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Immunoreactive Gonadotropin-Releasing Hormone-like Material in the Brain and the Pituitary Gland during the Perioviulatory Period in the Brown Trout (*Salmo trutta* L.): Relationships with the Plasma and Pituitary Gonadotropin

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In fish there are few data on the gonadotropin-releasing hormone (Gn-RH) neurosecretory activity, which could explain long- and short-term variations of the gonadotropin secretion. There is no biological species specificity between mammal and fish Gn-RH; although there is a structural difference, they are, on the contrary, characterized by a high immunological specificity which does not allow measurement of fish Gn-RH using radioimmunoassay for LH-RH. We have synthesized salmon Gn-RH according to the formula recently proposed by Sherwood (N. Sherwood, L. Eiden, M. Brownstein, J. Spies, J. Rivier, and W. Vale, 1983. *Proc. Natl. Acad. Sci. USA* 80, 2794–2798). Its activity has been tested by its ability to stimulate the gonadotropin hormone (GtH) secretion *in vivo* in testosterone-implanted juvenile rainbow trout, and for the recognition of synthesized Gn-RH (s-Gn-RH) perykaria by a specific antibody raised against the s-Gn-RH in regions of the brain described as containing LH-RH immunoreactive-like material. A radioimmunoassay has been developed for the salmon Gn-RH, and its specificity to measure trout Gn-RH has been tested. Using this assay, the brain and pituitary Gn-RH contents have been measured throughout the final phases of maturation and ovulation. Brain Gn-RH increases from the end of vitellogenesis (8.9 ± 0.76 ng/brain) to ovulation (more than 15 ng/brain). Pituitary Gn-RH is lower (1.58 ± 0.69 ng/pituitary) at the end of vitellogenesis and follows a similar profile as in the brain, except for a significant decrease just prior the beginning of oocyte maturation. The correlations between Gn-RH levels and GtH pituitary and plasma levels show that total brain Gn-RH is never correlated to the GtH, suggesting that the increase in the brain Gn-RH content is related to a Gn-RH system closely related to maturation and ovulation, which remains to be investigated. On the contrary, pituitary Gn-RH levels are well correlated with pituitary and plasma GtH levels, indicating that pituitary Gn-RH levels might represent a good index of the Gn-RH neurosecretory activity in the fish hypothalamohypophysial complex, given the absence of a portal system in teleost. © 1986 Academic Press, Inc.

Although there is much quantitative information about the endogenous levels of plasma and pituitary GtH in teleost fish, and especially in salmonids, it is still difficult to correlate them with variations of the hypophysiotropic activity in the central nervous system, whose secretions are the

primary chemical links between the fish and its environment for the control of most of the reproductive processes (gametogenesis, ovulation, spawning, reproductive behavior). It has been known for a long time that mammalian LH-RH and fish gonadotropin-releasing hormone (Gn-RH) are nearly similar regarding their biological activity (Breton *et al.*, 1972; Breton and Weil,

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1973; King and Millar, 1980). This property has been used for the development of a bioassay to measure the Gn-RH activity in salmonids (Crim and Evans, 1980), but this method, unlike similar *in vitro* determinations in rats, for example, is not sensitive enough and too complex to measure the Gn-RH content in isolated fish brain and pituitary in numerous samples. Only limited attempts have been made to develop a radioimmunoassay for fish Gn-RH because of the immunological species specificity between fish and mammalian releasing hormone (King and Millar, 1979; 1980), and few have been successful (Dufour, *et al.*, 1982; Schreibman, *et al.*, 1983). The aim of this study was to develop a specific radioimmunoassay for salmonid Gn-RH after the determination of its structure (Sherwood *et al.*, 1983) and to apply it to the determination of Gn-RH content in the brain and pituitary of the brown trout during the final stages of maturation.

MATERIALS AND METHODS

Peptide synthesis has already been described (Breton *et al.*, 1984), and its homogeneity was assessed after high-pressure liquid chromatography with a mixture of methanol–water–trifluoroacetic acid as mobile phase (50–50–0.1).

In addition, the characterization of the newly synthesized peptide was pursued by testing its ability to stimulate the *in vivo* GtH secretion and after identification of the brain structure reacting with an antibody raised against the synthesized Gn-RH (s-Gn-RH).

1. *Biological activity of the synthesized Gn-RH.* Biological activity was determined in May in 60-g immature rainbow trout pretreated by intraperitoneal testosterone implants to stimulate pituitary GtH synthesis (Crim and Evans, 1980; 1981). Testosterone Silastic implants (100 µg/fish) were set up 3 weeks before the intraperitoneal administration of Gn-RH. Six groups were constituted (Table 1).

Blood was sampled with heparinized syringes from a caudal vessel at 0, 5, and 24 hr after the beginning of the experiment. After centrifugation, plasma was preserved frozen until determination of GtH content by radioimmunoassay (Breton *et al.*, 1971, 1983).

2. *Immunocytochemical localization of Gn-RH.* The similarity of the synthetic Gn-RH to a biological peptide was also established by an immunocytochemical technique testing the ability of an antibody raised

against the s-Gn-RH to recognize nervous structures containing a LH–RH-like factor within the trout brain. The brains and pituitaries of immature rainbow trout were dissected and fixed for 1 hr in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.6. After rinsing, they were immersed overnight in 0.1 M phosphate buffer containing 12% sucrose. The sections were processed by the PAP technique, the antibody being diluted at 1/2000. The link antibody and the PAP complex (DAKO, Denmark) were diluted 1/200 and 1/600, respectively. The peroxidase activity was visualized using 3-3'-tetrahydrochloride diaminobenzidine (Sigma) and hydrogen peroxide.

3. *Radioimmunoassay procedure.* The acquisition of antibodies, the radioiodination, and purification technique have been previously described (Breton *et al.*, 1984). After tracer purification by polyacrylamide gel electrophoresis, the degree of iodination (iodine molecules fixed on the tyrosine group) was determined according to Caraty *et al.* (1980). The labeled s-Gn-RH was pepsin digested for 24 hr at 37°. The digested peptide was submitted to thin-layer chromatography on silica gel (Merck kiesegel 60 F 254) in an ethyl acetate, methanol 2 M, NH₄OH system (5-2-1) in the presence of 3-monoiodo-L-tyrosine (MIT) and 3,5-diiodo-tyrosine (DIT) as carriers. After migration, the spots were revealed by ninhydrin, scanned on a radiochromatogram scanner, and those corresponding to the radioactive MIT and DIT were aspirated and counted in a gamma counter.

The specificity of the assay to recognize s-Gn-RH was partially tested by measuring the cross reaction between the labeled s-Gn-RH and antibodies directed against mammalian LH–RH and bird Gn-RH (Gln⁸–LH–RH), and by studying the ability of brain and pituitary extracts, LH–RH and LH–RHa (D-Ala⁶–Des-Gly¹⁰ LH–RH) to compete the binding of s-Gn-RH to its specific antibody.

4. *Collection of samples.* The experiment was conducted on a group of 3-year-old female brown trout. Between November 16 and 24, they were checked three times for the detection of their stage of maturity, as described earlier (see Fig. 5) (Jalabert and Breton, 1980).

Some fish were kept for one additional month after ovulation, but were not stripped.

The animals were killed by decapitation, and the entire brain and pituitary were immediately frozen in liquid nitrogen and kept frozen at –30° until they were extracted for radioimmunoassay. All these operations were done between 9 and 11 PM.

Radioimmunoassay of Gn-RH was performed according to Breton *et al.* (1984) and of GtH according to Breton *et al.* (1971).

The results were analyzed using the Student *t* test, variance analysis, and couple method after logarithmic transformation of the data in order to homogenize the

TABLE I
ASSAY PROCEDURE FOR THE DETERMINATION OF THE BIOLOGICAL ACTIVITY OF THE
NEWLY SYNTHESIZED s-Gn-RH

Group number	Pretreatment	Treatment	Number of fishes
1	Control	Control	11
2	Empty implant	Saline	16
3	Testosterone implant	Saline	11
4	Testosterone implant	s-Gn-RH 50 µg/kg body wt + vehicle	15
5	Testosterone implant	s-Gn-RH 100 µg/kg body wt + vehicle	15
6	Testosterone implant	s-Gn-RH 500 µg/kg body wt + vehicle	14

variances. These tests were employed to compare pituitary and plasma GtH, and brain and pituitary Gn-RH. The correlations between GtH and Gn-RH were also calculated for each stage of gonadal development. The parallelism between the competition curves obtained with standards s-Gn-RH and analogs was compared by analysis of covariance of the slopes of the logit B/B_0 log dose curves.

RESULTS

Gn-RH Synthesis and Biological Activity

The amino acid analysis of the newly synthesized s-Gn-RH has already been given (Breton *et al.*, 1984).

The HPLC elution profile reveals only one symmetric peak, as shown in Fig. 1.

In fish not implanted with testosterone, pituitary GtH were, respectively, 2.84 ± 0.5 ng/pituitary in control fish and 15.3 ± 8.5 ng/pituitary in fish receiving an empty implant. In testosterone-implanted fish, pituitary GtH levels markedly increased to 1459 ± 187 ng/pituitary. Figure 2 shows the profiles of plasma GtH secretion in the experimental groups. At 0 and 5 hr, there were no statistical differences between the plasma GtH levels of fish which did not receive Gn-RH treatment (Fig. 2A). After 24 hr GtH plasma levels did not differ in fish from groups 1–3. Gn-RH alone induced a significant increase in the plasma GtH levels at 5 and 24 hr after the injection only in fish receiving the higher Gn-RH dosage ($P < 0.01$) (fig. 2B).

The biological activity of the s-Gn-RH

was further confirmed *in vitro* by its ability to stimulate the GtH secretion in trout pituitary cells cultures in a range of dosages between 10^{-6} to 10^{-9} M (Weil, personal communication).

In the brain, immunoreactive perikarya are located in the olfactory nerves and bulbs, the ventral telencephalon, the preoptic region, and the basal hypothalamus. Immunoreactive fibers are detected in many areas of the forebrain (Fig. 3A). The pituitary gland contains immunoreactive material located in the neurohypophysis bordering the infundibular recess and within digitations entering the proximal pars distalis (Fig. 3B).

Radioimmunoassay

The mean yield of the iodination was around 70%, corresponding to a sp act ranging from 60 to 75 µCi/µg of s-Gn-RH. The profiles of the radioactivity after PAGE of the four most radioactive fractions eluted from the LH-20 column are shown in Fig. 4. The main peaks appear homogeneous, but in all four fractions there is a small, more electronegative component which does not cross react with the antibody raised against s-Gn-RH. The summit of each main peak possesses a greater immunoreactivity than that of the corresponding fraction before electrophoresis; the mean increase in immunoreactivity is around 15%

FIG. 1. Profile of Gn-RH elution from reversed-phase HPLC. Column R.P. 18-5 m Bromlee labs 4.6 $\times$ 25 cm—mobile phase as MeOH 50% H₂O 49.9%—TFA 0.1% Flow 2.5 ml mn. Isocratic elution.

FIG. 2. Evolution of the plasma GtH levels in (A) fish which did not receive s-Gn-RH: ($\diamond$) control group 1, ($\bullet$) nonimplant fish + saline, ($\circ$) testosterone implanted + saline; and (B) in fish which received s-Gn-RH alone: $\square$ 50 μ g/kg body wt, $\triangle$ 100 μ g/kg body wt, $\blacktriangle$ 500 μ g/kg body wt.

FIG. 3. (A) Immunoreactive cell bodies (arrows) in the ventral telencephalon. $\times 240$. (B) Immunoreactive fibers in the pituitary gland at the level of the neurohypophysis (nh) adjacent to the proximal pars distalis (pdp). v, third ventricle. $\times 140$.

when the antibody is used as a 1:1000 final dilution.

After pepsin digestion and thin-layer chromatography, the scanning of the plate shows only one peak of radioactivity corresponding to the monoiodotyrosine. After aspiration, the radioactivity was shown to correspond only to the monoiodotyrosine spot.

The tracer diluted in glycerol can be preserved for at least 1 month at 0° without loss of immunoactivity (Table 2).

The labeled s-Gn-RH never binds more than 7.2% to eight different anti-LH-RH used at a 10^{-2} dilution, and 22.7% to anti-2-10 LH-RH used at the same dilution, with which labeled LH-RH binds more than 80%. In the same manner, s-Gn-RH binds less than 10% with the antibody raised against the chicken LH-RH. The slope of the standard curve was -1.30 and parallel to those obtained in competition studies with brain (-1.38) and brown trout pituitary extracts (-1.48) (Breton *et al.*, 1984), indicating a specificity for the assay of immunoreactive Gn-RH-like materials in these tissues. There was no competitive inhibition by a trout muscle extract, while that by LH-RH and D-Ala⁶-Des-Gly¹⁰

LH-RH was not specific. With the latter peptide, 5 ng/tube inhibited only 26% of the binding of ^{125}I Gn-RH, the same inhibition being produced by 70 pg of s-Gn-RH. Similarly 5 ng of LH-RH inhibited 35% of the binding compared to 100 pg of s-Gn-RH. The competition obtained with these two peptides was not specific, the slopes of the competition curves being nonparallel to that of the standard curve -0.94 for LH-RH against -1.30 for s-Gn-RH.

The intraassay variability is around 30% for the standard between 7 and 31 pg/assay tube and less than 10% from 62 to 1000 pg/assay tube. These values are higher for the interassay variability ranging from 10 to 25% between 62 and 1000 pg/assay tube. This part of the curve is generally that which is used for the determination of the s-Gn-RH content in brains and pituitaries, and Table 3 gives the variation coefficients (CV) for five brain samples; they are similar to those obtained with standard, in the same range of concentrations.

GtH and Gn-RH during the Periovulatory Period

Blood and pituitary GtH contents showed similar variations to those already

FIG. 4. Radioactive profiles of s-Gn-RH—LH-20 fractions after polyacrylamide gel electrophoresis at 7.5% 2.5 mA per tube—4 hr migration.

described in the same species and same strain (Breton *et al.*, 1983). There was no statistical variation between the plasma GtH levels of fish taken at the end of vitellogenesis and those undergoing ovulation (5 ng/ml), a sharp increase occurred in recently ovulated, nonstripped animals (12 ng/ml), and levels returned to basal levels (2 ng or less) 1 month after ovulation. The pituitary GtH increased significantly from the end of vitellogenesis to maturation. It remained stable during maturation and ovulation but sharply decreased after ovulation.

At the end of vitellogenesis the total brain Gn-RH content was 8.94 ± 0.76 ng/brain ($n = 8$). It significantly increased up to 15 ng/brain when fish possess oocytes

TABLE 2
EVOLUTION OF THE IMMUNOAFFINITY OF THE
LABELED s-Gn-RH WITH THE TIME OF PRESERVATION

Days of preservation	0	3	6	9	17	30
Immunoactivity in % of binding	78	74.2	83.4	77	73.4	84.2

Note. Antibody was used at a 1:1000 final dilution.

TABLE 3
INTRA- AND INTERASSAY VARIABILITY

Sample number	Intraassay variability		Interassay variability	
	Gn-RH/ng per brain	CV %	Gn-RH/ng per brain	CV %
1	1.946	6.3	1.758	22
2	3.42	3.5	3.668	24.6
3	1.339	7.5	1.617	29.4
4	1.70	28	2.37	34.3
5	0.23	56.8	2.20	64.0

Note. Intraassay variability was determined measuring five different samples in 10 replicates within the same assay. For the interassay variability, the former assay was repeated on five consecutive days.

with germinal vesicles at their periphery just prior to the onset of maturation. It did not vary during maturation and ovulation, as did blood GtH concentrations, and began to decrease after ovulation to return to values close to 10 ng/brain 1 month after, and less than 5 ng 2 months later. The general profile of Gn-RH brain content roughly followed that of the pituitary GtH contents (Fig. 5).

Pituitary Gn-RH levels were lower than those of the brain. From the end of vitellogenesis until oocytes were at the germinal vesicle (VG) stage, the pituitary Gn-RH content appeared to rise from 1.58 ± 0.69 ($n = 8$) to 3.25 ± 0.7 ($n = 5$) ng/pituitary. In fishes beginning maturation, it significantly decreased to 1.51 ± 0.33 ($n = 8$, $P < 0.05$), and rose in ovulating and recently ovulated fish ($P < 0.01$), in which it reached its maximum values at the same time as the plasma GtH levels. It returned to basal values 1 month after ovulation.

There was a positive and significant correlation between the pituitary Gn-RH and GtH levels in fish which had not yet begun maturation [i.e., end of germinal vesicle (FV) and VG stages] ($r = +0.81$, $P < 0.01$, $n = 13$), as well as in fish 1 month after ovulation ($r = 0.89$, $P < 0.01$, $n = 21$). These two values were, however, not correlated in fish in active maturation and ovulation. On the contrary, these were the only phases during which pituitary Gn-RH and

blood GtH concentration were positively correlated ($r = +0.89$, $P < 0.01$, $n = 12$). Brain Gn-RH and plasma GtH were never correlated at any stage of the periovulatory period, but brain Gn-RH and pituitary GtH were correlated before the beginning of oocyte maturation ($r = 0.55$ with a 5% level of significance).

DISCUSSION

In this work we have developed a radioimmunoassay for the measurement of immunoreactive Gn-RH-like material in the brain and in the pituitary gland of salmonids and applied it to the determination of Gn-RH variations during the periovulatory period in the brown trout.

The amino acid composition (Breton *et al.*, 1984) and the elution profile obtained after HPLC lead to the conclusion that our Gn-RH preparation was homogeneous and corresponds to the formula established by Sherwood *et al.* (1983). Its biological activity in testosterone-implanted juvenile brown trouts is similar to that of LH-RH in the juvenile rainbow trout (Crim and Evans, 1980), in the same concentration range. It is possible that only higher dosages are able to stimulate GtH secretion, as in mammals where native LH-RH is rapidly degraded by peptidase, as opposed to its analogs.

Evidence for a relationship between the

FIG. 5. Evolution of brain (open bars) and pituitary (hatched bars) Gn-RH contents during the periovulatory period in the brown trout. FV, end of vitellogenesis germinal vesicle in the center of the oocyte; VG, germinal vesicle at the periphery of the oocyte; maturation, oocyte entering and during maturation; ovulated, ovulations were detected twice a week, i.e., between 0 and 3 after ovulation.

newly synthesized s-Gn-RH and immunologically similar trout peptides has been obtained by immunohistochemistry. The localization of the immunoreactive structures in the trout brain is consistent with what is known in other species concerning regulation of gonadotropin secretion by the central system (Munz *et al.*, 1982; Kah *et al.*, 1985). At the level of the pituitary, the fibers reacting to anti-s-Gn-RH have a repartition similar to that described previously using antibodies to LH-RH (Dubois *et al.*, 1979). This indicates that an immunoreactive-like s-Gn-RH substance is

present in the brain and the pituitary of the trout. This has also been demonstrated after immunoassay and HPLC studies on rainbow trout brain extracts (Sherwood *et al.*, 1984) which contain a peptide chromatographically and immunologically identical to salmon s-Gn-RH. Our results confirm the immunological identity between brown trout Gn-RH and s-Gn-RH, as indicated by competitive studies.

Our Gn-RH does not specifically cross react with antibodies raised against mammalian and chicken Gn-RH, and the binding of s-Gn-RH to its antibodies is not specifically complete by LH-RH and D-Ala-Des-Gly⁶ LH-RH. Although this seems to indicate that our system does not specifically recognize these different forms of LH-RH, it remains possible that we could measure other forms of salmon Gn-RH, with similar immunoreactivity, as demonstrated after running rainbow trout brain extracts in HPLC (Sherwood *et al.*, 1984).

After labeling, the electrophoretic purification step increases by about 20% the immunoactivity of the labeled s-Gn-RH; since electrophoretic profile shows no degradation of the molecule, it can be postulated that rather than the elimination of degraded forms of s-Gn-RH, this step allows the separation of labeled and unlabeled s-Gn-RH, as already suggested for insulin (Berson and Yalow, 1966) and LH-RH (Caraty *et al.*, 1980), for example. This profile, as well as the result obtained after enzymatic degradation of the peptide, demonstrates the suitability of the iodination procedure for obtaining monoiodo s-Gn-RH.

There is very little information concerning the activity of the Gn-RH system in the fish brain in relation to that of the pituitary gonadotropic function. Cytological studies have shown that some brain nuclei exhibit variations related to the reproductive cycle (Peter, 1970); nevertheless, there is no evidence that these nuclei contain Gn-RH. Several studies have also reported variations in the ultrastructural as-

pects of the neurosecretory fibers innervating the gonadotrophs at the time of ovulation (Ekengren and Terlou, 1978; Knowles and Vollrath, 1966). At the quantitative level, the only available data have been obtained using heterologous radioimmunoassay (Dufour *et al.*, 1982; Idler and Crim, 1981; Schreibman *et al.*, 1983), and there has been no direct and specific measurement of brain and pituitary Gn-RH correlated with GtH levels in fish. This work shows, for the first time, that during the periovulatory period, the slow, prolonged increases in blood and pituitary GtH levels leading to ovulation are associated with a similar increase in the total Gn-RH content of the brain and pituitary in brown trout, and especially in the brain, which contains twice as much as Gn-RH as the pituitary.

The variations in the pituitary are not totally parallel to those of the brain. There is a decrease in the pituitary Gn-RH content in fish undergoing maturation when estradiol levels are lowest (Breton *et al.*, 1983) and 17α -hydroxy 20β -dihydroprogesterone has just begun to increase (Fostier and Jalabert, 1982; Scott *et al.*, 1983), which could mean that estradiol exerts a negative feedback on both brain and pituitary Gn-RH with Gn-RH being released from the pituitary only when estradiol levels reach their minimum values. This decrease in pituitary Gn-RH content may be linked to the stimulation of GtH secretion to induce maturation. This hypothesis could explain the existence of a correlation between pituitary Gn-RH and plasma GtH during this event. It must be emphasized that it is only during this period that the plasma GtH secretion follows a circadian rhythm in the trout (Zohar *et al.*, 1982). Perhaps Gn-RH would follow the same rhythm. On the contrary, before and after ovulation, during which time GtH secretion is pulsatile (Zohar *et al.*, 1982), there is no correlation between pituitary Gn-RH and plasma GtH, although the pituitary Gn-RH is correlated with the pituitary GtH.

Although the brain Gn-RH content profile varies with the pituitary GtH content, these two parameters are not correlated as well as Gn-RH and plasma GtH, suggesting that the increase in brain Gn-RH is related to a Gn-RH system closely related with maturation and ovulation. Immunoreactive material was found in many areas of the central nervous system and in the hypothalamopituitary complex. It is clear that the measurement of the total brain Gn-RH content partially reflects possible variations in specialized regions concerned with maturation and ovulation (i.e., the exact role of the different Gn-RH systems in the fish brain is still unknown concerning their control of maturation-ovulation or the acquisition of a sexual behavior, for example). The use of immunocytochemical techniques will be very helpful in the future in recognizing the Gn-RH systems and differentiating between them for the measurement of their Gn-RH content during typical stages of the reproductive cycle. Nevertheless, in the absence of a portal system allowing the determination of the Gn-RH directly supplying the pituitary gland, and as a first approach, the measure of the pituitary Gn-RH content appears to be a good index of the gonadotropic-releasing activity reaching the gonadotrophs in the trout.

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