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Bioactive forms of gonadotropin releasing hormone in the brain of an Indian major carp, *Catla catla* (Ham.)

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Abstract. Two forms of biologically active gonadotropin releasing hormones were isolated from the hypothalami of *Catla catla*. Gonadotropin releasing hormone activity was studied *in vitro* using enzymatically dispersed carp pituitary cell incubation system. Gonadotropin released into the medium was measured by carp gonadotropin-radio immuno assay. Acetic acid extracted hypothalamic material was subjected to acetone fractionation. Among the three protein pellets obtained at different time periods (AC I, AC II and AC III), AC II exhibited the gonadotropin releasing hormone activity. Gel filtration of AC II through Sephadex G-25 column showed three protein peaks (SG I, SG II SG III) and only SG II demonstrated strong gonadotropin releasing hormone activity. Elution of SG II through FPLC Mono Q column (an anion exchanger) in NaCl gradient programme showed one unadsorbed (MQ I) and three adsorbed (MQ II, MQ III and MQ IV) protein peaks. MQ III, which was eluted with 51% NaCl, exhibited gonadotropin releasing hormone activity. Surprisingly, unadsorbed fractions, MQ I, also showed gonadotropin releasing hormone activity. MQ 1 was therefore subjected to FPLC Mono S (a cation exchanger) column chromatography where a highly active gonadotropin releasing hormone enriched peak, i.e., MS III, could be eluted with 45% NaCl. These findings show that *Catla catla* hypothalamus has two forms of gonadotropin releasing hormones one anionic (carp gonadotropin releasing hormone I) and another cationic (carp gonadotropin releasing hormone II). These two forms of gonadotropin releasing hormones were also active in heterologous carp species, rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*) and an exotic common carp (*Cyprinus carpio*). Combined activity of two forms of gonadotropin releasing hormones was significantly greater as compared to any of the single form.

Keywords. Two forms of GnRHs; carp gonadotropin; pituitary cell.

1. Introduction

It is well known that gonadotropin releasing hormone (GnRH) plays a key role in the reproduction of vertebrates. Since the discovery of its decapeptide structure in 1970s (Matsu *et al* 1971; Burgus *et al* 1972), only one form of GnRH has been identified in mammals which is still considered to be the sole hormone causing the release of gonadotropins from the pituitary. Later, different forms of immunoreactive GnRHs have been identified, two forms in chicken, chicken GnRH I (King and Miller 1982a,b; Miyamoto *et al* 1982), chicken GnRH II (Miyamoto *et al* 1983); one form in salmon (Sherwood *et al* 1983); one form in ratfish (Lovejoy *et al* 1991); two forms in dog fish (Lovejoy *et al* 1992) and three forms in lamprey (Sower *et al* 1993). Further, there exists considerable variations in

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Abbreviations used: GnRH, Gonadotropin releasing hormone; GtH, Gonadotropin; cGtH, carp GtH; MEM, minimum essential medium; mGnRH, mammalian GnRH.

GnRH molecular forms. Immunoreactivity was the main criterion in identifying GnRHs during chromatographic separation. Although large number of teleostean species are available in India, nothing is yet known about the molecular forms of GnRH in them due to the lack of sensitive assay for detecting GnRH during purification. We have recently developed a sensitive assay based on incubating the fish pituitary cells *in vitro*, where addition of GnRH releases gonadotropin (GtH) in a dose dependent manner (Jamaluddin *et al* 1989; Bhattacharya *et al* 1990). In the present paper we report isolation of two bioactive forms of GnRHs from an Indian major carp (*Catla catla*) hypothalami. Their biological activity was also tested in heterologous species.

Materials and methods

2.1 *Collection of carp hypothalami*

Hypothalami were collected from sexually mature male and female *C. catla* (40–50 cm in size, 1·5–2·0kg body weight) in prespawning and spawning (April to July) stages of their reproductive cycle. The hypothalami were immediately stored in glass vials placed on ice and then transferred to a deep freezer (– 20°C).

2.2 *Hormones*

Synthetic mammalian GnRH (LH-FSH-RH, NIH, Lot No. 21-103-DH, Batch No. 2) was a gift from the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK), Bethesda, Maryland, USA. Purified carp gonadotropin (cGtH) and its antisera (anti-cGtH) were prepared as described previously (Banerjee *et al* 1989; Manna *et al* 1989).

2.3 *Determination of GnRH activity*

GnRH activity was determined by following the procedure described earlier from this laboratory (Jamaluddin *et al* 1989; Bhattacharya *et al* 1990). Briefly, carp pituitary cells were disaggregated by using 0·05% trypsin, 0·3% collagenase plus 3% BSA in oxygenated Earle's minimum essential medium (MEM, GIBCO Laboratories, USA). The cells were harvested by centrifugation at 500 g for 10 min at 4°C and washed several times with MEM to remove enzymes used. Cells were resuspended in 1 ml of MEM containing penicillin (100 U/ml) and streptomycin (100μg/ml) and gassed with 95% oxygen and 5% CO_2. The viability of cells were examined by trypan blue dye exclusion method. After counting the cells they were diluted with MEM and about 6×10^4 cells were taken in each well (microwell module, NUNC, Denmark, volume 400 μl/well) for incubation. Pituitary cells (6×10^4 cells/well) were incubated for 3 h without hormones (preincubation time) and continued for another 2 h with test materials at 30°C, hence total incubation time was 5 h. All pituitary cell incubation, contained 2 mM Ca^{2+} as GnRH requires extracellular Ca^{2+} to release GtH from the fish pituitary cells (Jamaluddin *et al* 1989; Bhattacharya *et al* 1990; Mukhopadhyay *et al* 1994, 1995). At the end of the incubation, the cells were pelleted by centrifugation at 500 g and medium was

collected from each well, stored at – 20°C for the determination of amount of GtH released with the help of carp GtH-radioimmuno assay (RIA) developed earlier in this laboratory (Manna *et al* 1989).

2.4 *Extraction and solvent fractionation*

The pooled frozen hypothalami was homogenized and extracted with 1 N acetic acid. The homogenate was passed through double folded cheese cloth to remove fat and cell debris. This was then subjected to chilled acetone fractionation. Three protein fractions – AC I, AC II and AC III were obtained. Of these only AC II fraction exhibited GnRH activity.

2.5 *Gel filtration*

AC II fraction was filtered through a Sephadex G-25 (Pharmacia, Sweden) column (2 × 36 cm) and was eluted with 0·05 M phosphate buffer saline (pH 7·4, 0·6% NaCl) at a flow rate of 15 ml/h. The elution profile of synthetic mammalian GnRH (mGnRH) was also obtained through this column with similar condition to identify the zone of decapeptide elution. Eluted fractions of AC II corresponding to the mGnRH peak were collected, pooled and lyophilized. Three protein peaks obtained in gel filtration were examined for GnRH activity. Sephadex G-25 peak II (SG II) fractions demonstrated GnRH activity.

2.6 *Fast performance liquid chromatography*

Fast performance liquid chromatographic system (FPLC, Pharmacia, Sweden) was used with Mono Q (HR 5/5) an anion exchanger column, and Mono S (HR 5/5) a cation exchanger column to further purify carp GnRH. Mono Q column was equilibrated by using 5 ml of start buffer (buffer A) in 20 mM TRIS, pH 8·0. SG II fraction was injected into the column. The column was eluted with 10 ml of eluting buffer (buffer B) containing 20 mM TRIS, pH 8·0 with 1 M NaCl and finally washed with buffer A until the base line of the recorder was stable. The gradient programme was started with 4 ml of buffer A when buffer B was 0%, for next 8 ml, B was 0% to 80% by continuous mixing with buffer A with a flow rate of 0·5 ml/min and chart speed of 0·5 cm/min. Hence for each run the total elution time and volume was 24 min and 12 ml respectively. Fractions were collected in an automatic fraction collector (1 ml/tube). Peaks obtained from eluted fractions were collected separately, pooled and lyophilized. GnRH activity of each pooled fractions was then determined. Mono Q chromatography provided four protein peaks, one unadsorbed (MQ I) and three adsorbed (MQ II, III, IV). MQ I and MQ III showed GnRH activity.

MQ I, the unadsorbed protein peak indicated the elution of large amount of protein. Lyophilized MQ I was therefore redissolved in ammonium acetate buffer (50 mM, pH 4 8) and passed through Mono S cation exchanger column. Column was equilibrated with buffer A containing 50 mM ammonium acetate, pH 4·8. MQ I material was injected and eluted with buffer B (buffer A plus 1 M NaCl) and finally washed with buffer A. The gradient programme was started with 5 ml of

buffer A when buffer B was 0%, for the next 10 ml it was 0 to 80% buffer B by continuous mixing with buffer A. The flow rate was 0·5 ml/min and chart speed 0·5 cm/min. Mono S elution provided one unadsorbed (MS I) and three adsorbed (MS II, III, IV) protein peaks, which were then examined for GnRH activity.

2.7 *Measurement of proteins*

The protein content of each chromatographic fraction was determined by measuring the absorbance at 280 nm in a UV spectrophotometer (Shimadzu, Japan). The protein of pooled fractions was estimated following the method of Lowry *et al* (1951), taking bovine serum albumin as standard.

2.8 *Statistical analysis*

Data were analysed by one-way analysis of variance (ANOVA); where the F value indicated significance, means were compared by a post hoc multiple range test. All values are expressed as mean ± standard error of the mean.

3. Results

Acetone fractionation of hypothalamic extract gave three protein fractions at different time level i.e., 15, 30 and 180 min. Figure 1 shows that only AC II fraction had

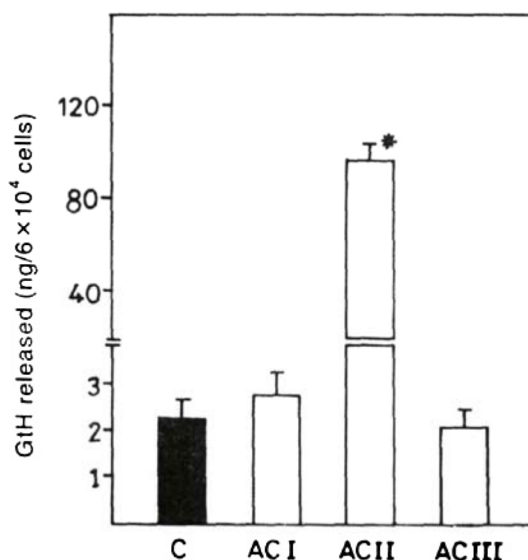

Figure 1. Examination of GnRH activity in the pellets of acetone fractionation.

100 μg protein from each pellet was added to pituitary cell incubation, except the control (C) where equal amount of medium was added as vehicle. After terminating the incubation, medium was collected and subjected to GtH RIA. Each value is the mean ± SE of five determinations.

*P<0·01 as compared to AC I, AC III and control.

GnRH activity. AC II fraction was gel filtered on Sephadex G-25 column. Elution profile clearly indicated three protein peaks, SG I, SG II and SG III. Elution of mammalian synthetic GnRH gave a peak corresponding to the SG II region. This suggests SG II to be a decapeptide related peptide (figure 2). SG II, which exhibited GnRH activity (table 1), was therefore subjected to FPLC Mono Q column chromatography. Figure 3 shows that there was one unadsorbed peak (MQ I) and three adsorbed peaks (MQ II, MQ III and MQ IV) which were eluted by NaCl gradient. MQ III, which exhibited GnRH activity (table 2), was eluted with 51% NaCl. Since MQ I, the unadsorbed peak, also contained appreciable GnRH activity (table 2), it was pooled, lyophilized to reduce the volume and subjected to FPLC Mono S column chromatography. Figure 4 depicts that there were four peaks, one unadsorbed (MS I) and three adsorbed (MS II, MS III and MS IV) peaks which were eluted by NaCl gradient. MS III had the GnRH activity (table 3) and this was eluted with 45% NaCl. MQ III was designated as carp GnRH I and MS III as carp GnRH II.

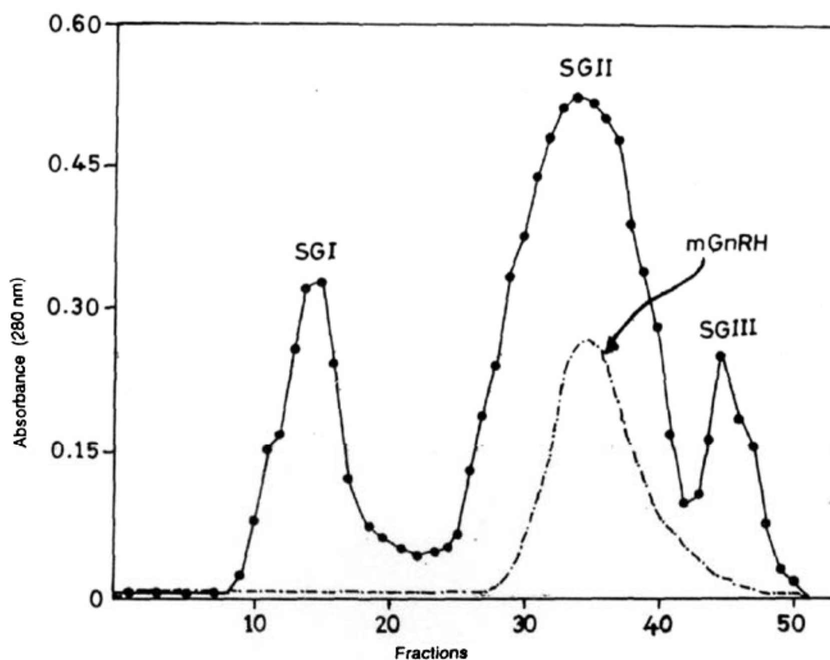

Figure 2. Gel Filtration of AC II fraction through Sephadex G-25 column. Fractions 11 to 16 from SG I, 29 to 40 from SG II and 45 to 48 from SG III peaks were separately pooled, lyophilized and tested for GnRH activity.

We have therefore obtained two forms of bioactive GnRHs, one anionic (carp GnRH I) and the other cationic (carp GnRH II) in nature. These two forms of GnRHs were tested in the *in vitro* pituitary cell incubation obtained from different species of carps and table 4 demonstrates that both forms were active in all the carps. Carp GnRH II was found to be more active as compared to carp GnRH I.

Table 1. GnRH activity of different peaks obtained from gel filtration through Sephadex G-25 column.

Fractions	GtH released (ng/6 × 10^4 cells)
Control (C)	2·4 ± 0·21
C + SG I	2·9 ± 0·22
C + SG II	32·3 ± 4·28*
C + SG III	2·3 ± 0·26

10 µg of protein from SG I or SG II or SG III was added to the pituitary cell incubation. Control (C) had equal amount of medium as vehicle. Each value is the mean ± SE of five determinations.
*P < 0·01 as compared to SG I or SG III or control.

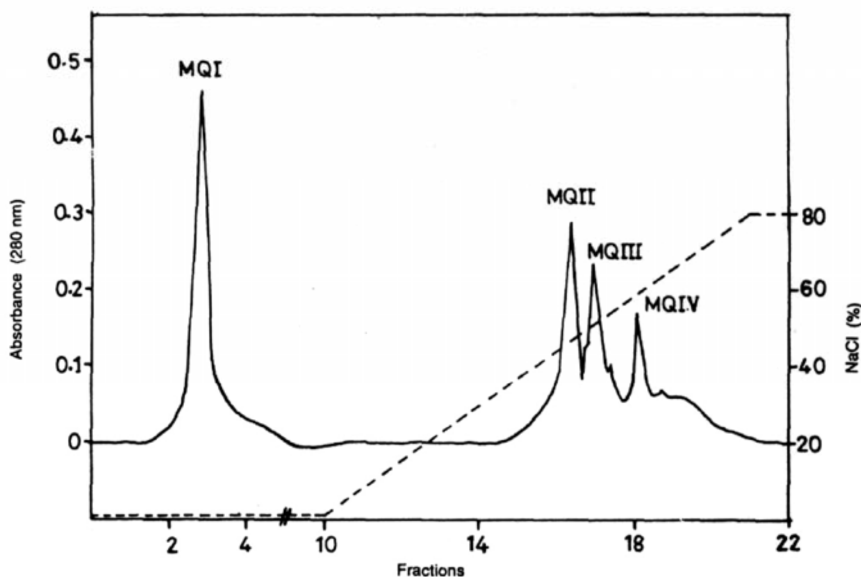

Figure 3. Chromatography of SG II pooled fractions on FPLC Mono Q Column with NaCl gradient programme. Fractions 3 (MQ I), 16 (MQ II), 17 (MQ III) and 18 (MQ IV) were separately collected, volume in each case was reduced by lyophilization and examined for GnRH activity.

Table 5 shows the comparison between combined effect of two forms and any one of the single form. There was statistically significant increase (P<0·01) in GtH release by combined carp GnRH I plus II as compared to only carp GnRH I or carp GnRH II. Equal amount of ovaprim although effected a significantly higher release of GtH in comparison to control but its activity was less than half as compared to carp GnRH I or carp GnRH II or their combinations. Synthetic mGnRH although stimulated GtH release as compared to control, its effect was significantly less (P <0·01) than that of carp GnRH I or II.

Table 2. Examination of GnRH activity in pooled fractions under different peaks eluted from Mono Q column.

Fractions	GtH released (ng/6 × 10^4cells)
Control (C)	2·3 ± 0·16
C + MQ I	48·2 ± 1·15
C + MQ II	2·6 ± 0·25
C + MQ III	334·5 ± 3·83*
C + MQ IV	2·4 ± 0·31

10µg protein from each Mono Q peak was added to test GnRH activity. Control (C) had equal amount of medium as vehicle. Each value is the mean ± SE of five determinations.
**P*< 0·01 in comparison to MQ I or MQ II or MQ IV or control.

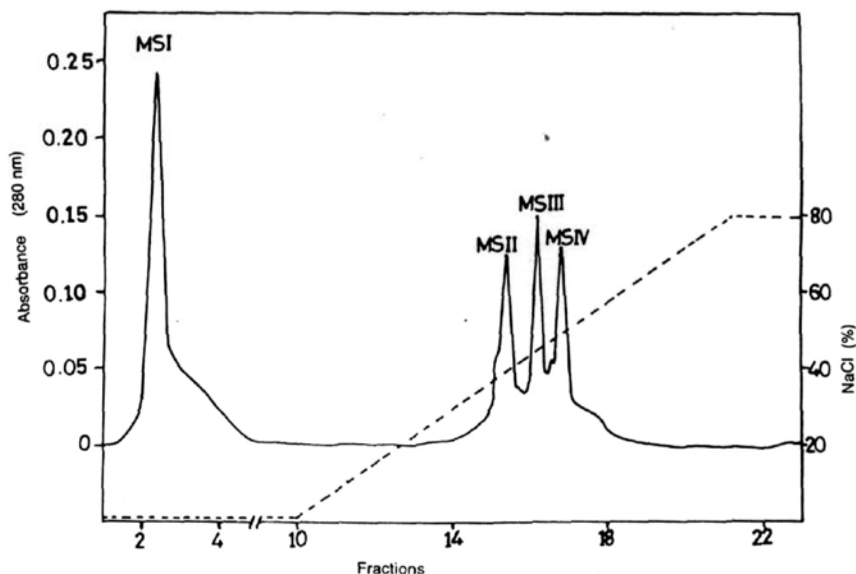

Figure 4. FPLC Mono S column chromatography of MQ I peak with NaCl gradient programme. Fractions 3 (MS I), 15 (MS III), 16 (MS III) and 17 (MS IV) were separately collected, volume was reduced by lyophilization and examined for GnRH bioactivity.

4. Discussion

Present study clearly shows the existence of two bioactive forms of GnRHs in *C. catla*. Both Mono Q and Mono S forms are more than 90% pure as compared to the crude material. Enzymatic disintegration of *Catla* pituitary cells and their *in vitro* incubation was a convenient and reliable system to detect GnRH activity. Prior to examining the GnRH activity of the test materials, the pituitary cell *in vitro*

Table 3. GnRH activity of pooled fractions under different peaks eluted through Mono S column.

Fractions	GtH released (ng/6 × 10^4 cells)
Control (C)	2·25 ± 0·12
C + MS I	2·2 ± 0·19
C + MS II	3·9 ± 0·24
C + MS III	378·8 ± 4·59*
C + MS IV	2·3 ± 0·09

10µg protein from each Mono S peak was added to examine GnRH activity. Control (C) had equal amount of medium as vehicle. Each value is the mean ± SE of five determinations.
* $P < 0{\cdot}01$ as compared to MS I or MS II or MS IV or control.

Table 4. Effect of carp GnRH I and II on heterologous carp pituitaries.

Carp species	Incubations	GtH released (ng/6 × 10^4 cells)
Cyprinus carpio	Control (C)	2·6 ± 0·19
	C + carp GnRH I	249·6 ± 3·69*
	C + carp GnRH II	316·6 ± 4·46**
Labeo rohita	Control (C)	2·2 ± 0·09
	C + carp GnRH I	232 ± 3·74*
	C + carp GnRH II	278·4 ± 2·87**
Cirrhinus mrigala	Control (C)	2·0 ± 0·13
	C + carp GnRH I	204·8 ± 1·96*
	C + carp GnRH II	273·4 ± 2·53**
Catla catla	Control (C)	2·3 ± 0·21
	C + carp GnRH I	318·0 ± 2·05*
	C + carp GnRH II	343·8 ± 2·58**

10 µg of carp GnRH I or carp GnRH II was added to each pituitary cell incubation. Control (C) had equal amount of medium as vehicle. Each value is the mean ± SE of 5, determinations.
*$P < 0{\cdot}01$ as compared to control.
**$P < 0{\cdot}0\ 1$ as compared to carp GnRH I.

system was checked by synthetic mammalian GnRH which provided a clear linear response in GtH release in a dose dependent manner (data not shown). Fish brain GnRH has not been purified so far by utilizing its biological activity. However, immunoreactive GnRH has been isolated from cod (Barnett *et al* 1982), salmon (Sherwood *et al* 1983), milkfish and rainbow trout (Sherwood *et al* 1984), catfish (Sherwood *et al* 1989), ratfish (Lovejoy *et al* 1991) and dogfish (Lovejoy *et al* 1992). Majority of the above mentioned fish brain GnRH is of one form except catfish (Sherwood *et al* 1989) and dogfish (Lovejoy *et al* 1992) where two forms

Table 5. Comparison of biological activity of carp GnRH I and II with ovaprim and mammalian GnRH.

Incubations	GtH released (ng/6 × 10^4 cells)
Control (C)	2·3 ± 0·13
C + carp GnRH I	329·6 ± 6·86*
C + carp GnRH II	370·8 ± 2·68*
C + carp GnRH I + carp GnRH II	394 ± 6·96**
C + ovaprim	186·5 ± 3·16***
C + mGnRH	20·13 ± 2·22***

10 µg of GnRH was added to each incubation. Control (C) had equal amount of medium as vehicle. 10 µg protein from ovaprim (Syndel Laboratories, Canada) and 10 µg of synthetic mammalian GnRH (mGnRH) was added to pituitary cell incubation separately for comparison. To observe the combined effect, carp GnRH I 5 µg and carp GnRH II 5 µg (total 10 µg) was added to the cell incubation. Each value is the mean± SE of five determinations.
*$P < 0{\cdot}01$ as compared to control.
**$P < 0{\cdot}0l$ as compared to carp GnRH I or II.
***$P < 0{\cdot}001$ as compared to carp GnRH I or II.

of GnRHs could be identified by immunological method. Lamprey brain contains three forms of immunoreactive GnRHs (Sower *et al* 1993).

There is no information on the forms of GnRHs in Indian teleosts. This is a first attempt to isolate GnRH from an Indian major carp brain using a bioassay method, a technique not applied so far for isolating fish GnRH. Isolation of GnRH by using gel filtration through Sephadex G-25 has been found to be very effective in cod (Barnett *et al* 1982) and salmon (Sherwood *et al* 1983). Gel filtration of *Catla* AC II eliminated 64% contaminating proteins. Sephadex G-25 chromatography of synthetic mammalian GnRH clearly corresponded to carp GnRH enriched peak, suggesting similarity in molecular size. Till this point carp GnRH appears to be of single form.

Ion exchange chromatography in FPLC Mono Q and Mono S distinctly separated two forms of carp GnRHs—anionic, carp GnRH I and cationic, carp GnRH II In the present assay system carp GnRH II showed greater activity than that of carp GnRH I. Combinations of these two forms caused higher release of GtH as compared to either carp GnRH I or carp GnRH II. This indicates the possibility of two different classes of receptors in the pituitary cells for carp GnRH I and carp GnRH II. Ovaprim, marketed by Glaxo India and manufactured by Syndel Laboratories Ltd., Canada is a salmon GnRH analogue, is used to induce maturation and breeding of Indian major carps and other fishes. Its efficacy was also examined in the present assay system to compare with the two forms of carp GnRHs. Release of GtH from the pituitary cells was less than half by ovaprim as compared to carp GnRH I or II. This suggests that carp GnRH forms are different than salmon GnRH. Both these forms caused remarkable amount of GtH release from rohu, mrigal and common carp pituitary cells as well. In these cases also carp GnRH II

exhibited higher activity than carp GnRH I. The present study demonstrates that two bioactive forms of GnRH molecules exist in the brain of *C. catla* and that both exhibit strong biological activity in homologous as well as heterologous bioassay systems.

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