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Seasonal variations in cellular expression of neuropeptide Y (NPY) in testis of the catfish, *Clarias batrachus* and its potential role in regulation of steroidogenesis

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ABSTRACT

The present study demonstrates seasonal variation in the cellular expression of neuropeptide Y (NPY), a known orexigenic neuropeptide, in the testis of the catfish, *Clarias batrachus* and its relation with testicular steroids. *In vitro* effects of NPY on androgen production and activities of steroidogenic enzymes were also analyzed to reaffirm the relation between NPY and steroids. NPY-immunoprecipitation was observed in Sertoli cells, interstitial cells and germ cells in recrudescing testis. Intensity of NPY-immunoreaction in the interstitial cells increased steadily with initiation of spermatogenesis and reached maximal in fully grown testes, and then decreased suddenly in the spermiating/spent testis. NPY was also expressed considerably in Sertoli cells in recrudescing testis, but disappeared in the fully grown testis. A moderate NPY-immunoreactivity was also seen in spermatogonial cells in recrudescing testis, but intense NPY-immunoprecipitation was detected in advanced germ cells (spermatids/spermatozoa) in fully mature testis. NPY-immunoreation intensity in interstitial cells showed positive correlation with increasing levels of testicular testosterone and 11-ketotestosterone, and with activities of 3β-HSD & 17β-HSD coinciding with advancing testicular activities. NPY treatment of testicular fragments *in vitro* stimulated the activities of 3β-HSD & 17β-HSD and increased testosterone & 11-ketotestosterone levels. This study for the first time demonstrates the existence of NPY peptide at cellular levels in fish testis, which stimulates androgen production by acting directly at testicular level.

1. Introduction

NPY, a 36-amino acid long orexigenic neuropeptide, is reported in several tissues, particularly in brain (hypothalamo-hypophysial axis) of vertebrates. Its role is implicated in regulation of various physiological processes, especially nutrition and reproduction [1–3]. Existence of NPY mRNA and its peptide has been shown in mammalian testis [4,5]. At cellular level, NPY mRNA and its peptide have been demonstrated in Leydig and Sertoli cells in rat testis [6,7]. NPY positive nerve fibers around the testicular tubules and vessels are also reported in rat [7]. NPY gene expression increases with testicular development in mouse [4]. NPY receptors have also been identified in mammalian testes [1,8,9] and its role in regulation of testicular steroidogenesis has been advocated [10].

The expression of NPY is extensively demonstrated in fish brain [2,3,11,12]. NPY and GnRH have been co-localized in different brain regions of *Clarias batrachus*, suggesting stimulatory role of NPY in secretion of GnRH and LH [13]. Some other studies have revealed that NPY plays an important role in gonadotropin secretion in fishes [14–16]. Recently the presence of NPY mRNA has also been shown in testis of cichlid fish, *Cichlasoma dimerus* [17] and catfish, *Clarias gariepinus* [18] without any information on its cellular origin and role in testicular activity. Moreover, no report is available on the existence of NPY peptide in fish testis till date. In the present study, therefore, we aimed to examine (a) does NPY peptide exist in the testis of *C.batrachus* and, if so, in which cell type?, (b) does NPY has any correlation with testicular androgens and steroidogenic enzymes? and, if so, does NPY influence androgen production independent of other steroidogenic factors?

2. Materials and methods

2.1. Chemicals

The polyclonal NPY-antibody (antibody raised in rabbit against porcine NPY, cat. No. N9528) and its peptide (human NPY, cat. No. N5017) were procured from the Sigma-Aldrich, USA. Goat anti-rabbit IgG-HRP secondary antibody (cat. No. 621140380011730) was

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purchased from GeNei, Bangalore, India. ELISA kit for testosterone (DKO002) was obtained from DiaMetra, Italy, while 11-ketotestosterone EIA kit from Cayman Chemical Comp., Michigan, USA (cat. No.582751). Routinely used other laboratory chemicals of AR grade were procured from Qualigens, Merck, SRL and HiMedia, India through authorized vendors.

2.2. Fish

The freshwater catfish, *C. batrachus* weighing 95–105 g were collected from pond in suburbs of Varanasi (28°8′N; 83°1′E), India in each month from January to August, covering reproductively inactive and active periods of the reproductive cycle. The catfish were acclimated for a fortnight to laboratory under ambient photoperiod and temperature in tanks (capacity–100 l) at the rate of fifteen fish per tank and were fed with chopped goat liver *ad libidum*. The reproductive cycle of *C. batrachus* has been divided into seven phases as per gonadal morphology and described elsewhere [19] i.e., mid-quiescence (January), late-quiescence (February), early-recrudescence (March), mid-recrudescence (April), late-recrudescence (May–June), spermiating (July) and post-spawning phase (August). All experiments were carried out as per guidelines of the Institutional Committee for Animal Ethics and Care of Banaras Hindu University, India (F. Sc./IAEC/2016-17/1135).

2.3. Seasonal study

Acclimated catfish, in mid of each month, were anaesthetized in ice-chilled water, weighed and then blood from male catfish was collected by caudal puncture. Blood was spinned at 5000 rpm under refrigerated centrifuge to separate serum. Testes were removed rapidly under aseptic condition and weighed to the nearest gram to calculate GSI. One of the testes of the individual catfish was stored at −70 °C until processed for the estimation of testosterone and 11-ketotestosterone contents and activities of steroidogenic enzymes like 5-ene-3β-hydroxysteroid dehydrogenases (3β-HSD) and 17β-hydroxysteroid dehydrogenases (17β-HSD). The other testis, however, was fixed in Bouin's fluid and processed for histological study and immunohistochemical localization of NPY.

2.4. In vitro study

To evaluate the effect of NPY on steroidogenesis, testicular fragments were incubated with human NPY *in vitro* during the quiescence and mid-recrudescence phases. During both the phases, fish were sacrificed, testes were quickly dissected out (n = 5), cleaned and cut into small fragments (approximately 10 mg/fragment) in culture Medium199 (Himedia) supplemented with 0.2% $NaHCO_3$, 100IU/ml penicillin, 100 µg/ml streptomycin and 40 µg/ml gentamycin. After initial incubation for 2 h at 25 °C, medium was discarded and testicular fragments (one fragment/well) were finally incubated in 1 ml of fresh medium with 1, 10, 100 nM of NPY separately in a humidified atmosphere with 95% air and 5% CO_2 at pH 7.4 for 24 h at 25 °C. Control incubations without NPY were also maintained simultaneously. Experiments were repeated thrice. The NPY concentration and duration of culture were decided based on the pilot experiment and literature. At the end of treatment, testicular fragments and corresponding Medium199 were collected, separately, stored at −70 °C for analysis of steroids in testicular fragments and medium along with the activities of 5-ene-3β-hydroxysteroid dehydrogenases (3β-HSD) and 17β-hydroxysteroid dehydrogenases (17β-HSD) in testicular tissue.

2.5. Immunohistochemistry of NPY

Immunohistochemical localization of NPY was performed in testicular sections of *C. batrachus* during different phases of the reproductive cycle as described previously [20]. In brief, testicular sections were deparaffinized in xylene, hydrated with descending series of alcohol and then endogenous peroxidase activity was quenched by H_2O_2 and methanol (ratio 1:40) for 25 min and then washed twice in PBS (0.05 M, pH 7.4). Blocking was done by incubating sections in 5% normal goat serum for 2.5 h at room temperature in a moist chamber followed by addition of primary antibody of NPY (1:400 dilution) for overnight at 4 °C. Then sections were washed in PBS thrice 10 min each followed by incubation with HRP tagged secondary antibody (dilution 1:100) for 1 h at room temperature. Thereafter, sections were washed in PBS thrice 10 min each and subjected to chromogen (0.06% DAB) with H_2O_2 and kept in dark for 10 min to develop color, reaction was stopped by dipping sections in distilled water. Sections were then dehydrated in ascending grade of alcohol and mounted in DPX. The images were captured by Leica DM2000 camera attached to microscope at 40× and 100× magnifications. Intensity of NPY immunoreactivity was measured by spot densitometry tool, Alpha EaseFC software (Alpha Innotech Corp., CA, USA) and expressed in term of integrated density values (IDV) as arbitrary unit [21]. For nonspecific immunoreactions, control sections were processed in parallel for which only PBS omitting the primary antibody was used. Pre-absorbed control sections were also developed by incubating sections with primary antibody, pre-incubated with 10^{-5}M NPY peptide for 24 h at 4 °C. The pre-adsorption of antibody with NPY peptide resulted in total loss of immunoreactivity in testicular sections (Fig. 3i, I & j, J).

2.6. Determination of androgens levels

Levels of testosterone and 11-ketotestosterone in serum, testis, testicular fragments and culture medium were measured using ELISA kits according to the manufacture's protocols. Detailed procedure for steroid extraction and estimation is described earlier from the author's laboratory [19].

2.7. Steroidogenic enzymes activities

Measurement of activities of 3β-HSD and 17β-HSD were determined using the protocols described elsewhere [20,21].

2.8. Statistical analyses

Data were presented as mean ± SEM (n = 5) and analyzed through one-way ANOVA followed by post-hoc, Duncan's multiple range test ($P < 0.05$) for comparisons amongst different groups. The correlation (r-value) between the intensity of NPY immunoreaction and levels of testosterone, 11-ketotestosterone as well as activities of steroidogenic enzymes in testis were computed at significance level of $P < 0.01$. All the statistical analyses were performed with the help of SPSS16 software (SPSS Inc., IL, USA).

3. Results

3.1. Seasonal variations in testicular histology and gonadosomatic index (GSI)

During the mid- and late-quiescent phases, the diameter of seminiferous tubules was small and tubules were primarily lined by Sertoli cells and spermatogonial stem cells, and lumen was full of cysts containing spermatogonia, the interstitium was poorly developed (Fig. 1a,A and b,B). In the early-recrudescence phase, germinal epithelium lining the seminiferous tubules exhibited dividing spermatogonial stem cells and interstitium started developing. (Fig. 1c,C). During the mid-recrudescence phase, seminiferous tubules were enlarged and filled with cysts, some cysts showed advanced germ cells and interstitium became prominent with conspicuous interstitial cells (Fig. 1d,D). In the late-recrudescence phase (May), testis showed well-developed interstitium with large number of interstitial cells.