Effects of ovariectomy and oestradiol-17 β replacement on brain tyrosine hydroxylase in the catfish *Heteropneustes fossilis*: changes in *in vivo* activity and kinetic parameters

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Abstract

In *Heteropneustes fossilis*, ovariectomy inhibited *in vivo* brain (hypothalamus–pituitary, telencephalon and medulla oblongata) tyrosine hydroxylase (TH) activity with significant effects in weeks 2, 3, 4 and 5 of the gonadal resting phase and in weeks 3, 4 and 5 of the prespawning phase ($P < 0.05$, Tukey's test). Oestradiol-17 β (OE₂) replacement in 3-week ovariectomised fish produced biphasic responses in both seasons; the low dosages of 0.05 and 0.5 $\mu\text{g/g}$ body weight (BW) elevated TH activity, whereas the high dosages of 1.0 and 2.0 $\mu\text{g/g}$ BW decreased it. The magnitude of the inhibition was higher in the resting phase than in the prespawning phase. The inhibitory effect of ovariectomy may be produced by elevating the apparent K_m values (decreased affinity) of the enzyme for both L-tyrosine (substrate) and dimethyltetrahydropteridine (cofactor) and consequently decreasing the $V_{\max}$. Significant changes ($P < 0.05$) in both these parameters were noticed but showed minor differences

with regard to the length of ovariectomy, season or brain regions. The biphasic effects of OE₂ replacement on TH activity seemed to be produced by differential effects on apparent K_m and $V_{\max}$. The stimulatory effect of the low dosages of OE₂ coincides with a decrease in the apparent K_m values (increased affinity) for both substrate and cofactor and an increase in the $V_{\max}$ of the enzyme. The inhibitory effect of the high dosages of OE₂ correlated with an increase in the apparent K_m values (decreased affinity) for both substrate and cofactor, and a decrease in the $V_{\max}$ compared with the lower dosage groups. The results strongly suggested that OE₂ can modulate brain catecholaminergic activity at the level of tyrosine hydroxylation which, in turn, may alter gonadotrophin secretion. OE₂ may elicit biphasic effects by differentially altering the enzyme affinity towards the substrate and cofactor.

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Introduction

In teleosts, neuroanatomical studies have demonstrated an extensive distribution of monoamines in the brain with a predominant concentration of neuronal perikarya in the hypothalamus (Hornby & Piekut 1990, Ekstrom *et al.* 1995). A unique feature of this distribution is that the hypothalamic aminergic system forms an important component of the hypothalamo–hypophyseal system, the aminergic fibres making direct or indirect contacts with secretory cells in the pituitary. The functional significance of the aminergic innervation has been investigated in a number of teleosts and indicates its strong involvement in the regulation of various pituitary hormones (Olivereau & Olivereau 1999a,b). In the regulation of gonadotrophin (GTH) secretion, it has been well documented that dopamine (DA) inhibits and noradrenaline (NA) stimulates spontaneous and/or gonadotrophin-releasing hormone-induced GTH-II (luteinizing hormone; LH) release (Schulz *et al.* 1995, Peter & Yu 1997, Joy 1999). Catecho-

lamine (CA) involvement is also indicated by significant variations in activity during the reproductive cycle (Guerrero *et al.* 1990, Senthilkumaran & Joy 1995, Hernandez-Rauda *et al.* 1999). In *Heteropneustes fossilis*, the hypothalamic catecholamines DA, NA and adrenaline show differential activity during the reproductive cycle; DA which dominates in the gonadal quiescent phase is hydroxylated to yield NA which predominates in the early recrudescence phase (preparatory and prespawning phases) and the latter (NA) is N-methylated to adrenaline which dominates the late recrudescence (spawning) phase (Senthilkumaran & Joy 1995). The activity of the CA-synthesising enzymes, DA- β -hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT), closely parallels the CA seasonal pattern and is stimulated by long photoperiods and/or high temperature (Senthilkumaran & Joy 1995), implying environmental modulation of LH secretion through the CAs.

Among endogenous factors, gonadal steroids exert positive or negative feedback control on LH secretion in

teleosts (Goos *et al.* 1999). In the feedback control, it has been shown that the steroids interact at different enzyme-catalysed steps in CA synthesis and degradation, such as oxidative deamination (including serotonin deamination) by monoamine oxidase (Senthilkumaran & Joy 1994, 1995), O-methylation of CA by catechol-O-methyltransferase (Joy & Senthilkumaran 1998), β -hydroxylation of DA by D β H and N-methylation of NA by PNMT (Senthilkumaran & Joy 1995). In our laboratory, we used ovariectomised and oestradiol-17 β (OE₂)-replaced *Heteropneustes fossilis* as experimental models to study the steroid-monoamine interaction and feedback regulation of LH secretion (Senthilkumaran & Joy 1994, 1995).

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in CA synthesis (Nagatsu *et al.* 1964). Because of these features, as well as its multi-factorial regulation, including end-product feedback inhibition (Ames *et al.* 1978, Zigmond *et al.* 1989), measuring TH activity is a more sensitive index to assess CA neuronal activity than measuring the amines *per se*. A functional interaction between gonadal steroids and TH activity (vis-a-vis CA) has been reported in mammals in the context of LH and prolactin regulation (Beattie *et al.* 1972, Beattie & Soyka 1973, Babu & Vijayan 1984, Blum *et al.* 1987, Gonzales *et al.* 1989). In teleosts, studies on TH have been largely confined to its use as a marker enzyme to demonstrate the neuroanatomical distribution of the CA system (Hornby & Piekut 1990), the quantitative distribution of TH activity in different brain regions (Saligaut *et al.* 1993, Linard *et al.* 1996a) and cDNA cloning and sequencing of TH in rainbow trout (Linard *et al.* 1998). In rainbow trout, oestrogen receptor-binding neurones are mostly TH positive in the preoptic area which implies that oestrogen feedback sites are located on CA(DA)-ergic neurones (Linard *et al.* 1996b). Gonadectomy inhibits mRNA levels in TH neurones and OE₂ treatment partially reverses the effect (Vetillard *et al.* 1999). We have recently shown significant seasonal, sex and brain regional differences in TH with high activity in the gonadal recrudescence phase and stimulation of enzyme activity on exposure to long photoperiods and high temperature (Chaube & Joy 2002a,b). Detailed experimental studies on oestrogen regulation of TH activity are lacking in teleosts.

In the present study, we have demonstrated ovariectomy and OE₂ replacement-induced changes in TH activity and kinetics in different brain regions in gonadal quiescent and recrudescence phases.

Materials and Methods

Chemicals

Catalase, L-tyrosine, OE₂, 6-7-dimethyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine (DMPH₄), bovine serum albumin (BSA), Sephadex G-25 and 3-amino-

benzoic acid ethylester (MS222) were purchased from Sigma Chemicals, St Louis, MO, USA. Sodium molybdate, 2-mercaptoethanol, sodium nitrite and Folin-Ciocalteu reagent were purchased from E-Merck (Mumbai, India). Other reagent chemicals were purchased from Hi-media or BDH (Mumbai, India).

Collection and acclimatisation of fish

Heteropneustes fossilis is a freshwater air-breathing catfish whose reproductive cycle can be divided into five phases: resting (November–January), preparatory (February–April), prespawning (May–June), spawning (July–August) and post-spawning (September–October) phases. Fish were collected from local fish markets in Varanasi (resting and prespawning phases). They were maintained in flow-through aquarium tanks under a normal photoperiod and ambient temperature (resting phase: 10.5 h light:13.5 h darkness, 18 $\pm$ 2 °C; prespawning phase: 12.5 h light:11.5 h darkness, 28 $\pm$ 2 °C). They were fed minced goat liver daily. After 15 days of acclimatisation, the following experiments were conducted.

Ovariectomy

About 120 acclimated fish were ovariectomised and 100 fish were sham-ovariectomised in the resting (second week of December) and prespawning (second week of May) phases of the reproductive cycle. The fish were anaesthetised with MS222 (100 mg/250 ml distilled water) by spraying it over the gills. A 4 cm long mid-ventral incision was made anterior to the urogenital pore to expose the paired ovary. The ovaries were carefully detached from the peritoneal covering and removed. The cut end of the oviduct was cauterised with a hot needle to prevent regeneration and the incision sutured. The fish were treated with benzaniline penicillin (16 000 IU/l) for 3–5 days to prevent skin infection. For sham-ovariectomy, all the above steps were followed except that the ovaries were not removed. The operated fish were maintained for 1, 2, 3, 4 and 5 weeks. Mortality was negligible (<3%). Completeness of ovariectomy and regeneration of gonads, if any, were checked by examining the peritoneal cavity of the fish at the time of sampling. Only tissues from completely ovariectomised fish were used for enzyme assay.

OE₂ supplementation

Five 3-week ovariectomised fish in both the resting and the prespawning phases were given OE₂ intraperitoneally in dosages of 0.05, 0.5, 1.0 and 2.0 μ g/g body weight (BW) daily for 3 days. Five ovariectomised and five

Figure 1 A diagrammatic representation (sagittal view) of the brain of *Heteropneustes fossilis* showing the plane of cuts to separate various regions used in the study. A, telencephalon; B, hypothalamus; C, medulla oblongata; P, pituitary; OT, olfactory tract; ON, optic nerve.

sham-ovariectomised fish were given an equal volume (0.1 ml) of vehicle (propylene glycol) as controls.

Tissue sampling and processing

At the end of the experiments, the fish were killed by decapitation between 1100 and 1200 h. Brains, along with the pituitary, were dissected out immediately on ice. Telencephalon (excluding olfactory tract and bulb) and hypothalamus along with pituitary and medulla oblongata were separated as shown in Fig. 1. The tissues were stored at -70°C . After 24 h, the tissues were thawed and homogenised in 30 mM sucrose containing 10 mM Tris-HCl buffer (pH 7.3) in a Potter-Elvehjem homogeniser with a loose fitting Teflon pestle. The rotor speed was 300–500 r.p.m. and the pestle was raised and lowered four to five times. The homogenate was centrifuged at 105 000 *g* for 1 h and passed through a Sephadex G-25 column (1 ml column, flow rate 1 ml/40 min) at 4°C to remove endogenous catecholamines as described by Yamauchi & Fujisawa (1978). The eluate containing TH activity was stored up to 1 week at -20°C and used as the enzyme preparation for the assay. Enzyme activity was not affected by storage up to 1 week (data not shown).

TH assay

TH activity was measured by the method of Shiman *et al.* (1971). To 150 μl of the enzyme fraction, 0.25 ml L-tyrosine (2 mM) in distilled water, potassium phosphate-buffered saline (PBS) buffer (2.0 M, pH 6.2), 0.01 ml catalase (1 mg/3 ml in PBS buffer), 0.05 ml 2-mercaptoethanol (0.28 M) in distilled water and 0.05 ml DMPH₄ (6 mM) in 0.005 M HCl (prepared just before use and kept in ice) were added in the same order. The reaction mixture was incubated in a test tube at 30°C for

25 min. Reaction was stopped by adding 0.5 ml 0.5 M HCl. Freshly prepared nitrite-molybdate (1 ml) reagent was added to the mixture and allowed to stand for at least 5 min. The colour was stable for 30 min and 0.5 ml 2 M NaOH solution was quickly added and mixed. Absorption was immediately determined at 510 nm in a UV-VIS spectrophotometer (Systronics, ASE Ltd, Ahmedabad, India). To express enzyme activity, tissue protein content in each aliquot was measured by the method of Lowry *et al.* (1951) using BSA as standard. Enzyme activity was expressed in nmol L-DOPA formed/mg protein per h.

Determination of K_m and maximum velocity (V_{max}) for L-tyrosine (substrate) and DMPH₄ (cofactor)

Michaelis-Menten constants (K_m) for the substrate and cofactor, and maximum velocity (V_{max}) of the enzyme were determined from double reciprocal Lineweaver-Burk (LB) plots (Price & Stevens 1999). The enzyme preparation was incubated in different concentration ranges of L-tyrosine (0.1–0.5 mM) and DMPH₄ (1–8 mM) to measure TH activity as stated above. The K_m and V_{max} were calculated from the intercepts ($-1/K_m$ and $1/V_{max}$ respectively) on the x and y axes of the LB plots.

Statistical analysis

All data are expressed as means $\pm$ S.E.M. and were analysed by two-way (ovariectomy) and one-way ANOVA (OE₂ replacement) followed by Tukey's test ($P < 0.05$).

Results

Effects of ovariectomy on brain TH activity

Ovariectomy produced overall significant effects on TH activity in different regions of the brain in resting

(hypothalamus $F=8.33$, telencephalon $F=11.46$ and medulla oblongata $F=6.24$) and pre spawning (hypothalamus $F=16.28$, telencephalon $F=20.35$ and medulla oblongata $F=8.62$) phases (Fig. 2; $P<0.001$, two-way ANOVA), when measured under saturating conditions of substrate and cofactor. The analysis also showed a highly significant effect of ovariectomy and duration, and of the interaction between these two factors in all brain regions in the resting and pre spawning phases ($P<0.001$). Multiple comparisons by Tukey's test showed that, in the resting phase, TH activity did not vary significantly in week 1 but decreased significantly from week 2 onwards in a duration-dependent manner ($P<0.05$; Fig. 2a, c and e). In the pre spawning phase, the ovariectomy-induced inhibition of enzyme activity was significantly different in weeks 3, 4 and 5 ($P<0.05$, Fig. 2b, d and f).

Effects of OE_2 replacement on brain TH activity

Administration of OE_2 in 3-week ovariectomised fish produced an overall significant effect on brain TH activity (Fig. 3; $P<0.001$, one-way ANOVA; resting phase: hypothalamus $F=146.37$, telencephalon $F=109.71$, medulla oblongata $F=79.23$; pre spawning phase: hypothalamus $F=168.76$, telencephalon $F=137.65$ and medulla oblongata $F=79.32$), when measured under saturating conditions of substrate and cofactor. In both phases, OE_2 produced dosage-dependent biphasic effects on enzyme activity. The low dosages (0.05 and $0.5 \mu\text{g/g BW}$) not only restored TH activity, but also elevated it significantly ($P<0.05$). The high dosages (1.0 and $2.0 \mu\text{g/g BW}$) decreased the enzyme activity significantly ($P<0.05$). The magnitude of the inhibition was higher in the resting phase (December) than in the pre spawning phase (May). In the resting phase, the enzyme inhibition for the $2.0 \mu\text{g/g}$ dosage was 71% (hypothalamus), 58% (telencephalon) and 69% (medulla oblongata) and in the pre spawning phase it was 31%, 30% and 31% respectively.

Effects of ovariectomy on enzyme kinetics

Figure 4 shows that ovariectomy affected both apparent K_m and V_{max} of TH and the inhibition appeared to be of the mixed or uncompetitive type. Ovariectomy produced overall significant effects on the apparent K_m and V_{max} of the enzyme (Figs 5 and 6; $P<0.001$, one-way ANOVA). The apparent K_m values for the substrate (L-tyrosine) and cofactor (DMPH_4) showed an increasing trend over the period of ovariectomy in both resting and pre spawning phases. In the resting phase, the apparent K_m values for the substrate (Fig. 5) were significantly higher in weeks 4 and 5 in the hypothalamus, weeks 3, 4 and 5 in the telencephalon and weeks 2, 3, 4 and 5 in the medulla oblongata ($P<0.05$; Tukey's test) in comparison to the sham-ovariectomised group. In the pre spawning phase, the values were significantly high in weeks 2, 3, 4 and 5

in all the brain regions. The apparent K_m values for the cofactor (Fig. 6) were significantly higher in weeks 3, 4 and 5 in the hypothalamus, weeks 2, 3, 4, and 5 in the telencephalon and at all times in the medulla oblongata in the resting phase ($P<0.05$). In the pre spawning phase, the values were significantly higher in weeks 3, 4 and 5 in all the brain regions.

V_{max} of the enzyme for the substrate and cofactor showed overall significant changes in both seasons following ovariectomy (Figs 5 and 6; $P<0.001$, one-way ANOVA) showing a decreasing tendency over the duration of ovariectomy. For the substrate, the values decreased significantly in all regions except in the hypothalamus in week 1 in the resting phase and at all times in the pre spawning phase when compared with the sham-ovariectomised group ($P<0.05$). In the resting phase, V_{max} for the cofactor was reduced significantly at all times except in week 1 in the telencephalon and hypothalamus. In the pre spawning phase, V_{max} values for the cofactor were decreased significantly from week 2 onwards in medulla oblongata and week 3 onwards in the telencephalon and hypothalamus ($P<0.05$).

Effects of OE_2 replacement on enzyme kinetics

OE_2 replacement produced significant overall effects in both apparent K_m and V_{max} values of the enzyme (Figs 7 and 8; $P<0.001$; one-way ANOVA). The K_m values showed dosage-dependent biphasic effects. The low dosages (0.05 and $0.5 \mu\text{g/g}$) decreased the K_m values significantly in all the brain regions in both seasons ($P<0.05$), compared with the ovariectomised, vehicle-treated control groups. The high dosages (1.0 and $2.0 \mu\text{g/g BW}$) increased the values significantly in all the brain regions in both seasons ($P<0.05$).

OE_2 replacement elicited dose-related effects on the V_{max} . The low dosages (0.05 and $0.5 \mu\text{g/g}$) elevated it maximally. In the high dosage (1.0 and $2.0 \mu\text{g/g}$) groups, the values were significantly higher than the ovariectomised, vehicle-treated groups (control) in all the groups except the values of hypothalamus and telencephalon for the substrate (Fig. 7) in the resting phase and that of the medulla oblongata in the pre spawning phase which were significantly lower than the control value.

Discussion

In the catfish *Heteropneustes fossilis*, ovariectomy and OE_2 replacement procedures have been used previously to investigate oestrogen-monoamine interaction in the control of LH secretion. These studies indicate that OE_2 interacts with the hypothalamic CA system at different enzyme-catalysed metabolic steps ($\text{D}\beta\text{H}$, PNMT, COMT and monoamine oxidase (MAO)) to alter CA activity

Figure 2 Effects of ovariectomy (Ovx)/sham-ovariectomy (Sham OvX) on TH activity in hypothalamus (a and b), telencephalon (c and d) and medulla oblongata (e and f) in *Heteropneustes fossilis* in resting and pre-spawning phases. Values are means $\pm$ S.E.M.; $n=5$. Asterisks show significant differences from sham-ovariectomised groups (Tukey's test, $P<0.05$).

Figure 3 Effects of OE₂ replacement on TH activity in hypothalamus, telencephalon and medulla oblongata in 3-week ovariectomised (Ovx) *Heteropneustes fossilis* in resting and prespawning phases. Values are means $\pm$ S.E.M.; $n=5$. Asterisks show significant differences between sham+vehicle control (VC) and Ovx+VC, and OE₂-treated groups and Ovx+VC groups (Tukey's test, $P<0.05$).

Figure 4 LB plots of TH activity for substrate (L-tyrosine) and cofactor (DMPH₄) in the hypothalamus (resting phase) showing apparent (app.) K_m (km)/ V_{max} of ovariectomised (OVX) and sham-ovariectomised (control) groups. The control data are pooled from sham-ovariectomised groups of all weeks (WK). $\frac{1}{V}$ denotes reciprocal of velocity of enzyme (measured activity) reaction.

Figure 5 Effects of ovariectomy (OVX)/sham-ovariectomy (Sham OVX, pooled data of weeks 1, 2, 3, 4 and 5) on kinetics of brain TH activity for substrate (L-tyrosine) (K_m (A) and V_{max} (B)) in *Heteropneustes fossilis* in resting and prespawning phases. Values are means $\pm$ S.E.M.; $n=5$. Groups bearing the same numbers or letters are not significantly different from sham-ovariectomised groups and those bearing different numbers or letters are significantly different (Tukey's test, $P<0.05$).

Figure 6 Effects of ovariectomy (OVX)/sham-ovariectomy (Sham OVX, pooled data of weeks 1, 2, 3, 4 and 5) on kinetics of brain TH activity for cofactor (DMPH₄) (K_m (A) and V_{max} (B)) in *Heteropneustes fossilis* in resting and prespawning phases. Values are means $\pm$ S.E.M.; $n=5$. Groups bearing the same numbers or letters are not significantly different from sham-ovariectomised groups and those bearing different numbers or letters are significantly different (Tukey's test, $P<0.05$).

Figure 7 Effects of OE_3 replacement on kinetics of brain TH activity for substrate (L-tyrosine) (K_m (A) and V_{max} (B)) in 3-week ovariectomised (OVX) *Heteropneustes fossilis* in resting and prespawning phases. Values are means $\pm$ S.E.M.; $n=5$. Groups bearing the same numbers or letters are not significantly different and those bearing different numbers or letters are significantly different (Tukey's test, $P<0.05$).

Figure 8 Effects of OE₂ replacement on kinetics of TH activity for cofactor (DMPH₄) (K_m (A) and V_{max} (B)) in 3-week ovariectomized (OVX) *Heteropneustes fossilis* in resting and prespawning phases. Values are means $\pm$ S.E.M.; $n=5$. Groups bearing the same numbers or letters are not significantly different and those bearing different numbers or letters are significantly different (Tukey's test, $P<0.05$).

which, in turn, regulates LH secretion (Senthilkumaran & Joy 1994, 1995, Joy & Senthilkumaran 1998). The results of the present study conducted in an identical manner in the corresponding seasons have demonstrated that tyrosine hydroxylation is yet another step in CA metabolism influenced by OE₂. In the recrudescence phase (prespawning phase), ovariectomy, which produced a duration-dependent decrease in plasma OE₂ level and consequent OE₂ feedback-induced increase in plasma LH (Senthilkumaran & Joy 1994, 1995), caused an inhibitory effect on TH activity. The response was greater in the hypothalamus and telencephalon than in the medulla oblongata, suggesting a strong involvement of the forebrain CA system in the OE₂ feedback regulation of LH. Comparable studies are lacking in other teleosts but in rainbow trout a significant decrease in mRNA level was reported after ovariectomy in nucleus preopticus-anteroventralis (Vetillard *et al.* 1999). In contrast, studies in the rat showed that gonadectomy led to an increase in TH activity in the hypothalamus of females (Beattie *et al.* 1972) and the median eminence of males (Kizer *et al.* 1974, Babu & Vijayan 1984). Nakahara *et al.* (1976), however, showed that the response to castration varied with the hypothalamic nuclei studied.

The ovariectomy-induced inhibition of TH activity was reversed by the administration of low dosages (0.05 and 0.5 µg/g BW) of OE₂. In rainbow trout, OE₂ partially reversed the effect of ovariectomy on TH mRNA level (Vetillard *et al.* 1999). In the rat, a combination of OE₂ and progesterone increased the *in situ* molar activity of TH in the rat median eminence (Anguila-Mansilla *et al.* 1991). OE₂ elevated hypothalamic TH activity in the ovariectomised rat and progesterone decreased it in ovariectomised and ovariectomised+oestrogen-treated rats (Beattie *et al.* 1972, Babu & Vijayan 1984). In the ovariectomised rat, low-dose OE₂ implants for 3 days reduced TH mRNA levels but large-dose OE₂ implants for 20 days depressed the mRNA levels further, removal of the implants resulted in the TH mRNA levels returning to a level higher than ovariectomised control values (Rasmussen *et al.* 1992). In the catfish, the effect of OE₂ was biphasic depending on the dosage. The low dosages elevated enzyme activity significantly above the control values, but the high dosages (1.0 and 2.0 µg/g BW) inhibited it. Similar biphasic effects of OE₂ have been demonstrated on hypothalamic DβH, PNMT, MAO and COMT activities in the catfish after ovariectomy (Senthilkumaran & Joy 1994, 1995, Joy & Senthilkumaran 1998). Such graded effects from stimulatory to inhibitory, as the dosage is increased, represent a non-monotonic or inverted-U response which is characteristic of the actions of oestrogen in other biological systems (Vom Saal *et al.* 1997).

The functional significance of the effects of OE₂ on brain TH activity during the gonadal recrudescence (prespawning) phase can be related to the CA control of LH

secretion (DA inhibits and NA/adrenaline stimulates LH secretion) in the catfish (Senthilkumaran & Joy 1995, Goos *et al.* 1999, Joy 1999). During ovariectomy, plasma LH levels were elevated with the peak at week 4 by depressing DA content and turnover, and elevating NA/adrenaline content and turnover. Further OE₂ treatment in 3-week ovariectomised catfish reversed the patterns in LH secretion and CA activity in a dose-dependent manner. The differential changes in CA activity could also be correlated with changes in DβH and PNMT activity (Senthilkumaran & Joy 1995). Since we sampled the tissues only at 7 days after ovariectomy, a stimulatory response prior to it, if there was one, was missed in this study. The insignificant change observed at weeks 1 and 2 may be the course of changes from a stimulatory to an inhibitory one during ovariectomy. This needs to be verified in further studies. The elevated TH activity in low OE₂ dosage groups suggests increased CA synthesis/affinity of the enzyme for the substrate and cofactor. The inhibitory effect due to long-term ovariectomy (4, 5 and 6 weeks) and high dosages of OE₂ (1.0 and 2.0 µg/g BW) suggests decreased synthesis/affinity of the enzymes for both substrate and cofactor. The OE₂ effect on TH activity may be produced directly (see below) or indirectly by altering CA activity which may exert end-product feedback control of the enzyme.

The significance of changes in TH activity due to ovariectomy and OE₂ replacement in the gonadal quiescent (resting) phase is not clear at present. In fact, the inhibition to OE₂ replacement was stronger in the resting phase. Perhaps the changes may indicate CA (DA) involvement in maintaining the pituitary-gonad axis in a quiescent state. During this phase, DA is the principal CA, and NA, adrenaline and their synthesising enzymes are absent or show low activity (Senthilkumaran & Joy 1995). In our earlier studies, we could not show any effect of ovariectomy or OE₂ replacement on hypothalamic CAs or on DβH, PNMT, COMT or MAO in the resting phase (Senthilkumaran & Joy 1994, 1995, Joy & Senthilkumaran 1998). The TH response may indicate that it is a more highly sensitive index of CA metabolism than the other parameters. Apart from LH regulation, DA is also involved in the regulation of GH, prolactin and melanocyte-stimulating hormone secretion in teleosts (Peter & Yu 1997, Olivereau & Olivereau 1999a,b) and the actions of OE₂ on TH activity can modify these DA-dependent functions, apart from other neural functions.

The kinetic data show that both ovariectomy and OE₂ replacement influenced the kinetic properties of the enzyme and the observed *in vivo* activity changes in the enzyme can be correlated. Ovariectomy resulted in an increase in the apparent K_m values for both substrate and cofactor with a consequent decrease in V_{max} over its duration. These results indicate that ovariectomy decreased the affinity of the enzyme for the substrate and cofactor. Since both apparent K_m and V_{max} were altered

by OE₂, the interaction may be uncompetitive or mixed (Price & Stevens 1999). Ovariectomy in the rat increased hypothalamic TH activity with a concurrent decrease in apparent K_m value for DMPH₄ (cofactor; Beattie *et al.* 1972). Progesterone did not alter the K_m and V_{max} of hypothalamic TH for tyrosine but that for the cofactor was altered (K_m increased, V_{max} decreased; Beattie & Soyka 1973). However, Morgenroth *et al.* (1974) have reported changes in the K_m and V_{max} for substrate as well. In the catfish, we have reported changes in apparent K_m values for both substrate and cofactor across the seasons, sexes and brain regions (Chaube & Joy 2002a,b). It is possible that the concentration of the substrate may be a critical factor for TH activity in fish.

OE₂ replacement produced biphasic effects on the kinetic parameters. The low steroid dosages decreased the apparent K_m values and increased the V_{max} concurrently, which may explain the stimulatory effect on *in vivo* TH activity. On the other hand, the high dosages of the steroid produced effects opposite to that of the low dosages, resulting in TH inhibition *in vivo*. In rainbow trout, OE₂ did not affect apparent K_m and V_{max} of TH in the hypothalamus and telencephalon, but 2-hydroxyoestradiol decreased the affinity for cofactor (elevated K_m and decreased V_{max}) (Saligaut *et al.* 1993) as has been reported in mammals (Foreman & Porter 1980).

It is known that the regulation of TH activity involves two mechanisms: acute regulation in which the rapid increase in activity upon nerve stimulation is attributed to a decrease in end-product inhibition and activation by phosphorylation, and long-term regulation in which the delayed response is related to changes in protein amount (Joh *et al.* 1978, Zigmond *et al.* 1989). OE₂ has been demonstrated to modify TH synthesis by regulating transcriptional activity (Blum *et al.* 1987, Zigmond *et al.* 1989, Selmanoff *et al.* 1991, Rasmussen *et al.* 1992, Vetillard *et al.* 1999). Further, it has been demonstrated that OE₂ can act at the membrane level (Thomas 1999, Russell *et al.* 2000, Cambiasso & Carrer 2001) to modulate signal transduction pathways that alter the phosphorylation status of the enzyme (Arbogast & Voogt 1991, Du & Iacovitti 1997, Arbogast & Hyde 2000). Therefore, it is likely that the alteration in the TH activity due to ovariectomy and OE₂ replacement may involve both genomic and non-genomic actions.

In conclusion, the present results clearly show that OE₂ exerts biphasic effects on brain TH activity and is an important mechanism of the steroid regulation of CA activity in the catfish.

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