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ORIGINAL ARTICLE

Estradiol-17β modulates dose-dependently hypothalamic tyrosine hydroxylase activity inhibited by α-methylparatyrosine in the catfish *Heteropneustes fossilis*

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Abstract The brain is a target for organizational and activational effects of oestrogens synthesized de novo or transported from the peripheral organs. A neuroprotective role of oestrogens has been documented in a variety of vertebrates. In the present study in the catfish *Heteropneustes fossilis*, we have demonstrated that estradiol-17β (E_2), the major circulating oestrogen at low dosages (0.05 and 0.1 μg/g body weight of fish for 3 days) stimulated hypothalamic tyrosine hydroxylase (TH) activity, and countered the negative effects of ovariectomy (3-week) or α-methylparatyrosine (α-MPT: 250 μg/g body weight, a competitive inhibitor of TH). In contrast, high dosages of E_2 (1 and 2 μg/g body weight of fish for 3 days) were inhibitory and further amplified the inhibitory effects of ovariectomy and α-MPT. The inhibiting role of E_2 was higher in gonad-active (prespawning) phase than gonad-inactive (resting phase) phase. The dual roles of E_2 may ensure a tight regulation of catecholaminergic activity, activating and inhibiting the system against wide fluctuations that are characteristic of seasonally breeding animals.

Keywords α-Methylparatyrosine · Tyrosine hydroxylase · Ovariectomy · Hypothalamus · Estradiol-17β

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Introduction

Involvement of steroid hormones in differentiation, development, and physiology of the central and peripheral nervous systems has been well documented in vertebrates [1]. It has long been held that the steroids of peripheral origin mediate these processes. The demonstration of aromatization of androgens by the central nervous system [2, 3] has highlighted the potential of the neural tissues to synthesize steroid hormones for local actions. The concept of neurosteroidogenesis has been established by the pioneering study of Beaulieu and others [4] and has further broadened the chemistry, metabolism and physiology of steroid hormones. The term neurosteroids has been used to describe neuroactive steroids synthesized in the brain, which have been the subject of intense research across vertebrates in recent times [1, 4–6]. Neurosteroids exert both protective and deleterious effects on the CNS, depending on the chemical nature and concentration [7–9]. (Neuro-) Oestrogens have both organizational and activational roles in a number of neural systems controlling reproductive and non-reproductive functions of the brain [10]. The neuroendocrine-pituitary axis is an important target/site of oestrogen synthesis, controlling reproductive activity and behaviour in vertebrates including teleosts [5, 11]. Brain monoaminergic system is one of the targets of oestrogen actions, affecting monoamine-dependent neural functions.

Hypothalamic monoamines (catecholamines (CAs) and serotonin) have been implicated in the regulation of pituitary hormone secretions, in particular gonadotropin secretion in vertebrates. Neuroanatomical studies in teleosts have demonstrated an extensive distribution of monoamines in the hypothalamus [12, 13] with distinct afferent projections to the adenohypophysis, forming direct or indirect contacts (synaptic or synapse-like) with the

hormone-producing cells. The functional involvement of the pituitary aminergic innervation in the regulation of various pituitary hormones has been investigated in different species [11, 14]. Based on the extensive study in the catfish *Heteropneustes fossilis* and perch *Channa punctatus* [15], it has been demonstrated that the hypothalamic aminergic system serves as a common neurophysiological substrate for the mediation of external (photoperiod and temperature) and internal environmental (steroid feedbacks) signals that regulate reproductive activity. In rainbow trout, Linard et al. [16] reported that TH-immunoreactive neurons in the preopticus pars anteroventralis were immunoreactive for oestrogen receptors, implying a functional interaction between the two systems.

Amongst endogenous factors, gonadal steroids exert a positive or negative feedback control on LH secretion in teleosts [17]. It has been shown that E_2 interacts at different enzyme-catalyzed steps in CA biosynthesis and degradation, such as β-hydroxylation of dopamine (DA) by dopamine-β-hydroxylase (DβH) and N-methylation of noradrenaline (NA) by phenylethanolamine-o-methyltransferase (PNMT) [18], oxidative deamination (including serotonin deamination) by monoamine oxidase (MAO) [19–22] and O-methylation of CAs by catechol-*O*-methyltransferase (COMT) [23]. Tyrosine hydroxylase (TH) which catalyses the hydroxylation of L-tyrosine to L-DOPA is considered the rate- limiting enzyme in CA biosynthesis [24]. It has been shown in the catfish that TH activity varies in relation to season, and is influenced by changes in environmental photoperiod, temperature or circulating titre of E_2 [25–27].

The tyrosine analogue, α-methylparatyrosine (α-MPT) has been widely used as a potent competitive inhibitor of tyrosine (substrate) for TH, which inhibits product (CA) formation in adrenergic neurons in the brain and sympathetic system of mammals [28, 29] and brain of teleosts [16, 18]. Gonadectomy inhibited mRNA levels in TH neurons and E_2 replacement partially reversed the effect [30]. Senthilkumaran and Joy [31] showed that α-MPT inhibited differentially CA content and turnover depending on the reproductive phase. The changes in CA activities following the α-MPT treatment have impaired the functioning of the neuroendocrine–gonadal axis and decreased spawning activity [31].

In view of the neuroprotective role of oestrogens and adverse impact of inhibition by α-MPT of tyrosine hydroxylation (CA biosynthesis) in the neuroendocrine control of reproduction in fish, the present study was undertaken to examine whether E_2 treatments override the α-MPT inhibitory effect. Ovariectomized and E_2-replaced catfish model, as described earlier [18, 20, 26, 31, 32] was used in the present study. The results indicate that E_2 indeed reverses the effect of the drug at low concentrations.

Materials and methods

Adult female catfish were purchased from local fish markets in Varanasi in resting (December) and prespawning (May) phases. The fish were maintained in flow-through aquarium tanks in the laboratory under natural photoperiod and ambient temperature conditions (resting phase: 10.5 h light, 13.5 h darkness, 18 ± 2°C; prespawning phase: 12.5 h light, 11.5 h darkness, 28 ± 2°C) for 15 days. They were fed with minced goat liver daily during acclimatization and experiments were conducted in accordance with local/national guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind.

Catalase, L-tyrosine, α-MPT, 6,7-dimethyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine ($DMPH_4$), bovine serum albumin (BSA), Sephadex-G-25 and 3-aminobenzoic acid ethylester (MS222) were purchased from Sigma Chemicals, St. Louis, MO, USA. Sodium molybdate, 2-mercaptoethanol, sodium nitrite and folin-cicoalteu reagent were purchased from E-merck (Mumbai, India). Other chemicals were purchased from Hi-media and BDH (Mumbai, India).

About 200 acclimatized fish were ovariectomized and 60 fish were sham ovariectomized in the resting (second week of December) and prespawning (second week of May) phases. The fish were anaesthetized with MS222 (3-aminobenzoic acid ethylester; 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 cauterized with a hot needle to prevent regeneration and the incision sutured. The fish were treated with benzanthine 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 3 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 ovariectomized fish were used for enzyme assay.

In both resting and the prespawning phases, 3-week ovariectomized fish were given E_2 intraperitoneally in dosages of 0.05, 0.1, 1.0 and 2.0 μg/g body weight (BW) daily for 3 days (group size = 5 fish). As control, five fish each from the ovariectomized and sham ovariectomized groups were given an equal volume (0.1 ml) of vehicle (propylene glycol).

α-MPT was dissolved in acidic saline [18] and then neutralized with 5 N NaOH (pH 7.8). Five fish each from sham, ovariectomized, and E_2 replaced (0.05, 0.1, 1.0 and 2.0 μg/g BW daily for 3 days) groups were injected intraperitoneally with α-MPT (250 μg/g BW) for 3 days.

The dosage was optimized in our previous studies [26, 27]. As control, saline was administered in sham, ovariectomized, and E_2 replaced fish. The enzyme values did not vary significantly in the control groups and hence pooled in the respective control groups.

At the end of the experiments, all fish were sacrificed by decapitation between 11.00 am and 12.00 noon to avoid interferences because of circadian changes in TH activity [27]. Brains, along with pituitary were dissected out immediately on ice and hypothalamus along with pituitary was separated and stored at −70°C. After 24 h, the tissues were thawed and homogenized in 30 mM sucrose containing 10 mM Tris–HCl buffer, pH 7.3 with a Potter–Elvehjem homogenizer and loose fitting Teflon pestle. The rotor speed was 300–500 rpm and the pestle was taken up and down four to five times. The homogenate was centrifuged at 105,000×*g* for 1 h and passed through Sephadex G-25 column (1 ml column flow rate 1 ml/40 min) at 4°C to remove endogenous CAs, as described by Yamauchi and Fujisawa [33]. The elute containing TH was stored up to 1 week at −70°C and used as the enzyme preparation for the assay. The storage did not affect enzyme activity significantly (data not shown) [26].

Enzyme activity was measured by the method of Shiman et al. [34]. 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 0f 0.28 M 2-mercaptoethanol in distilled water and 0.05 ml $DMPH_4$ (6 mM, dissolved in 0.005 N 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. The reaction was stopped by adding 0.5 ml 0.5 N 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. 2 N NaOH (0.5 ml) solution was quickly added and mixed. Absorption was immediately determined at 510 nm in a Systronics UV–vis spectrophotometer. To express enzyme activity, tissue protein content of each aliquot was measured by the method of Lowry et al. [35] using BSA as a standard. The enzyme activity was expressed in nmoles/mg protein/h. The assay was validated for enzyme concentration, incubation time, pH, temperature, substrate, cofactor, catalase, removal of endogenous CAs by Sephadex G-25 column chromatography and after addition of DA on Sephadex G-25 purified preparations. TH activity was linear with time, enzyme concentration and cofactor ($DMPH_4$) concentration. The activity increased significantly up to 0.4 mM concentration of the substrate L-tyrosine. Subsequent increase (0.5–5 mM) in the substrate concentration did not alter the activity significantly. The activity showed an overall significant effect with the pH ranges used. The activity was low at 4.5–5.0 and 7.0–7.8, and was significantly high in the range between 6.0 and 6.8. Catalase increased the enzyme activity concentration-dependently. The presence of endogenous CAs decreased TH activity in comparison to the Sephadex G-25 column-eluted samples. Dopamine (0.5–100 µg/ml) decreased TH activity significantly in a concentration-dependent manner in Sephadex G-25 purified preparations. The enzyme activity increased linearly up to 35°C of incubation temperature and decreased at higher temperatures [27].

All data were expressed as mean ± SEM and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test ($P < 0.05$).

Results

Ovariectomy and the administration of α-MPT in 3-week ovariectomized fish produced overall significant effects on hypothalamic TH activity in both resting ($F = 123.15$) and prespawning ($F = 241.09$) phases (Fig. 1; one way ANOVA, $P < 0.001$; df = 24). TH activity decreased significantly in the 3-week ovariectomized fish compared to the sham control group. The magnitude of the ovariectomy-induced inhibition was higher in the resting phase than prespawning phase and percentage inhibition was 36 and 32%, respectively. The α-MPT treatment decreased enzyme activity in both sham and ovariectomized groups in both phases ($P < 0.05$; Tukey's test; df = 5). In the α-MPT alone groups, the inhibition was 58 and 53%, respectively, in the resting and prespawning phases. In the combination groups (ovariectomy + α-MPT), the inhibition was 70 and 56%, respectively, in the two seasons.

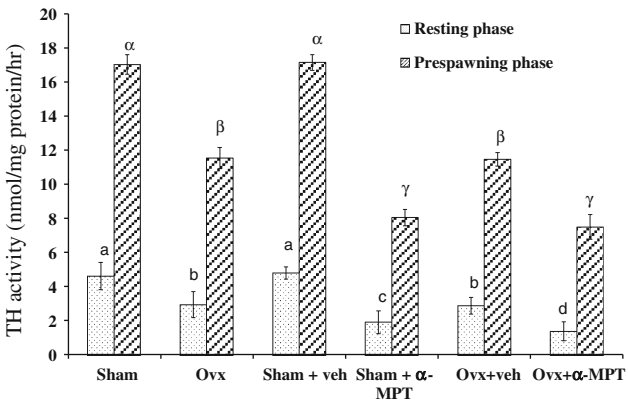

Fig. 1 Effects of 3 week ovariectomy and α-MPT (250 µg/g BW) on hypothalamic TH activity in the female catfish *Heteropneustes fossilis* (mean ± SEM, $n = 5$) in resting and prespawning phase. Data were analysed by one way ANOVA ($P < 0.001$) and Tukey's test ($P < 0.05$)

The administration of α-MPT significantly altered TH activity in both sham and ovariectomized fish administered with E_2 (Fig. 2; $P < 0.001$, one way ANOVA; df = 56; Tukey's test, $P < 0.05$; df = 13). The E_2 replacement produced dosage-dependent effects on the α-MPT effect. The low E_2 dosages (0.05 and 1.0 μg/g BW) reversed the effect of the drug treatment and elevated enzyme activity significantly compared to that of the ovariectomized group. However, the high E_2 dosages (1.0 and 2.0 μg/g BW) amplified the inhibitory effect of the drug and the response was higher in the prespawning phase.

Discussion

In our previous study [26], we have shown that TH activity was inhibited time-dependently after ovariectomy in different brain regions and E_2 replacement has produced dosage-dependent biphasic effects. In this study, we used the 3-week ovariectomized and E_2 replaced fish to demonstrate whether α-MPT effects were modified by the steroid environment. The present results demonstrate that both ovariectomy and α-MPT treatment inhibited hypothalamic TH activity in a season-dependent manner with a higher inhibition in the resting phase. The seasonal effect may be because of several factors such as the differences in the circulating titre of E_2 [20], and the apparent K_m and V_{max} of the enzyme [27]. Alpha-MPT competes for the substrate and co-factor [36], and the apparent K_m values for the enzyme varied with season and brain regions [27]. During the ovariectomy-induced enzyme inhibition, the kinetics parameters varied significantly; K_m values for both the substrate and cofactor, and V_{max} altered differentially [25, 27]. These workers have shown further that α-MPT increased the apparent K_m value of the substrate several folds, suggesting perhaps a decrease in the binding (affinity) of the enzyme towards the substrate and/or cofactor. In the resting phase, the apparent K_i value was significantly higher than that of the preparatory/prespawning phase which could be related to the significantly higher inhibition of enzyme activity in the α-MPT group. Alpha-MPT along with ovariectomy further inhibited enzyme activity; in the resting phase, the inhibition was 70% and in the preparatory phase, it was 56%. Since the effect was cumulative, both treatments (ovariectomy and α-MPT) might have strongly affected the kinetic properties of the enzyme.

The present data further showed that E_2 exerted biphasic responses on hypothalamic TH activity depending on the concentration as reported earlier [26]. The low dosages up to 0.1 μg/g BW were stimulating and restored TH activity in ovariectomized fish, the lowest dosage 0.05 μg/g BW was highly effective. On the other hand, dosages ≥1.0 μg/g BW lowered the activity greater than ovariectomy. The biphasic action of E_2 has been explained by its differential effects on the kinetic properties of the enzyme. [26]. Biphasic actions of E_2 may point to the feedback regulation of enzyme activity that has both facilitative and inhibiting components. The low dosages (facilitative) could counteract the inhibitory action of α-MPT and the high dosages (inhibiting) acted cumulatively with the drug to suppress TH function. The antioxidant role of E_2 (the monophenolic ring acts as a free radical scavenger) [37] may help in the protective action of

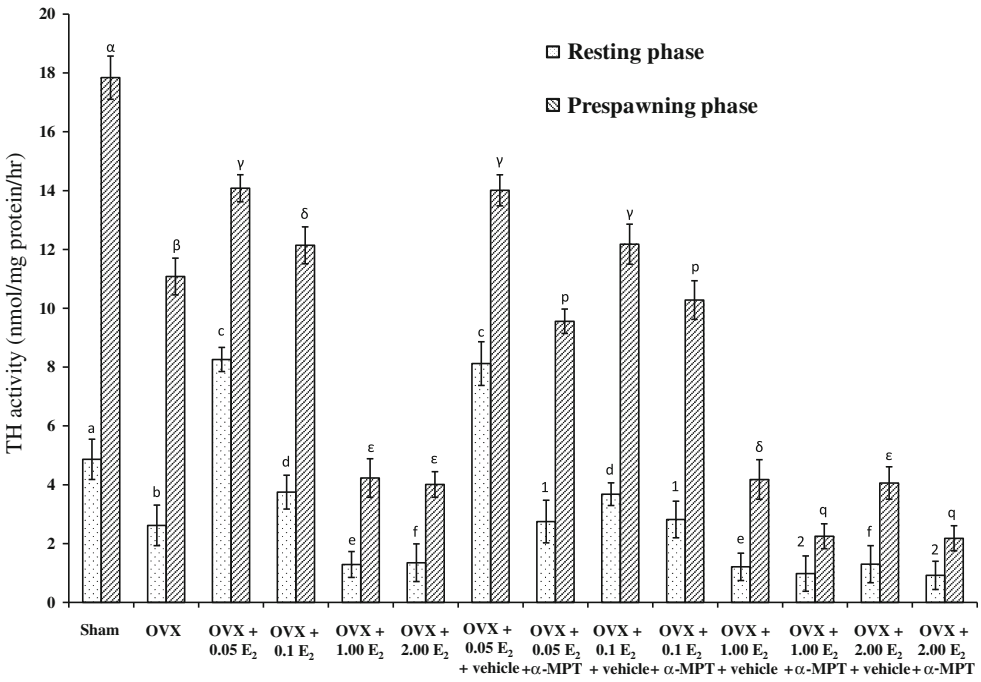

Fig. 2 Effects of α-MPT (250 μg/g BW) on ovariectomy and E_2-induced changes on hypothalamic TH activity in the female catfish *Heteropneustes fossilis* (mean ± SEM, $n = 5$) in resting and prespawning phase. Data were analysed by one way ANOVA ($P < 0.001$) and Tukey's test ($P < 0.05$)

the steroid. Brain aromatase and neuroestrogens have been characterized in a variety of vertebrates [2, 3, 38, 39]. Neuroestrogens are implicated in neuroprotection, neuronal proliferation, neurogenesis and neuronal signalling in mammals [40]. Oestrogens synthesized at synapses may quickly regulate neurotransmission. Brain aromatase activity is significantly higher in teleosts than other vertebrates [2] and high levels of E_2 is linked to very high neurogenic activity which occurs throughout the lifespan of fishes, a phenomenon that is restricted to limited regions in mammals [40]. Thus, E_2 can act locally to influence CA activity. The mechanism by which the low dosage of E_2 countered the inhibition of α-MPT is not clear at present. It is likely that the steroid may prevent the binding of the drug with the enzyme through an unknown mechanism or the steroid may stimulate enzyme activity by phosphorylation especially at Ser-40 by protein kinase A [41].

Previous studies in teleosts have shown that hypothalamic CA activity is influenced by the circulating levels of E_2 [21, 22, 42, 43]. In *H. fossilis*, ovarian E_2 varies seasonally and exerts feedback effects on hypothalamic CA activity depending on the season and concentration of the steroid administered [20, 23, 31, 32]. These studies indicate that E_2 interacts with the hypothalamic CA system at different enzyme-catalyzed metabolic steps (Dβ H, PNMT and COMT) to alter its activity, which, in turn, regulates LH secretion [44]. The present data confirm tyrosine hydroxylation as a yet another site of action of E_2 in CA metabolism. In the catfish as in other teleosts, DA inhibits and NA stimulates LH secretion [18, 31, 32]. Since α-MPT can alter DA and NA activities differentially depending on the reproductive stage of the fish (season), it can be used as a pharmacological agent to understand the seasonal regulatory mechanism of gonadotropin secretion and breeding activities.

In conclusion, the biphasic effects of E_2 may ensure a tight regulation of catecholaminergic activity at TH level, activating and inhibiting the system against wide fluctuations that are common in seasonally breeding animals.

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Conflict of interests The authors declare that they have no conflict of interests.

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