Periovulatory changes in hypothalamic and pituitary monoamines following GnRH analogue treatment in the catfish *Heteropneustes fossilis*: a study correlating changes in plasma hormone profiles

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Abstract

In *Heteropneustes fossilis*, administration of a single dose (0.15 µg/g body weight, i.p.) of [D-Ala⁶, Gly¹⁰]-gonadotrophin-releasing hormone analogue (GnRHa) induced ovulation (in 35 of 35 fish) when mild-stripped at 16 h. Plasma gonadotrophin II (GTH II) levels showed a highly significant increase at 2, 4, 8, 12 and 16 h with the peak at 8 h. Plasma cortisol, progesterone and testosterone showed significant elevations at 2, 4, 8 and 12 h with peaks at 8 h (cortisol and testosterone) and 4 h (progesterone). The levels declined to control values at 16 and 48 h except that of testosterone which decreased even further. In contrast, plasma levels of oestradiol-17β decreased significantly at 2, 4, 8 and 12 h, with the lowest value at 8 h, but increased at 16 and 18 h. The contents of hypothalamic and pituitary serotonin and noradrenaline increased at 8 h, coinciding with the peak GTH II rise, and decreased at 16 h. In contrast, dopamine content

declined at 8 h in both the hypothalamus and pituitary, but increased at 16 h only in the hypothalamus. The hypothalamic adrenaline level decreased at 8 h but increased significantly at 16 h. Hypothalamic levels of monoamine oxidase, catechol O-methyltransferase and dopamine β-hydroxylase were elevated significantly at 8 h; the dopamine β-hydroxylase activity decreased at 16 h. Phenylethanolamine N-methyltransferase activity was elevated only at 16 h, coinciding with the rise in adrenaline content. It is inferred that the preovulatory decrease in dopamine content concomitant with rises in serotonin and noradrenaline levels, triggered by the low titre of oestradiol, might have potentiated the GnRHa/GnRH (endogenous)-induced release of GTH II for a prolonged period.

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Introduction

In teleosts, hypothalamic monoamines (dopamine (DA), noradrenaline (NA) and serotonin (5-HT)) exert a stimulatory or inhibitory effect on gonadotrophin II (GTH II) secretion (Peter *et al.* 1991). DA is the most dominant inhibitory mechanism that inhibits basal or gonadotrophin-releasing hormone (GnRH)-induced GTH II release in several species including the catfish (Chang & Peter 1983, de Leeuw *et al.* 1987, Goos *et al.* 1987, Trudeau & Peter 1995, Senthilkumaran & Joy 1996). NA and 5-HT have been shown to facilitate GTH II release (Somoza & Peter 1991, Khan & Thomas 1992, Yu & Peter 1992, Trudeau & Peter 1995, Senthilkumaran & Joy 1996). The use of GnRH and its superactive analogues alone or in combination with DA receptor antagonists has become the basis of a simple and reliable method of induced breeding in teleosts (Peter *et al.* 1991, Yaron 1995). However, apparent seasonal or species variations with regard to dosage of GnRH, degree of DA inhibition of GTH II release, latency period of spawning response, etc. have

been found in spawning trials (Peter *et al.* 1991, Yaron 1995, Tharakan & Joy 1996). Tharakan & Joy (1996) have shown that a single injection of 0.5 µg/g body weight [D-Ala⁶-Gly¹⁰]-gonadotrophin-releasing hormone analogue (GnRHa) could induce ovulation in the catfish *Heteropneustes fossilis* without the use of a DA antagonist. In such instances, the question arises how the exogenously administered GnRHa overcomes the endogenous DA inhibition of the preovulatory GTH II surge and whether the treatment triggers endogenous mechanism(s) controlling GTH II secretion as well. In the present study, we report the periovulatory changes in hypothalamic and pituitary monoamine metabolism in catfish induced to ovulate by GnRHa treatment, and correlate them with plasma levels of GTH II, sex steroids and cortisol.

Materials and Methods

Adult mature female catfish weighing 90–100 g were purchased from local fish markets in and around Varanasi

in the first week of July (early spawning phase). They were checked for spontaneous ovulation. About 110 females were injected with 0.15 µg/g body weight GnRHa (Sigma, St Louis, MO, USA) i.p. in the evening (18 h). This dose was selected because, in pilot dose-determination studies, it could induce a high rate of ovulation in this species. As controls, 110 fish were given 0.1 ml vehicle (0.7% NaCl containing 0.25% BSA, fraction V; Sigma). Fish in the GnRHa and control groups were divided into seven batches of five and three batches of 25 each. The seven batches of fish from treated and control groups were bled at 0, 2, 4, 8, 12, 16 or 48 h. Each batch of fish was used only once for blood collection to avoid stress. Blood was drawn from the caudal vein using a heparinized syringe with a 22-gauge needle. Plasma was separated by centrifuging at 800 *g* for 20 min at 4 °C. They were checked for ovulation 16 h after injection by mild hand-stripping. Fish that yielded a copious stream of translucent green-brown eggs were rated as having ovulated and scored individually. Twenty-five fish from each of the remaining batches were killed at 0, 8 and 16 h (after stripping of eggs) for the estimation of monoamines and related enzymes. Brains were quickly dissected out on ice and the hypothalami were separated, as described previously (Senthilkumaran & Joy 1993), and stored at -20 °C for 2–3 days. Pituitaries were similarly removed and stored at -20 °C for estimation of amine contents. Blood had not been previously withdrawn from these fish to avoid stress.

RIA of GTH II

Plasma GTH II levels were estimated by a heterologous assay (Senthilkumaran & Joy 1994) following the procedure of Goos *et al.* (1986). Intra- and interassay coefficients of variation were 5.2 and 8.9% respectively. The minimum sensitivity limit of the assay was 800 pg/ml. Other details of the assay were as described previously. Since only a single gonadotrophin corresponding to GTH II of other teleosts is present in catfish (Koide *et al.* 1992), we use GTH II to describe the GTH measured in this study.

RIA of testosterone, oestradiol-17β (OE₂) and progesterone

Plasma levels of steroid hormones were measured by the RIA method of Abraham (1974). Details of testosterone and OE₂ assays were as described previously (Senthilkumaran & Joy 1994). The coefficients of intra- and interassay variation for the OE₂ assay were 1.5 and 2.9% respectively. The progesterone assay was performed using [1,2,6,7-³H]progesterone (specific radioactivity 85 Ci/mmol; ICN, CA, USA) and its antiserum (ICN). The assay procedure was similar to that of OE₂, as described previously (Senthilkumaran & Joy 1994). The cross-reactivity of the antibody was 3% with 17α-hydroxyprogesterone and 1% with 17α,20β-

dihydroxyprogesterone (17α,20β-P). The sensitivity limit of the assay was 10 pg/ml. The intra- and interassay coefficients of variation were 3.5 and 4.7% respectively.

Enzyme immunoassay (EIA) of plasma cortisol

Plasma cortisol was estimated by the double-antibody EIA method of Shrivastav *et al.* (1988). The details are as follows. Cortisol EIA kit was a gift from Dr G L Kumari, NIHFW, New Delhi, India. Cortisol-21-hemisuccinate was coupled with penicillinase by the carbodi-imide method of Mattox *et al.* (1979), as described by Shrivastav *et al.* (1988). Different concentrations of the standard (10–2000 pg/100 µl in 10 mM sodium phosphate buffer (pH 7.0) containing 154 mM NaCl (PBS), 15 mM sodium azide and 1 g/l gelatin or BSA (assay buffer)) and 10 µl of the reconstituted plasma (extracted with ethanol, dried and reconstituted in PBS) were assayed in duplicate. Cortisol antibody (100 µl at 1:4000 dilution in the assay buffer containing normal rabbit serum (1:500 dilution)) and 100 µl of the enzyme conjugate (1:600 dilution) were mixed and incubated at 4 °C overnight. Anti-rabbit IgG (1:80 dilution in 200 µl of assay buffer) was added and incubated for 2 h at 37 °C. After the incubation, 30 µl celite solution (1 g/l in assay buffer) and 1 ml PBS were added to the tubes. They were centrifuged at 800 *g* for 10 min and the pellets were washed twice with 2 ml PBS. The process was repeated and the supernatant discarded. The sides of the tubes were wiped off; 200 µl freshly prepared 1 M penicillin V (substrate) in PBS was then added to each tube and the mixture vortex-mixed thoroughly. They were again incubated at 37 °C for 1 h. At the end of the incubation, 2 ml starch-iodine solution (prepared by mixing with constant stirring 0.25 ml 18 mM iodine in 3.325 M potassium iodide in distilled water with 190 ml 0.2 M PBS, containing 3 ml 20 g/l hydrolysed starch) were added to each tube and the enzymatic reaction was stopped within 10 min by adding 1 ml 5 M HCl. The end point of the reaction was the appearance of blue colour which was read at 620 nm in a Kontron spectrophotometer. The sensitivity limit of the assay was 10 pg/ml. In our assay system, the cross-reactivity of the antibody was <0.7% for progesterone, 5% for 17α-hydroxyprogesterone, 22% for cortisone, 3% for corticosterone, <0.5% for testosterone and <0.5% for OE₂. The intra- and interassay coefficients of variation were 7.7 and 9.3% respectively. In order to evaluate further the specificity of the assay, plasma from a group of five fish given metopirone (2 µg/g body weight i.p. for 7 days) was used for the cortisol assay. The control fish received 0.6% NaCl. The treatment caused a significant reduction of cortisol (about 56%) when compared with that of the control.

Estimation of hypothalamic and pituitary monoamines

5-HT was assayed by the spectrofluorimetric method of Snyder *et al.* (1965). For assay of pituitary 5-HT, three

pituitaries were pooled to make a sample. Catecholamine contents were measured radioenzymatically by the method of Ben-Jonathan & Porter (1976) as modified by them later (1981, personal communication). The details of both the assays including validation were explained previously (Senthilkumaran & Joy 1994, 1995). For assay of catecholamines in the pituitary, two pituitaries were pooled to make a sample.

Estimation of monoamine oxidase (MAO), catechol O-methyltransferase (COMT), phenylethanolamine N-methyltransferase (PNMT) and dopamine β -hydroxylase (DBH)

Hypothalamic MAO, COMT and PNMT were measured by the radioisotopic method of Parvez & Parvez (1973). DBH was assayed by the coupled radiometric procedure of Coyle & Axelrod (1972). The details of these assays including validation were described previously (Senthilkumaran & Joy 1994, 1995).

Statistical Analysis

All data are expressed as mean $\pm$ S.E.M. The data for monoamines and related enzymes in the GnRHa and control groups were tested for significance by Student's *t*-test. The data for plasma hormone levels were analysed by a two-way ANOVA followed by Newman-Keuls' multiple range test.

Results

Ovulation

Of 35 fish checked for ovulation in the GnRHa group, all ovulated upon mild stripping at 16 h. Even handling and holding the fish 12 h after the GnRHa injection led to extrusion of eggs from the ovipore. No fish in the control group ovulated even after hard stripping.

Changes in plasma GTH II and steroid hormone levels (Fig. 1)

Plasma GTH II (Fig. 1A) showed a highly significant elevation at all durations up to 16 h with the peak at 8 h (Newman-Keuls' test, $P<0.05$). The GTH II level was low at 48 h similar to that of the control group. A two-way ANOVA of the data demonstrated a highly significant effect of GnRHa treatment ($F=31187.6$, $P<0.001$), duration ($F=3095.3$, $P<0.001$) and interaction between these two factors ($F=3059$, $P<0.001$). Cortisol levels (Fig. 1B) were significantly increased at 2, 4, 8 and 12 h of the GnRHa treatment with maximum elevation at 8 h, and declined significantly at 16 and 48 h (Newman-Keuls'

test, $P<0.05$). A two-way ANOVA of the data showed a significant effect of the treatment ($F=100.8$, $P<0.001$), duration ($F=23.8$, $P<0.001$) and interaction between these two factors ($F=13.5$, $P<0.001$). According to the Newman-Keuls' analysis, the levels at 2, 4 and 12 h did not vary significantly among themselves in the GnRHa group. Plasma progesterone and testosterone levels (Figs 1C and E) were elevated significantly after the GnRHa treatment at 2, 4, 8 and 12 h with the peak increases at 4 and 8 h respectively. The testosterone level declined significantly at 16 and 48 h (Newman-Keuls' test, $P<0.05$). A two-way ANOVA of the data showed a significant effect of the treatment (testosterone, $F=7687.4$, $P<0.001$; progesterone, $F=3578.6$, $P<0.001$), duration (testosterone, $F=1268.5$, $P<0.001$; progesterone, $F=1565.3$, $P<0.001$) and interaction between these two factors (testosterone, $F=3787.9$, $P<0.001$; progesterone, $F=1061.7$, $P<0.001$). Plasma OE₂ levels (Fig. 1D) declined significantly in the GnRHa groups at 2, 4, 8 and 12 h with the greatest reduction at 8 h (Newman-Keuls' test, $P<0.05$). However, a sharp and significant increase was noted at 16 and 48 h (Newman-Keuls' test, $P<0.01$). A two-way ANOVA of the data showed a significant effect of the treatment ($F=1467.3$, $P<0.001$), duration ($F=1294.4$, $P<0.001$) and interaction between these two factors ($F=1221.1$, $P<0.001$).

Changes in hypothalamic and pituitary monoamine contents (Figs 2 and 3)

5-HT contents in the hypothalamus and pituitary were elevated significantly at 8 h ($P<0.01$; $P<0.001$) and declined to control levels 16 h after the GnRHa administration. The NA contents also increased in both ($P<0.01$) at 8 h. In the hypothalamus, the NA content at 16 h was significantly lower than the control level ($P<0.001$). The DA content showed a significant reduction at 8 h in both the hypothalamus and pituitary ($P<0.01$). At 16 h the hypothalamic DA content showed a significant increase ($P<0.01$) but the pituitary content was significantly lower than the control group ($P<0.01$). The concentration of hypothalamic adrenaline, on the other hand, did not differ significantly at 8 h but registered a significant rise at 16 h ($P<0.01$). In the pituitary, adrenaline was not detectable.

Changes in hypothalamic MAO, COMT, PNMT and DBH activities (Fig. 4)

In the GnRHa group, activities of MAO, COMT and DBH were significantly elevated ($P<0.05$, $P<0.001$, Student's *t*-test) at 8 h and declined to control levels at 16 h, except DBH activity which was decreased below the control value ($P<0.001$). Conversely, hypothalamic PNMT activity did not vary significantly at 8 h but increased at 16 h after the treatment ($P<0.05$).

Figure 1 Periovulatory effects of a single injection of a mammalian GnRH (0.15 µg/g body weight) on plasma levels of gonadotrophin (A), cortisol (B), progesterone (C), oestradiol-17β (D) and testosterone (E) in the catfish *Heteropneustes fossilis* (means ± S.E.M.; $n=5$; two-way ANOVA–Newman–Keuls' test).

Discussion

The present data show that a single injection of 0.15 µg/g body weight of GnRH can cause a very high rate of ovulation in the catfish after a latent period of 16 h. The injected dose was only three times higher than that used normally with a DA antagonist to induce ovulation in catfish species (de Leeuw *et al.* 1985, Goos *et al.* 1987, Manickam & Joy 1989, Tharakan & Joy 1996). The GnRH treatment resulted in a sustained and significant increase in plasma GTH II for 16 h with the peak at 8 h. The prolonged action of GnRH analogues has usually been attributed to their long half-life and increased receptor affinity and/or occupancy (Van der Kraak *et al.* 1983).

However, the roles of other factors such as changes in endogenous neuroendocrine mechanisms have not been examined in such studies. It has been shown that DA is a GTH II-release inhibitor, but its effect varies considerably among species: strong, moderate or no effect (Peter *et al.* 1991, Yaron 1995). In *H. fossilis*, hypothalamic DA content and turnover have been reported to be lowest in the spawning phase, implying a toning down of the DA-inhibitory mechanism at this period (Senthilkumaran & Joy 1995, control data). Nevertheless, spontaneous spawning does not occur in the catfish, which means that the residual DA activity seems to prevent the GTH II surge and spawning. During the GnRH-induced ovulation, profound changes occurred in both hypothalamic

Figure 2 Periovulatory effects of a single injection of GnRHα on hypothalamic 5-HT, DA, NA and adrenaline (A) contents in the catfish *Heteropneustes fossilis* (means $\pm$ S.E.M.; $n=5$). Comparisons with respective vehicle control: ** $P<0.01$, *** $P<0.001$; Student's t -test.

Figure 3 Periovulatory effects of a single injection of GnRHα on pituitary levels of 5-HT, DA and NA (means $\pm$ S.E.M.; $n=5$). Comparisons with respective vehicle control groups: ** $P<0.01$, *** $P<0.001$; Student's t -test.

and pituitary monoamine metabolism. These changes were (1) depression of DA content at 8 h with concurrent elevation of 5-HT and NA contents, (2) increase in DA and decrease in NA contents at 16 h after extrusion of eggs, and (3) decrease in adrenaline at 8 h and subsequent increase at 16 h. Although turnover of the amines was not examined in this study, the time-course changes in the activities of related enzymes in the hypothalamus could be a pointer to the nature of the events. The decrease in the DA content with a simultaneous increase in NA and DBH at 8 h clearly indicate increased conversion of DA into NA, as evident from increased DBH activity. The decrease in the NA content at 16 h might be due to

decreased DA conversion, since DBH activity was lowered, or increased conversion of NA into adrenaline, since PNMT activity was elevated at this time point. The increased 5-HT content might be due to its increased synthesis. The elevated activity of the MA-degrading enzymes, MAO and COMT, at 8 h suggests increased degradation of the amines (increased neuronal activity). Thus, coincident with the GTH II surge at 8 h, there was a depression of the inhibitory DA mechanism and stimulation of the NA and 5-HT mechanisms, a combination found to favour GTH II secretion (Senthilkumaran & Joy 1996). The present study also reports the changes in pituitary amine contents in this species, which were

Figure 4 Periovulatory effects of a single injection of GnRHa on hypothalamic MAO, COMT, DBH and PNMT activities in the catfish *Heteropneustes fossilis* (means $\pm$ S.E.M.; $n=5$). Comparisons with respective vehicle control groups: * $P<0.05$, ** $P<0.001$; Student's t -test.

similar to those observed in the hypothalamus (in teleosts the hypothalamic monoamine neurons innervate the pituitary directly (Peter & Fryer 1983)). The DA content was still low at 16 h, unlike in the hypothalamus, and may explain the high level of GTH II at 16 h. It is interesting to note that adrenaline was not detected in the pituitary and may not have any hypophysiotrophic role. The increased level of adrenaline at 16 h in the hypothalamus may be part of a mechanism to remove the excess NA to restore GTH II secretion to control levels.

The factors involved in the changes in hypothalamic and pituitary monoamine during the GnRHa treatment need to be considered next. The changes in plasma levels of gonadal steroids give some indications. It is evident from the data that there was a clear shift in steroid metabolism during the periovulatory period. These changes are (1) significant decrease in plasma OE_2 at 2, 4, 8 and 12 h with the ebb at 8 h and its significant elevation after stripping of eggs at 16 and 48 h and (2) OE_2 maintained an inverse relation with the testosterone levels. The rate of aromatization may be altered to maintain this inverse relationship. The decreased response of OE_2 during the periovulatory period was similar to that reported in salmonids (Fostier & Jalabert 1982, Scott *et al.* 1983, Van der Kraak *et al.* 1984, Kime & Dolben 1985, Levavi-Zermansky & Yaron 1986, Liley *et al.* 1986), bitterling (Shimizu *et al.* 1985) and white sucker (Scott *et al.* 1984), which exerts a negative feedback effect on brain and pituitary GnRH activity during induction of spawning (Breton *et al.* 1986). In rainbow trout, during the periovulatory period coinciding with the low titre of OE_2 , a decrease in both hypothalamic and pituitary DA turnover and an increase in hypothalamic 5-HT turnover have been reported (Saligaut *et al.* 1992). It was further reported that

oestrogen receptors were expressed in a subset of tyrosine hydroxylase-positive (dopaminergic) neurons of the pre-optic region, thus participating directly in DA inhibition of GTH II release (Linard *et al.* 1996). Thus the fall in OE_2 removes the DA inhibition, allowing the preovulatory surge of GTH II. We have already demonstrated in the catfish that OE_2 mediates the negative feedback control of GTH II release by acting differentially on hypothalamic monoamine activity: DA activity is inhibited and NA and 5-HT activities enhanced, and vice versa (Senthilkumaran & Joy 1994, 1995). Such inverse relationships of DA with NA and 5-HT underlying GTH II secretion have been reported in the catfish under natural conditions (seasonal data) or after ovariectomy and replacement with various doses of OE_2 (references as above). In 3-week ovariectomized catfish (prespawning phase), OE_2 produced a steady and significant increase in DA content and turnover and significant decrease in NA and adrenaline contents and turnover in a dose-related manner; plasma GTH II level declined correspondingly (Senthilkumaran & Joy 1995). Therefore it is reasonable to assume that, during the periovulatory period, the low titre of OE_2 altered the monoaminergic activity differentially to stimulate endogenous GnRH secretion, which together with the exogenously administered GnRHa produced the sustained and significant increase in GTH II release for about 16 h. This endogenous inhibition of DA may explain the prolonged ability of GnRHa to induce ovulation without using a DA antagonist. The participation of other sex steroids that showed elevated titres in the activation of the GTH II regulatory mechanism is not clear. Dulka *et al.* (1992) have reported that $17\alpha,20\beta$ -P which acts as a pheromone in goldfish, lowered DA turnover in the pituitary, favouring GTH II release. However,

in the catfish, $17\alpha,20\beta$ -P is a poor odorant (K P Joy, B Tharakan & S B Deshpande, unpublished observations).

During the GnRH α -induced ovulation of the catfish, plasma levels of both cortisol and progesterone exhibited significant rises, with the cortisol pattern following closely the GTH II levels. The periovulatory rise in the cortisol level is probably not due to stress since the control fish were handled similarly at all time points. A periovulatory rise in cortisol has also been demonstrated in goldfish (Cook *et al.* 1980), brown trout (Pickering & Christie 1981), carp (Kime & Dolben 1985) and white sucker (Scott *et al.* 1984). The elevated cortisol level has been ascribed to stimulation of interrenal activity by GTH (Sundararaj & Goswami 1977). Barry *et al.* (1995) have reported that $17\alpha,20\beta$ -P could be a physiological regulator of cortisol secretion in periovulatory salmonids. However, the present data on changes in monoamine metabolism suggest a central effect on adrenocorticotrophin secretion to stimulate cortisol secretion. Unlike the GTH II- and cortisol-release patterns, a prominent 'surge' of progesterone secretion was not evident. This may be due to its rapid conversion into 17α -hydroxyprogesterone and $17\alpha,20\beta$ -P; the latter steroids were not monitored in this study because of technical constraints. $17\alpha,20\beta$ -P is considered to be the most active maturation-inducing hormone in several teleosts, including the catfish (Sundararaj *et al.* 1985, Nagahama 1991).

In conclusion, during the GnRH α -induced ovulation of the catfish, the changes in the steroid milieu, especially the decreased OE₂ level, caused significant changes in hypothalamic and pituitary monoamine contents, modulating GnRH-induced GTH II release for a prolonged period.

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