Gonadotropin-releasing hormones in the African catfish: molecular forms, localization, potency and receptors

H.J.Th. Goos[1], P.T. Bosma, J. Bogerd, C.P. Tensen[2], K.W. Li[3], M.A. Zandbergen and R.W. Schulz
[1]Research Group for Comparative Endocrinology, University of Utrecht, P.O. Box 80.058, 3508 TB Utrecht, The Netherlands; [2]present address: Leiden/Amsterdam Institute for Immunology, Department of Dermatology, Free University Hospital, De Boelelaan 1117, 1087 HV Amsterdam, The Netherlands; [3]present address: Graduate School Neurosciences Amsterdam, Research Institute Neurosciences, Vrije Universiteit, Faculty of Biology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

Abstract

Two gonadotropin releasing hormones (GnRHs) were identified in the African catfish: chicken GnRH-II (cGnRH-II) and catfish GnRH (cfGnRH). Immunological screening of HPLC fractions from pituitary extracts indicated a third GnRH which co-eluted with lamprey GnRH-III. However, mass determination and amino acid sequencing identified this material as isotocin. This underlines the risk of "identifying" multiple forms of GnRH in tissue extracts on the basis of immunoreactivity in HPLC fractions. *In vivo* and *in vitro* studies demonstrated that cGnRH-II is an over 100-fold more potent gonadotropin (GTH) secretagogue than cfGnRH. This correlates with the respective receptor affinities. The presence of both GnRHs in the pituitary gland suggests that they may modulate each other's GTH release activity. Sub-threshold or low doses of cGnRH-II partly inhibited cfGnRH-induced GTH II secretion. Conversely, combinations of sub-threshold or low doses of cfGnRH with effective doses of cGnRH-II led to increases in GTH II levels similar to those induced by cGnRH-II alone. Combinations of submaximally effective dose of the 2 peptides resulted in additive effects. Hence, both GnRHs participate in the regulation of GTH II release, and their relative concentrations may determine the overall effect. Immunocytochemistry, using anti-bodies against the respective recombinant GnRH associated peptides (GAPs), as well as *in situ* hybridization showed that cfGnRH neurones are scattered in the ventral forebrain and project into the pituitary gland, while cGnRH-II neurones are confined to the midbrain tegmentum and without projections to the pituitary gland. Transfection experiments with GnRH receptor cDNA shows ligand activation characteristics similar to those of the native GnRH-R. Autoradiographic studies and hormone release studies indicate that GnRH-Rs in the African catfish pituitary gland are restricted to the gonadotrophs.

Introduction

Nine members of the gonadotropin-releasing hormone peptide family have been characterized so far (Powell et al. 1994). All GnRHs are decapeptides, showing conserved amino acids in positions 1, 2, 4, 9 and 10. At position 8, the highest variability is observed with 7 different amino acids, while 2 or 3 different amino acids were found on positions 3 and 5–7. Highly evolved placental mammals seem to express a single form of GnRH which can be present in 2 molecular variants (Gautron et al. 1992), while 2 forms of GnRH were found in metatherian species (King et al. 1990a). The presence of at least 2 different forms of GnRH is a common feature for all sub-mammalian vertebrates, including teleost fish (Powell et al. 1994; Sherwood et al. 1993; Muske 1993).

Teleost fishes do not have a functional hypothalamo-hypophyseal portal system. Instead, GnRH nerve fibers terminate in the vicinity of the

Correspondence to: H.J.Th. Goos, at the above address.

pituitary gonadotrophs (Muske 1993; Peter et al. 1990; Kah et al. 1993) and, as a consequence, the GnRHs are present in brain, as well as in pituitary extracts (Yu et al. 1988; King et al. 1990b). However, in a number of teleosts, one of the forms of GnRH found in the brain is not detectable in the pituitary gland (Powell et al. 1994; Somosa et al. 1994; Okuzawa et al. 1990; Amano et al. 1992). In these fishes, chicken GnRH-II (cGnRH-II), which is present in all teleosts studied so far (Powell et al. 1994; Sherwood et al. 1993), is restricted to the brain. The differential distribution of the GnRHs suggests different functions for the distinct peptides. GnRH forms not localized in the pituitary gland are most likely not directly involved in the release of gonadotropin (GTH) and might function as neurotransmitter and/or neuromodulator elsewhere in the brain (Sherwood et al. 1993; Muske 1993; Kah et al. 1993; Peter et al. 1990).

In brain (Bogerd et al. 1992) and pituitary (Schulz et al. 1993) extracts of the African catfish (*Clarias garipinus*) two forms of GnRH have been identified: chicken GnRH-II ([His^5,Trp^7,Tyr^8]-GnRH, cGnRH-II) and catfish GnRH ([His^{5}m, Asn^8] GnRH, cfGnRH. The latter has been found to date in the African and the Thai catfish (*Clarias macrocephalus*) only (Bogerd et al. 1992; Ngamvongchon et al. 1992). Surprisingly, pituitary extracts appeared to contain a third GnRH immunoreactive compound (Schulz et al. 1993).

A third GnRH-immunoreactive substance

To identify this compound, pituitary glands from adult male and female African catfish were collected in liquid nitrogen, powdered and extracted as described previously (Sherwood et al. 1989). The final aqueous phase was passed twice through a Supelclean LC-18 cartridge (Supelco, Leusden, The Netherlands), which was washed with 7.5 mM TFA and eluted with 6 ml 80% acetonitrile in 7 mM TFA. The eluate was concentrated by lyophilization and was applied to reverse phase HPLC. Synthetic GnRHs (mammalian GnRH (mGnRH), cfGnRH, chicken GnRH-I ([Gln^8]GnRH, cGnRH-I), cGnRH-II, lamprey GnRH-I ([Tyr^3,Leu^5,Glu^6, Trp^7,Lys^8]GnRH, lGnRH-I), lamprey GnRH-III ([His^5,Asp^6,Trp^7,Lys^8]GnRH, lGnRH-III), and salmon GnRH ([Trp^7,Leu^8] GnRH, sGnRH)) and

synthetic isotocin were used as standards. Immunoscreening of the HPLC fractions was performed using a cGnRH-II antiserum as described previously (Schulz et al. 1993). Three peaks of GnRH-immune reactive (ir) material were found, with retention times similar to synthetic cfGnRH, lGnRH-III and cGnRH-II, respectively. Fractions containing the unidentified GnRH-ir material (the ones that co-eluted with lGnRH-III) were reapplied to HPLC for further purification. Amino acid sequencing, by automated repetitive Edman degradation yielded the sequence: [Cys]-Tyr-Ile-Ser-Asn-[Cys]-Pro-Ile-Gly. This was supported by mass determination of the peptide (Li et al. 1994), showing a protonated mass of 967.3 Da, as compared to the calculated protonated mass of 967.15 Da. Furthermore, the isolated GnRH-ir material was present in the fractions that coeluted with synthetic isotocin. We conclude, therefore, that the peptide is isotocin, with the sequence: Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-Gly-amide. Thus, in catfish pituitary extracts 3 peaks of GnRH-ir material were detected, with HPLC retention times similar to cfGnRH, isotocin, lGnRH-III and cGnRH-II; only minor differences were found between the sexes.

In brain extracts from males as well as females, however, 4 peaks of GnRH-ir material were detected. HPLC retention times corresponded to cfGnRH, isotocin, lGnRH-III, cGnRH-I and cGnRH-II. With another GnRH-antiserum, an additional fifth peak was observed in brain extracts from both sexes, which did not coelute with any of the standards used. The ir material with cGnRH-I corresponding retention time and the unknown material have not been analyzed as yet.

The combination of HPLC and GnRH-RIA is commonly used to trace new forms of GnRH. With a GnRH antiserum, immunoreactive HPLC fractions are detected, and after subsequent purification the amino acid sequence of the new form is determined. Using these techniques the primary structures of 8 of the 9 GnRH forms were established (King et al. 1982; Sherwood et al. 1983, 1986; Miyamoto et al. 1984; Lovejoy et al. 1992; Ngamvongchon et al. 1992; Sower et al. 1993; Bogerd et al. 1994; Powell et al. 1994). In several cases, already known forms of GnRH have been purified and sequenced to prove their presence in a certain species (Lovejoy et al. 1991a,b, 1992; Lescheid et al. 1995; Powell et al. 1995).

In many species, purification and amino acid sequencing were not performed following HPLC fractionation and screening for GnRH-ir material. Our experiences with 3 different GnRH antisera (30-3, 34-3, and GF-4, Schulz et al. 1993) recognizing isotocin, which co-elutes from HPLC with lGnRH-III, exemplify the possibility of a false identification of a substance as GnRH when relying solely on the elution time of GnRH-ir compounds. Similarly, in lamprey (*Petromyzon marinus*), isolation and characterization of a GnRH-ir compound resulted in the identification of somatostatin (Sower et al. 1993, 1994). Regarding the two peaks of GnRH-ir in catfish brain extracts, we are therefore not optimistic to find additional GnRH forms.

Distribution and localization of catfish GnRHs

To investigate the distribution of cfGnRH and cGnRH-II over the brain and pituitary, 20 male and female catfish (6 months old) were decapitated. Brain and pituitary tissue was snap-frozen on dry ice separately and stored at –80 °C. Extractions were performed as described above. The concentrated extracts were applied to HPLC and eluted with an acetonitrile gradient of 10–80% in 7 mM TFA. Fractions (1 ml/min) were collected and tested for GnRH, using two different GnRH antisera: sGnRH antiserum 30-3 and cGnRH-II antiserum 34-3.

Antiserum 30-3 which cross-reacts with several GnRH forms binds 20% of 15,000 cmp of [^{125}I]cGnRH-II when diluted 1:11,000. The cross-reactivity of antiserum 30-3 with cfGnRH was measured by comparing the displacement of [^{125}I]cGnRH-II in incubations with increasing amounts of cfGnRH (1 pg to 10 ng) and cGnRH-II (5 pg to 5 ng). This resulted in 50% displacement of [^{125}I]cGnRH-II with 74 and 360 pg of radioinert cGnRH-II and cfGnRH, respectively. As we do not have a homologous cfGnRH RIA, amounts of immunoreactive cfGnRH (ir cfGnRH) in male and female brain and pituitary glands were estimated in a heterologous RIA using antiserum 30-3 and [^{125}I]cGnRH-II with a standard curve of cfGnRH (10 pg to 10 ng). The displacement curves for serially diluted cfGnRH-ir HPLC fractions and the cfGnRH standard curve were parallel.

Chicken GnRH-II was mainly located in the brain, and very little was present in the pituitary gland, whereas cfGnRH was mainly found in the pituitary gland, and a relatively small amount was present in the brain. In male fish, the brain contained 625 pg of ir-cfGnRH and 3225 pg of cGnRH-II, whereas the pituitary gland contained 12690 pg of ir-cfGnRH and 18 pg of cGnRH-II. Thus, cGnRH-II is present in a 5-fold excess over ir-cfGnRH in the male brain, whereas ir-cfGnRH is present in a 700-fold excess over cGnRH-II in the pituitary gland of males.

In female fish, the brain contains 525 pg of ir-cfGnRH and 3600 pg of cGnRH-II, while the pituitary gland contains 6,380 pg of ir-GnRH and 9 pg of cGnRH-II. Thus, cGnRH-II is present in a 7-fold excess over ir-cfGnRH in the female brain, whereas ir-cfGnRH is present in a 750-fold excess over cGnRH-II in the pituitary gland of females. Thus, in the African catfish, more than 90% of the total brain plus pituitary cfGnRH was present in the pituitary gland, which suggests that cfGnRH is mainly stored in the nerve endings close to the pituitary gonadotrophs.

In goldfish (*Carassius auratus*), sGnRH and cGnRH-II were present in brain and pituitary extracts; in juvenile fish, the pituitary contained equal amounts of both GnRHs, whereas in the adults, the pituitary sGnRH amounts were approximately 8-fold higher than those of cGnRH-II (Rosenblum et al. 1994). In European eel (*Anguilla anguilla*), mGnRH and cGnRH-II were detected in brain and pituitary extracts (King et al. 1990b). However, cGnRH-II was undetectable in individually sampled pituitary glands of eel suggesting that is present in very low amounts (Montero et al. 1995). Likewise, in rainbow trout (*Oncorhynchus mykiss*) (Okuzawa et al. 1990) and in masu salmon (*Oncorhynchus masu*) (Amano et al. 1992), where sGnRH and cGnRH-II were found in the brain, only sGnRH could be detected in single pituitary glands. In sea bream (*Sparus aurata*), sGnRH, cGnRH-II and sea bream GnRH ([Ser^8]GnRH, sbGnRH) were found in the brain (Powell et al. 1994). In the pituitary gland, cGnRH-II was undetectable, whereas sbGnRH, assumed to be responsible for GTH release, was present in a 500-fold excess over sGnRH. Interestingly, in the sea bream, sbGnRH is a less potent GTH secretagogue

than sGnRH (Zohar et al. 1995). Similarly, cfGnRH is less potent than cGnRH-II in the African catfish (Schulz et al. 1993).

In the African catfish, the cDNAs encoding prepro-cfGnRH and prepro-cGnRH-II have been cloned and characterized (Bogerd et al. 1994). This procedure allowed the localization of the GnRHs by means of *in situ* hybridization. Moreover, the respective GnRH-associated peptides (GAPs) were expressed in *E. coli* and used to raise antibodies (Zandbergen et al. 1995). Cell bodies expressing prepro-cfGnRH mRNA were found scattered in 2 lines lateral to the midline of the brain extending from the ventral telencephalon to the caudal hypothalamus in the medial olfactory tract, *area ventralis telencephali pars ventralis*, in the area of the *nucleus preopticus periventricularis*, in the area of the *nucleus anterioris periventricularis* and in the medial basal hypothalamus. Prepro-cGnRH-II mRNA expressing neurones were restricted to the midbrain tegmentum in the area of the *fasciculus longitudinalis medialis*. By using the respective anti-GAPs in an immunocytochemical study, the *in situ* hybridization results were completely confirmed. In addition, numerous fibres from the cfGnRH neurones project into the rostral pars distalis of the pituitary gland where the gonadotrophs are situated. From the chGnRH-II neurones in the midbrain tegmentum only a few descending fibres were observed along the border of the third ventricle but none were found in the pituitary gland. Hence, the colocalization of cfGnRH and cGnRH-II observed in a previous study using GnRH antisera (Schulz et al. 1993) may reflect a crossreaction of anti-cGnRH-II with the locally high concentration of cfGnRH. The apparent absence of cGnRH-II in fibres in the pituitary gland opens the question as to what extent the two GnRHs in the catfish are involved in the regulation of GTH II secretion. Both *in vivo* and *in vitro* cGnRH-II and cfGnRH have been shown to stimulate GTH II secretion. Calculating the GnRH's concentration on the basis of 5 mg pituitary wet weight corresponding to 5 µl volume, the concentration of cfGnRH and cGnRH-II are 2 µM and 3 nM, respectively. These concentrations are similar to the ED_{50} concentrations observed for cfGnRH-stimulated GTH II secretion of $[Ca^{2+}]_i$ increases in catfish gonadotrophs (Rebers et al. 1995). Hence, it appears reasonable to assume that both cfGnRH and cGnRH-II are participating in the regulation of GTH II in the African catfish. Also, in other fish species, no cGnRH-II fibres were detected in the pituitary gland, despite the presence of cGnRH-II perikarya in the midbrain tegmentum (Amano et al. 1991; Montero et al. 1994; Millar and King 1994). In goldfish, however, a tract between the cGnRH-II neurones and the pituitary gland was found in tracer experiments with Di-I (Anglade et al. 1993). It has been suggested by Schulz et al. (1993) that in feral catfish the role of cGnRH-II may be related to gonadotropin surges, such as those associated with ovulation and spawning. The present study was carried out on laboratory bred animals in which spontaneous ovulation does normally not occur. This situation may be correlated, or even be caused, by a low activity of the cGnRH-II synthesis and transport system.

Interaction between two endogenous GnRHs

Chicken GnRH-II is about 160-fold more potent than cfGnRH regarding GTH secretion or $[Ca^{2+}]_i$ increase (Rebers et al. 1995). A similar difference in potency has recently been found in GnRH-stimulated inositol phosphate production by gonadotrophs *in vitro*. The differences in GTH release activity is well correlated with difference in receptor affinities. The low affinity displayed by cfGnRH, however, may be compensated for by its large excess over cGnRH-II in the catfish pituitary gland. The simultaneous presence of cfGnRH and cGnRH-II in the pituitary gland of the African catfish allows speculations on a mutual modulation of the GnRH's GTH II release activity, which were tested experimentally. When clearly effective doses of cfGnRH were combined with subthreshold or slightly effective doses of cGnRH-II, the effect of cfGnRH was markedly impaired. Combinations of subthreshold or low doses of cfGnRH with clearly effective doses of cGnRH-II, however, induced a GTH II release similar to that of cGnRH-II alone. Combinations of effective but submaximal doses of both GnRHs resulted in a higher GTH release than either of the peptides induced separately. The modulating effects of the two peptides on each other's GTH release activity is directly on the gonadotrophs since *in vivo* and *in*

vitro results are essentially the same (Bosma et al., unpublished data). These data support the notion that both cfGnRH and cGnRH-II participate in regulating pituitary GTH II secretion in African catfish, the overall effect depending on the relative concentration of the two peptides in the vicinity of the gonadotrophs.

GnRH receptors

In goldfish, it has been demonstrated that the endogenous GnRHs (sGnRH and cGnRH-II) not only induce the release of GTH II, but also of growth hormone (GH) (Marchant et al. 1989) and GnRH receptors have been shown to be present on gonadotrophs as well as on somatotrophs. A GnRH-stimulated release of GH was also recorded in tilapia (*Oreochromis* hybrid) (Melamed et al. 1995) but not in rainbow trout (Blaise et al. 1995) or in African catfish, where an autoradiographic study showed that GnRH receptors are confined to the pituitary cells immunoreactive for the GTH II ß-subunit; moreover, cfGnRH as well as cGnRH-II induced an increase of the $[Ca^{2+}]_i$ in the gonadotrophs, but not in the somatotrophs (Bosma et al. 1995). It is also interesting to note that goldfish gonadotrophs and somatotrophs appear to carry pharmacologically distinct GnRH receptors (Murthy and Peter 1994).

In contrast to goldfish (Habibi et al. 1987), a single class of GnRH receptors was characterized by radio-receptor assays of the catfish pituitary gland (De Leeuw et al. 1988). This receptor was originally characterized as a high affinity receptor type. However, more recent studies showed an affinity towards the native cGnRH-II in the nM-range, but the affinity towards cfGnRH is at least 100 fold lower (Schulz et al. 1993).

Recently, a cDNA encoding a putative GnRH receptor was cloned from the pituitary of the African catfish (Tensen et al. 1997). When the isolated cDNA was transiently transfected in human embryonic kidney cells (HEK 293), which were then challenged with cfGnRH or cGnRH-II, both peptides stimulated inositol phosphate production in a dose-dependent manner, cGnRH-II again being about 100-fold more potent than cfGnRH. Moreover, both peptides showed similar ED50 values as in primary culture of catfish gonado-

trophs. This pattern is in accordance with previous results from *in vivo* and *in vitro* experiments on GnRH-induced GTH II released and suggests a single cognate GnRH receptor mediates the effects of both cfGnRH and cGnRH-II.

References cited

Amano, M., Oka, Y., Aida, K., Okumoto, N., Kawashima, S. and Hasegawa, Y. 1991. Immunocytochemical demonstration of salmon GnRH and chicken GnRH-II in the brain of Masu salmon (*Oncorhynchus masou*). J. Comp. Neurol. 314: 587–597.

Amano, M., Aida, K., Okumoto, N. and Hasegawa, Y. 1992. Changes in salmon GnRH and chicken GnRH-II contents in the brain and pituitary, and GTH contents in the pituitary in female masu salmon, *Oncorhynchus masou*, from hatching through ovulation. Zool. Sci. 9: 375–386.

Anglade, I., Zandbergen, M.A. and Kah, O. 1993. Origin of pituitary innervation in the goldfish. Cell Tiss. Res. 273: 345–355.

Blaise, O., Le Bail, P.Y and Weil, C. 1995. Lack of gonadotropin releasing-hormone action on *in vivo* and *in vitro* growth hormone release in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. 110C: 133–141.

Bogerd, J., Li, K.W., Janssen-Dommerholt, C. and Goos, H. 1992. Two gonadotropin-releasing hormones from African catfish (*Clarias gariepinus*). Biochem. Biophys. Res. Commun. 187: 127–134.

Bogerd, J., Zandbergen, T., Andersson, E. and Goos, H. 1994. Isolation, characterisation and expression of cDNAs encoding the catfish-type and chicken-II-type gonadotropin-releasing hormone precursors in the African catfish. Eur. J. Biochem. 222: 541–549.

Bosma, P.T., van Dijk, W., van Haren, S.F., Kolk, S.M., Lescroart, O., Schulz, R.W., Terlou, M. and Goos, H.J.Th. 1995. GnRH receptors are restricted to gonadotropes in male African catfish. *In* Proc. 5th Int. Symp. Reproductive Physiology of Fishes. pp. 55–57. Edited by F.W. Goetz and P. Thomas. Fish Symposium 95, Austin.

De Leeuw, R., Conn, M.P., van 't Veer, C., Goos, H.J.Th. and van Oordt, P.G.W.J. 1988. Characterization of the receptor of gonadotropin-releasing hormone in the pituitary of the African catfish, *Clarias gariepinus*. Fish Physiol. Biochem. 5: 99–107.

Gautron, J.P., Pattou, E. and Kordon, C. 1992. Precursor and deaminated forms of both luteinizing hormone-releasing hormone (LHRH) and [hydroxyproline^9]LHRH are present in the rat hypothalamus. Biochem. Biophys. Res. Commun. 189: 1368–1373.

Habibi, H.R., Peter, R.E., Sokolowska, M., Rivier, J.E. and Vale, W.W. 1987. Characterization of gonadotropin releasing hormone (GnRH) binding to the pituitary receptors in goldfish (*Carassius auratus*). Biol. Reprod. 36: 844–853.

Kah, O., Anglade, I., Leprêtre, E., Dubourg, P. and de Monbrison, D. 1993. The reproductive brain in fish. Fish Physiol. Biochem. 11: 85–98.

King, J.A. and Millar, R.P. 1982. Structure of chicken hypo-thalamic luteinizing hormone-releasing hormone. II. Iso-lation and characterization. J. Biol. Chem. 257: 10729–10732.

King, J.A., Hinds, L.A., Mehl, A.E., Saunders, N.R. and Millar, R.P. 1990a. Chicken GnRH II occurs together with mamma-lian GnRH in a South American species of marsupial (*Monodelphis domestica*). Peptides 11: 521–525.

King, J.A., Dufour, S., Fontaine, Y.A. and Millar, R.P. 1990b. Chromatographic and immunological evidence for mamma-lian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary. Peptides 11: 507–514.

Lescheid, D.W., Powell, J.F.F., Fischer, W.H., Park, M., Craig, A., Bukovskaya, O., Barannikova, I.A. and Sherwood, N.M. 1995. Mammalian gonadotropin-releasing hormone (GnRH) identified by primary structure in Russian sturgeon, *Acipenser gueldenstaedti*. Reg. Pept. 55: 299–309.

Li, K.W., Hoek, R.M., Smith, F., Jiménez, C.R. van der Schors, R.C., van Veelen, P.A., Chen, S., van der Greef, J., Parish, D.C., Benjamin, P.R. and Geraerts, W.P.M. 1994. Direct peptide profiling by mass spectrometry of single identified neurones reveals complex neuropeptide-procesing pattern. J. Biol. Chem. 269: 30288–30292.

Lovejoy, D.A., Fischer, W.H., Parker, D.B., McRory, J.E., Park, M., Lance, V., Swanson, P., Rivier, J.E. and Sherwood, N.M. 1991a. Primary structure of two forms of gonadotropin-re-leasing hormone from brains of the American alligator (*Alli-gator mississippiensis*). Reg. Pept. 33: 105–116.

Lovejoy, D.A., Sherwood, N.M., Fischer, W.H., Jackson, B.C., Rivier, J.E. and Lee, T. 1991b. Primary structure of gonado-tropin-releasing hormone from the brain of a Holocephalan (ratfish: *Hydrolagus colliei*). Gen. Comp. Endocrinol. 182: 152–161.

Lovejoy, D.A., Fischer, W.H., Ngamvongchon, S., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E. and Sherwood, N.M. 1992. Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolu-tion. Proc. Nat. Acad. Sci. USA 89: 6373–6377.

Marchant, T.A., Chang, J.P., Nahorniak, C.S. and Peter, R.E. 1989. Evidence that that gonadotropin releasing hormone also functions as growth hormone-releasing factor in goldfish. Endocrinology 124: 2509–2518.

Melamed, P., Eliahu, N., Levavi-Sivan, B., Ofir, M., Farchi-Pisanty, O., Rentier-Delrue, F., Smal, J. and Yaron, Z.Z. 1995. Hypothalamic and thyroidal regulation of growth hormone in tilapia. Gen. Comp. Endocrinol. 97: 13–30.

Millar, R.P. and King, J.A. 1994. Plasticity and conservation in gonadotropin-releasing hormone structure and function. *In* Perspectives in Comparative Endocrinology. pp. 129–136. Edited by K.G. Davey, R.E. Peter and S.S. Tobe. NRC, Canada, Ottawa.

Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K. and Matsuo, H. 1984. Identification of the sec-ond gonadotropin-releasing hormone in chicken hypo-thalamus: Evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proc. Nat. Acad. Sci. USA 81: 3874–3878.

Montero, M., Vidal, B., King, J.A., Tramu, G., van de Sande, F., Dufour, S. and Kah, O. 1994. Immunocytochemical localiza-tion of mammalian GnRH (gonadotropin releasing hormone) and chicken GnRH-II in the brain of the European eel (*Anguilla anguilla* L.). J. Chem. Neuroanat. 7: 227–241.

Montero, M., LeBelle, N., King, J.A., Millar, R.P. and Dufour, S. 1995. Differential regulation of the two forms of gonado-tropin-releasing hormone (mGnRH and cGnRH-II) by sex steroids in the European female silver eel (*Anguilla anguilla*). Neuroendocrinology 61: 525–535.

Murthy, C.K. and Peter, R.E. 1994. Functional evidence regard-ing receptor subtypes mediating the action of native gonado-tropin-releasing hormones (GnRH) in goldfish (*Carassius au-ratus*). Gen. Comp. Endocrinol. 94: 78–91.

Muske, L.E. 1993. Evolution of gonadotropin-releasing hormone (GnRH) neuronal systems. Brain Behav. Evol. 42: 215–230.

Ngamvongchon, S., Lovejoy, D.A., Fischer, W.H., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E. and Sherwood, N.M. 1992. Primary structures of two forms of gonadotropin-re-leasing hormone, one distinct and one conserved, from catfish brain. Mol. Cell. Neurosci. 3: 17–22.

Okuzawa, K., Amano, M., Kobayashi, M., Aida, K., Hanyu, I., Hasegawa, Y. and Miyamoto, K. 1990. Differences in salmon GnRH and chicken GnRH-II contents in discrete brain areas of male and female rainbow trout according to age and stage of maturity. Gen. Comp. Endocrinol. 80: 116–126.

Peter, R.E., Yu, K.L., Marchant, T.A. and Rosenblum, P.M. 1990. Direct neural regulation of the teleost adenohypophysis. J. Exp. Zool. Suppl. 4: 84–89.

Powell, J.F.F., Zohar, Y., Elizur, A., Park, M., Fischer, W.H., Craig, A.G., Rivier, J.E., Lovejoy, D.A. and Sherwood, N.M. 1994. Three forms of gonadotropin-releasing hormone char-acterized from brains of one species. Proc. Nat. Acad. Sci. USA 91: 12081–12085.

Powell, J.F.F., Fischer, W.H., Park, M., Craig, A.G., Rivier, J.E., White, S.A., Francis, R.C., Fernald, R.D., Licht, P., Warby, C. and Sherwood, N.M. 1995. Primary structure of solitary form of gonadotropin-releasing hormone (GnRH) in cichlid pitui-tary; three forms of GnRH in brain of cichlid and pumpkin-seed fish. Reg. Pept. 57: 43–53.

Rebers, F.E.M., Bosma, P.Th., Willems, P.W.G.M., Tensen, C.P., Leurs, R., Schulz, R.W. and Goos, H.J.Th. 1995. GnRH-in-duced changes of $[Ca^{2+}]_i$ in African catfish (*Clarias garie-pinus*) gonadotropes. *In* Proc. 5th Int. Symp. Reproductive Physiology of Fishes. p. 76. Edited by F.W. Goetz and P. Thomas. Fish Symposium 95, Austin.

Rosenblum, P.M., Goos, H.J.Th. and Peter, R.E. 1994. Regional distribution and *in vitro* secretion of salmon and chicken-II gonadotopin-releasing hormones from the brain and pituitary of juvenile and adult goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. 93: 369–379.

Schulz, R.W., Bosma, P.T., Zandbergen, M.A., van der Sanden, M.C.A., van Dijk, W., Peute, J., Bogerd, J. and Goos, H.J.Th. 1993. Two gonadotropin-releasing hormones in the African catfish, *Clarias garipinus*: localization, pituitary receptor binding, and gonadotropin release activity. Endocrinology 133: 1569–1577.

Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J. and Vale, W. 1983. Characterization of a teleost gonadotropin-releasing hormone. Proc. Natl. Acad. Sci. USA 80: 2794–2798.

Sherwood, N.M., de Leeuw, R. and Goos, H. 1989. A new mem-ber of the gonadotropin-releasing hormone family in teleosts: catfish gonadotropin-releasing hormone. Gen. Comp. Endo-crinol. 75: 427–436.

Sherwood, N.M., Lovejoy, D.A. and Coe, I.R. 1993. Origin of

mammalian gonadotropin-releasing hormones. Endocr. Rev. 14: 241–254.

Sherwood, N.H., Sower, S.H., Marshak, D.R., Fraser, B.A. and Brownstein, M.J. 1986. Primary structure of gonadotropin-releasing hormone from lamprey brain. J. Biol. Chem. 261: 4812–4819.

Somoza, G.M., Stéfano, A., d'Eramo, J.L., Canosa, L.F. and Fridman, O. 1994. Immunoreactive GnRH suggesting a third form of GnRH in addition to cIIGnRH and sGnRH in the brain and pituitary gland of *Prochilodus lineatus* (Characiformes). Gen. Comp. Endocrinol. 94: 44–52.

Sower, S.A., Chiang, Y.-C., Lovas, S. and Conlon, J.M. 1993. Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. Endocrinology 132: 1125–1131.

Tensen, C., Okuzawa, K., Blomenröhr, M., Reners, F., Leurs, R., Bogerd, F., Schulz, R. and Goos, H.J.Th. 1997. Distinct efficiencies for two endogenous-ligands on a single cognate gonadoliberin receptor. Eur. J. Biochem. 243: 134–140.

Yu, K.L., Sherwood, N.M. and Peter, R.E. 1988. Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). Peptides 9: 625–630.

Zandbergen, M.A., Kah, O., Bogerd, J., Peute, J. and Goos, H.J.Th. 1995. Differential expression of prepro-catfish GnRH mRNA and prepro-chicken GnRH-II mRNA in a GnRH-immunoreactive cell population in the brain of the African catfish. Neuroendocrinology 62: 571–578.

Zohar, Y., Elizur, A., Sherwood, N.M., Powell, J.F.F., Rivier, J.E. and Zmora, N. 1995. Gonadotropin-releasing activities of the three native forms of gonadotropin-releasing hormone present in the brain of gilthead seabream, *Sparus aurata*. Gen. Comp. Endocrinol. 97: 289–299.