in male rough-skinned newts (Zoeller and Moore, 1985). Although the species is different, these results in newts are comparable to ours in frogs that GnRH intensities and the number of GnRH neurons were highest during ovulation periods and were relatively higher during vitellogenesis period. In mares, LHRH concentration in the brains also changed seasonally and correlated with reproductive stages in mares (Hart *et al.*, 1984).

The present results and other reports revealed that GnRH is implicated in the control of the seasonal reproductive cycle in amphibians,mammals and probably in other seasonally breeding vertebrates.

Acknowledgments

The authors thank Dr. Judy A. King (South Africa) for her generous gift of anti-sGnRH.

References

Crim. J.W., 1985. Immunocytochemistry of luteinizing hormone-releasing hormone and sexual maturation of the frog brain: Comparisons of juvenile land adult bullfrog (*Rana catesbeiana*) *Gen. Comp. Endocrinol.* **59:** 424-433.

Daniels, E. and P. Licht, 1980. Effects of gonadotropin-releasing hormone on the levels of plasma gonadotropins (FSH and LH) in the bullfrog, *Rana catesbeiana. Gen. Comp. Endocrinol.* **42:** 455-463.

Dierickx, K., 1964. The structure and activity of the hypophysis of *Rana temporaria* in normal and in experimental conditions. *Z. Zellfoforsch.* **61:** 920-939.

Dierickx, K., 1966. Experimental identification of a hypothalamic gonadotrophic centre. *Z. Zellforsch.* **74:** 53-79.

Hart, P.J., E.L. Squires, K.J. Imel and E.L. Besch, 1984. Seasonal variation in hypothalamic content of gonadotropin-releasing hormone (GnRH), pituitary recepotors for GnRH and pituitary content of luteinizing hormone and follicle-stimulating hormone in the mare. *Biol. Reprod.* **30:** 1055-1062.

Hsu, S.M., L. Raine and H. Fanger, 1981. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique: A comparison between ABC abd unlabeled antibody (PAP procedures). *J. Histochem. Cytochem.* **29:** 577-580

Iela, L., R. Pierantoni and R.K. Rastogi, 1980. Effect of temperature and light on the production of androgens in the male *Rana esculenta, Experientia* **36:** 256-257.

Jokura, Y. and A. Urano, 1985. An immunohistochemical study of seasonal changes on luteinizing hormone-releasing hormone and vasotocin in the forebrain and the neurohypophysis of the toad, *Bufo japonicus. Gen. Comp. Endocrinol.* **59:** 238-245.

Kelly, D.B., 1982. Female sexual behiviors in the South African clawed toad, *Xenopus laevis*: Gonadotropin-releasing, gonadotropic, and steroid holrmones. *Horm. Behav.* **16:** 158-174.

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. Millar, 1987. Phylogenetic diversity LHRH. In: Vickery and J.J. Nestor (eds), LHRH and Its analogs. Contraceptive and Therapeutic Application. Books. 2. MTP Press Limited, Lancaster, pp.53-74.

Kubo, S., K. Watanabe, Y. Ibata and Y. Sano, 1979. LH-RH neuron system of the newt by immunohistochemical study. *Arch. Histo.* Japan **42:** 235-242.

Kwon, H.B., H.H. Choi, R.S. Ahn and Y.D. Yoon, 1991. Steroid production by amphibian (*R. nigromaculata*) ovarian follicles at different developmental stages. *J. Exp. Zool.* **260:** 66-73.

Licht, P., B.R. McCreey, R. Barnes and R. Pang, 1983. Seasonal and stress related changes in plasma gonadotropins, sex steroids and corticosterone in the bullfrog, *Rana catesbeiana. Gen. Comp. Endocrinol.* **50:** 124-145.

McCreery, B.R., P. Licht, R. Barnes, J.E. Rivier and W.W. Vale, 1982. Actions of agonistic and antagonistic analogs of gonadotropin releasing hormone (Gn-RH) in the bullfrog *Rana catesbeiana. Gen. Comp. Endocrinol.* **46:** 511-520.

Moore, F.L., L.J. Millar, S.P. Spielvogel, T. Kubiak and K. Folkers, 1982. Luteinizing hormone-releasing hormone involvement in the reproductive behavior of a male amphibian. *Neuroendocrinologly* **35:** 212-216.

Nozaki, M. and H. Kobayashi, 1979. Distribution of LHRH-like substance in the vertebrate brain as revealed by immunohistochemistry. *Arch. Histol.* Japan **42:** 201-209.

Pierantoni, R., L. Iela, M. d'Istria, S. Fasano, R.K. Rastogi and g. Chieffi, 1984. Seasonal testosterone profile and testicular responsiveness to pituitary factors and gonadotropin releasing hormone during two different phases of the sexual cycle of the frog (*R. esculenta*). *J. Endocrinol.* **102:** 387-392.

Rastogi, R.K., L. Tammaro, M. Di Meglio, L. Iela, L. Di Matteo and G. Chieffi, 1981. Circannual testicular rhythm in the green frog, *Rana esculenta. Biol. Zool.* **48:** 97-105.

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

Thornton, V.F. and J.J. Geschwind, 1974. Hypothalamic control of gonadotropin release in amphibia: Evidence from studies of gonadotropin release in vitro and in vivo. *Gen. Comp. Endocrinol.* **23:** 294-301.

Zoeller, R.T. and F.L. Moore, 1985. Seasonal changes in

luteinizing hormone-releasing concentrations in microdissected brain regions of male rough-skinned newts (*Taricha granulosa*). *Gen. Comp. Endocrinol.* **58:** 222-230.

(Accepted February 25, 1994)

3종의 개구리 뇌에서 생식주기에 따른 GnRH 면역반응성의 변화

임욱빈 · 김정우 · 최완성* · 배동규 · 김미승 · 권혁방(전남대학교 자연과학대학 생물학과, *경상대학교 의과대학 해부학실)

산란기가 다른 3종의 개구리, 참개구리, 북방산개구리, 옴개구리 뇌에서 생식소 자극 호르몬 분비 호르몬(GnRH)의 계절적 변화를 면역조직화학법으로 조사하여 GnRH 유전자 발현과 생식 기능과의 상호 관계를 연구하였다. 3종의 개구리에서 공통적으로 GnRH 면역 반응성과 GnRH 신경세포의 수가 계절과 관계없이 산란전 시기에 비교적 많았으며 산란기에 뚜렷하게 증가하였다. 산란기 이후 감소하기 시작하여 동면초기 동안 낮은 상태가 계속되었다.

이러한 결과는 개구리 뇌에서 GnRH 발현은 생식 주기와 깊이 연관되어 있음을 뜻한다.