Research Article

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Journal of Translational Neurosciences

2016
Vol. 1 No. 1:2

# *In situ* Localization of Vasotocin and Isotocin Precursor mRNA in Brain and Ovary of the Catfish Heteropneustes Fossilis and Estrogen Regulation of the Gene Expression

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**Citation:** Banerjee P, Chaube R, Joy KP. *In situ* Localization of Vasotocin and Isotocin Precursor mRNA in Brain and Ovary of the Catfish Heteropneustes Fossilis and Estrogen Regulation of the Gene Expression. J Transl Neurosci. 2016, 1:1.

## Abstract

In the catfish, vasotocin and isotocin precursor (pro-VT and pro-IT) transcripts were localized in brain and ovary by RNA *in situ* hybridization and estrogen control of gene expression studied by qRT-PCR. Both pro-VT and pro-IT transcripts were detected in nucleus preopticus (NPO) of the brain. Within the NPO, the VT and IT neurons displayed distinct spatial distribution and neuronal organization. Apart from the NPO, pro-VT transcripts were also detected in the anterior nucleus lateralis tuberis. In the ovary, the follicular envelope (theca and granulosa layers) of ovarian follicles showed strong positive transcript signals, which was reported for the first time in teleosts. The regulation of expression of pro-VT and pro-IT genes by estrogen was investigated in vitro using brain preoptic area and ovarian slices in presence of exemestane, a known endogenous estrogen (E2) synthesis blocker, alone or in co-incubation with E2. Exemestane elicited differential effects on the nonapeptide expression in the brain and ovary. Exemestane mildly down-regulated pro-IT expression but did not influence the pro-VT expression in the brain. In the ovary, exemstane inhibited both pro-VT and pro-IT expression about 4 folds higher than the control groups. The E2 supplementation increased the expression of both pro-VT and pro-IT in the brain differentially with the high E2 dose eliciting about 3-fold stimulation of the pro-VT expression. In the ovary, only the high dose of E2 supplementation restored and increased the pro-VT and pro-IT expression compared to the exemstane group. The steroid-induced expression was still significantly lower than the control level. The presence of pro-VT and pro-IT transcripts in the follicular envelope points to de novo synthesis of the neuropeptides in the ovary, which is influenced by the locally synthesized E2. The pro-VT and pro-IT neurons seem to be responsive to the E2 feedbacks rather than to the locally produced E2.

**Keywords:** Catfish; Estrogen; Exemstane; Gene expression; *In situ* hybridization; Nonapeptides

**Received:** November 23, 2015; **Accepted:** January 04, 2016; **Published:** January 07, 2016

## Introduction

The nonapeptides are a family of structurally-related peptide hormones with conserved structural features of two half-cysteine residues at 1 and 6, an amidated glycine at the 9th position and varying amino acid residues at 3, 4 and 8 positions [1]. The nonapeptides are classified into two families: basic and neutral families based on the amino acid at the 8th position. In teleosts, the basic and neutral nonapeptides are vasotocin (VT) and isotocin (IT), respectively, and are synthesized in distinct neurons in the nucleus preopticus (NPO) and axonally transported to the neurohypophysis from where they are released into the circulation [2]. The circulating nonapeptides perform major functions like osmoregulation, reproduction, metabolism, behavior, cardiovascular function etc., [3] mediated through specific receptors [4]. In addition to the brain, nonapeptides are also synthesized in peripheral organs like ovary, testis, uterus, adrenal, thymus, pancreas, etc., of higher vertebrates [5-11]. Such studies are meager in lower vertebrates. Using immunohistochemical and HPLC methods, VT was characterized in the follicular envelope of ovarian follicles in the catfish

Heteropneustes fossilis [12]. The ovarian VT shows seasonal and periovulatory changes and is involved in steroidogenesis and prostaglandin secretion, and follicular hydration, maturation and ovulation [12-17]. In the testis, VT secretion is relatively low and does not show any seasonal significant variation [12]. Ramallo et al. [18-20] demonstrated VT immunoreactivity in the interstitial tissue of the testis of the cichlid Cichlasoma dimerus. In rainbow trout [21] reported that VT stimulates in vitro testosterone production by the immature testis. However, information on the role of IT is scarce and it has a lesser role in reproductive functions [13,14,22]. The non-availability IT-specific antibody is a constraint for localization and expression studies. Bobe et al. [23] reported a high expression of both pro-VT and pro-IT genes in rainbow trout during oocyte maturation. In the catfish, cloning and characterization of pro- VT and pro-IT genes showed that both genes are expressed exclusively in the brain and gonads [24]. The development of molecular probes in the catfish has enabled the study of nonapeptides at the gene level.

Gonadal steroid hormones are involved in the regulation of nonapeptide secretion in vertebrates [25-29]. Brain pro-VT and pro-IT mRNA levels were correlated with sex steroid hormone levels in salmons [2]. In the catfish, ovariectomy decreased brain and plasma VT levels and estradiol-17β (E2) replacement in 3-week ovariectomized fish elicited a dose-dependent increase in the VT levels [30-33]. E2 produced season- and dose-dependent effects on VT secretion in the catfish ovary in vitro [30]. While, in the preparatory phase, E2 produced a dose-dependent modulation: low dose stimulated, and high dose inhibited VT levels, in the prespawning phase, E2 inhibited VT secretion in a dose-dependent manner. The question whether gonadal steroids modulate pro-VT and pro-IT gene expression is not investigated so far. Further, the teleost brain is an important source of neurosterogens [34]. The catfish brain expresses both ovarian and brain type P450 aromatase genes and synthesizes E2 [33,35]. The role of neuroestrogens in the modulation of nonapeptide gene expression and peptide secretion is not yet investigated although estrogen receptors are occur on the nonapeptide neurons [36,37]. Ovariectomy and E2 replacement can, at best, show the involvement of peripheral estrogens on nonapeptide secretion.

Keeping the above in mind, in the present study we demonstrated the cellular localization of pro-VT and pro-IT transcripts in the brain and ovary of the catfish to confirm de novo peptide synthesis. Secondly, we used an in vitro model system to demonstrate the role of locally produced E2 on the expression of pro-VT and pro-IT. For this, the preoptic area (POA) and ovary slices were incubated with exemstane, an irreversible steroidal inhibitor of P450 aromatase [38], to block endogenous E2 synthesis and the gene expression was measured in the presence of the inhibitor and E2 supplementation.

# Material and Methods

## Animal collection and acclimation

Heteropneustes fossilis (40–50 g) were collected from local fish markets in Varanasi in January (resting phase) and mid-May (pre-spawning phase). They were maintained in the laboratory for 48 h under natural photoperiod: January (11.0L:13.0D), May (13.0L:11.0D) and temperature (25 ± 2°C) to overcome stress due to transportation and fed daily with goat liver ad libitum. All experiments were performed in accordance with the guidelines of the Animal Ethics Committee, Banaras Hindu University, Varanasi.

## Chemicals and reagents

Guanidine thiocyanate-phenol solution (Qiagen), Revert-Aid H Minus First Strand cDNA Synthesis Kit (Fermentas), 2X PCR Master Mix (Fermentas), Nucleo-pore PCR clean- up gel extraction kit (Genetix), InsTAclone PCR Cloning Kit (Thermo), Maxiscript in vitro transcription kit (Ambion), dextran sulfate (Amresco), Denhardt's reagent (Invitrogen), veriquest SYBR green qPCR master mix (Affymetrix) and DNase I (Ambion) were purchased through local suppliers. Dig-11 UTP, yeast t-RNA, blocking reagent, antidigoxigenin AP Fab fragments and NBT/ BCIP stock solution were purchased from Roche. Agarose, tris base, glacial acetic acid, EDTA–Na2, proteinase K and other chemicals were of molecular grade, purchased from E-Merck, Mumbai. LB broth, LB agar, ampicillin, X-Gal and IPTG were purchased from Hi Media, Mumbai. Diethyl pyrocarbonate (DEPC), 3-aminopropyl triethoxysilane, formamide (molecular biology grade) and estradiol-17β (E2) were purchased from Sigma-Aldrich, New Delhi. Exemestane (FCE-24304) with the trade name of aromasin (Pfizer, Italy) was purchased from a local medical store. The primers used were synthesized by Integrated DNA Technology (IDT), Faridabad, India.

## Cloning and generation of probes

Heteropneustes fossilis VT (acc. no. JX035928.1) and IT (acc. no. JX669009.1) precursor genes were cloned and used for the generation of riboprobes. The pro-VT (505 bp) and pro-IT (569 bp) were amplified by PCR using the primers spanning the 5' and 3' UTR regions **(Table 1)** and the brain cDNA as the template. The pro-VT and pro- IT share an identity of only 53.47% so that the probes are expected not to cross-react. The PCR products were cloned into the TA cloning vector pTZ57R/T of InsTAclone™ PCR Cloning Kit (Thermo), using the manufacturer's protocol and competent cells of DH5α strain of E coli. Plasmids were extracted from the recombinant (white) colonies and screened for the orientation of ligation of the PCR product by using a PCR strategy. Briefly, the protocol included using a VT and IT UTR FP/ UTR RP and T7 promoter primer and the extracted plasmid as template in a PCR. Plasmids giving a positive amplification with VT/IT UTR FP and T7 primer were used for the generation of antisense probes. Plasmids giving a positive amplification with the VT/IT UTR RP and T7 primer were used for the generation of sense probes. These PCR products were purified using the Nucleo-pore PCR clean- up gel extraction kit (Genetix) and 250 ng of the products were used as the template for in vitro transcription using MAXIscript® T7 in vitro transcription kit (Ambion). The in vitro transcription reaction was carried out in a 20 µL reaction volume using T7 polymerase and 1 µL of 10 mM stock each of ATP, CTP, GTP and UTP. In this reaction, 1 µL of digoxigenin-11-UTP (Roche) (3.5 mM stock) was used as the labeled UTP. The reaction was carried out at 37°C for 1 h. 1 µL of the resulting reaction was checked by agarose gel electrophoresis to confirm the formation of the riboprobes and

**Table 1** Details of the primers used for generating the riboprobes.

| Primers | Sequences |
|---|---|
| VT FP | TGTTACATCCAGAACTGCCCCAGA |
| VT RP | CAGCCCAGTCCTTCTCCACAGCA |
| VT H FP | GTTACATCCAGAACTGCCCCAGA |
| IT FP | TCAATCTTCTGCATGCTGTGTCT |
| IT RP | CACACGCCATGCACTGTCTATTG |
| IT H FP | ACATCTCCAACTGTCCCATC |
| β actin FP | TGGCCGTGACCTGACTGAC |
| β actin RP | CCTGCTCAAAGTCAAGAGCGAC |
| T7 primer | TAATACGACTCACTATAGGG |
| VT UTR FP | GTCCAGTGAGAGACAGACCTCCGG |
| VT UTR RP | TAGAATGGACCGCGTGCTCTGC |
| IT UTR FP | CATCAGCTACTGAAGCTACTGATTCGT |
| IT UTR RP | AGGACATCAGAAGGTTCGGCTG |

stored at -80°C for *in situ* hybridization.

## *In situ* hybridization

*In situ* hybridization was performed in both whole brain and tissue sections. All solutions and reagents were prepared in diethyl pyrocarbonate (DEPC)-treated water to maintain RNase- free conditions at all steps prior to hybridization. The procedure for whole brain *in situ* hybridization (WISH) and *in situ* hybridization on tissue sections was almost the same, only differing in tissue pretreatment, composition of hybridization buffers and post-hybridization washing solution **(supplementary Table 1).** The brains dissected out from the resting phase fish were washed in ice - cold phosphate buffered saline (PBS) and allowed to harden in 4% paraformaldehyde (PFA) for 3 h. The hardened tissues were trimmed to remove the cerebellum and medulla oblongata and were put into fresh 4% PFA to fix overnight at 4°C. For the WISH on the fixed tissues, the protocol of [39] was followed with some modifications. In short, the fixed tissues were first washed in PBS/ 0.01% Tween- 20 (PBS-T), dehydrated through a series of methanol diluted in the PBS-T at room temperature (RT). The last step (100% methanol treatment) was repeated once and the tissues were stored in 100% methanol at -20°C overnight. The dehydrated tissues were rehydrated, washed in the PBS-T, digested with proteinase K (10 µg/mL) for 15 min, refixed in 4% PFA, washed in the PBS-T, pre-hybridized at 70°C in hybridization buffer without probe, followed by hybridization with probes (sense/antisense) diluted 1:500 in the hybridization buffer overnight at 70°C. Overnight hybridization was followed by stringency washes by incubating the tissues in successively lower salt solutions at the hybridization temperature. Next, the tissues were washed in 1 x maleic acid buffer-0.1% tween 20 (MAB-T) at RT, blocking in blocking solution for 2 h, then incubated in 1:2000 diluted anti-digoxigenin AP antibody (Roche) at 4°C. The antibody incubation was followed by washing in MAB-T and alkaline phosphatase buffer. Detection was done by applying a 1% solution of the NBT/BCIP stock solution (Roche) prepared in the alkaline phosphatase buffer and allowing the reaction to proceed in dark for 40 min (the reaction development time was standardized for optimal intensity with the least background).

For *in situ* hybridization on tissue sections, the fixed tissues were dehydrated in graded series of ethanol. 100% ethanol was replaced by xylene, followed by a 50:50 mixture of xylene and paraffin wax before embedding. Eight µm thick transverse and sagittal sections of the brain were cut on a Leica semi-automatic microtome and spread on silane-coated slides to affix the sections. The ovary was sectioned in the transverse plane. The slides were dewaxed in xylene and processed similarly as the whole brain for hybridization, washing, antibody incubation (carried out at RT instead of 4°C) and detection.

## *In vitro* effects of exemestane and E2 supplementation

Preparation of test compounds and incubation medium- A stock solution of E2 was made in propylene glycol after first dissolving the required amount in 50 µL of ethanol. A stock solution of exemestane was prepared in a 1:1 solution of PBS:DMSO, after dissolving the required amount in 100 µL of ethanol. Just before the incubation, the stock solutions were diluted with the incubation medium to make working concentrations. Leibovitz's L-15 medium (AT011A-1L, Hi Media Laboratories Pvt. Ltd., Mumbai, India), supplemented with 10% fetal bovine serum and 100 µg/mL streptomycin was prepared for the *in vitro* study. The medium was always prepared fresh and filtered in a sterilized syringe-driven filter having a nylon hydrophilic membrane of pore size 0.45 µm and 30 mm diameter before use.

In vitro incubations of brain and ovary- The organs were removed under aseptic conditions quickly from adult female fish in the pre-spawning phase after decapitation. The dissected brain and ovary were washed in ice-cold fish saline. For the preparation of brain slice, an anterior cut was made at the level of the anterior commissure to remove the telencephalic area and a posterior cut was made behind the optic chiasm with the help of a clean sharp razor blade. This slice contained the POA with the nucleus preopticus (NPO) and was used for the experiment. A slice from the middle part of the ovary was used for the experiment. Five treatment groups were made each for brain and ovary incubations: Group 1 (vehicle control), the tissue slices were incubated with the vehicle for 8 h. Group 2 (EM) was incubated with exemestane (200 nM) alone for 8 h. Group 3 (EM + vehicle) was incubated with exemestane (200 nM) for 3h and then co-incubated with exemestane and propylene glycol. Group 4 (EM + low E2) was incubated with exemestane (200 nM) for 3 h and then co-incubated with exemestane and 1 nM E2 for 5 h. Group 4 (EM+ high E2) was incubated with exemestane (200 nM) for 3 h and then co-incubated with exemestane and 10 nM E2 for 5 h. The incubations were carried out in 5 mL of culture medium at 25°C in a CO2 incubator with 5% CO2. Five fish were used in each group. The exemestane dose was chosen, as reported in [40] for fish ovarian tissue. The dose is about 100 times more than the IC50 (concentration giving 50% inhibition) values for the inhibition of human Cyp191a1 [41]. After the incubation period, the tissues were collected in RNA later and stored at -20°C until further processing.

## qPCR for nonapeptide precursor gene expression

A two-step qPCR was conducted to study the expression of pro-VT and pro-IT genes. Total RNA was isolated by the single-step method of RNA isolation. To remove genomic DNA contamination

from the preparation, DNAase (Ambion) treatment (2 units/10 µg RNA) was given and subsequently DNAase was heat inactivated at 75°C in presence of EDTA. RNA purity was checked by A260/A280 ratio. Samples having a ratio above 1.8 were only considered for reverse transcription. Two µg of the total RNA was reverse transcribed using random hexamer primers and Revert Aid M-MuLV reverse transcriptase in a 20 µL reaction volume (first strand cDNA synthesis kit, Fermantas) using the manufacturer's protocol. The resulting cDNA was diluted 10 times and 1µL was used in a PCR reaction of 20 µL containing veriquest SYBR green 2X master mix, VT/IT FP and IT FP/ RP, using the manufacturer's protocol in an Applied Biosystem 7500 machine with a thermal condition of 50º C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The specificity of the PCR product was checked by dissociation curve analysis of the amplicon and was also checked by agarose gel electrophoresis. The relative gene expression in the different experimental groups was calculated using the comparative CT method with catfish β-actin (accession number FJ409641.2) used as the endogenous control. The vehicle control cDNA was used as the calibrator sample. Each reaction was set up in duplicate and the average CT value was taken for the calculation. Graphs were plotted with the mean RQ (relative quantity) values (2-ΔΔCT) as calculated from five fish each [42].

### Statistical analysis

Data were expressed as mean ± SEM and were analyzed for statistical significance by one way ANOVA ($p < 0.001$), followed by Newman-Keuls' test ($p < 0.05$).

## Results

### Localization of pro-VT and pro-IT transcripts in brain

The whole brain preparation demonstrated the gross distribution of the precursor genes in the NPO **(Figure 1).** Within the NPO, the pro-VT and pro-IT transcripts exhibited a spatial distribution. The pro-VT neurons were present in the ventral part of the NPO, and not seen from the dorsal side **(Figures 1a and 1b).** The pro-IT neurons were present on the dorsal part of the NPO, and not seen from the ventral part **(Figures 1c and 1d).**

The brain sections hybridized with the sense probe for pro-VT and pro-IT did not give any positive signal **(Figures 2a-2c).** The regional distribution of pro-VT and pro-IT transcripts was clearly evident on probing the sections with the antisense pro-VT and pro-IT RNA probes. In the catfish, the NPO can be divided into two divisions: [ventral pars parvocellularis (ppc) and dorsal pars magnocellularis (pmc)]. The pro-VT neurons are distributed in both the subdivisions; smaller neurons in the NPO-ppc and larger neurons in the NPO-pmc **(Figures 2d and 2e).** The examination of the NPO in serial sections from the anterior to the posterior end shows that the VT neurons extend along the antero-ventral region of the NPO-ppc laterally to a more dorso-medial NPO-pmc caudally. The pro-IT neurons occupy the dorsal aspect of the NPO, throughout the rostro-caudal axis **(Figures 2f and 2g)**. The magnocellular subdivision has more IT neurons than the parvocellular subdivision. In addition to the NPO, pro-VT signal was also noticed in the ventral hypothalamus in a few neurons of the anterior nucleus lateralis tuberis (NLT) **(Figure 2h).**

### Neuron morphology and contacts

In the NPO, the VT neurons show a ventro-dorsal gradient in the size of the neurons with smaller neurons occupying a ventral position and larger ones occupying a dorsal position **(Figure 3a).** The size difference was not apparent in the dorsally positioned IT neurons. Many pro-VT and pro-IT neurons occur in clusters forming contacts with their perikarya or processes especially in the NPO-pmc **(Figures 3b and 3c).** The IT neurons are clustered in large numbers than the VT neurons. While a large number of IT neurons cluster by perikaryal appositions **(Figure 3c),** fewer VT neurons form such cell body contacts **(Figure 3a).** However, a large number of VT neurons make contacts through their processes **(Figure 3b).** Due to perikaryal clustering, neurite contacts in IT neurons are not clear. The neurons belong to the bipolar, tripolar and multipolar types. The perikaryon is round, oval, triangular or club-shaped. The transcripts were mainly detected in the cell bodies and at the proximal part of the axons and dendrites **(Figure 3b).** Consequently, there was no staining of the axonal fiber tracts or their terminals in the neurohypophysis. The IT neurons can be seen interdigitating with the ependymal cells or lying in the ependyma **(Figure 3d).** The VT neurons though present around the preoptic recess was seen beneath the ependymal layer and no interdigitating neurons were observed.

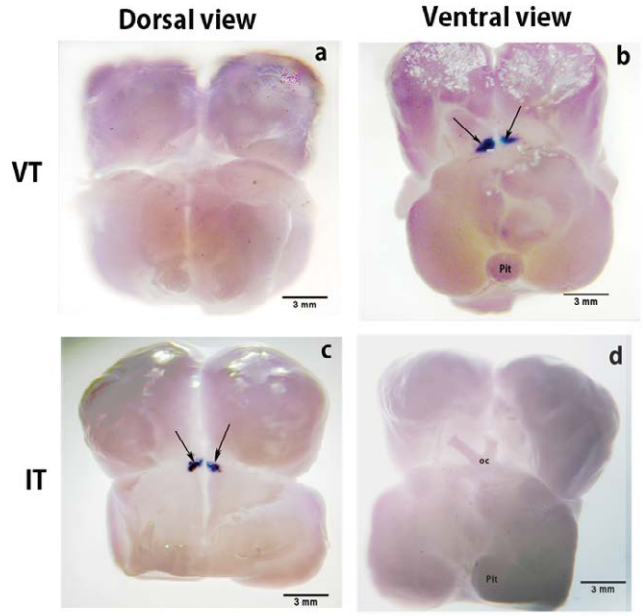


**Figure 1** Brain whole- mount *in situ* hybridization of VT and IT transcripts. (a) Dorsal view of the brain probed with the VT antisense RNA. (b) The same brain in the ventral view. (c) Dorsal view of the brain probed with the IT antisense. (d)The same brain in the ventral view. Arrows indicate the paired NPO. oc- optic chaism, pit- pituitary.

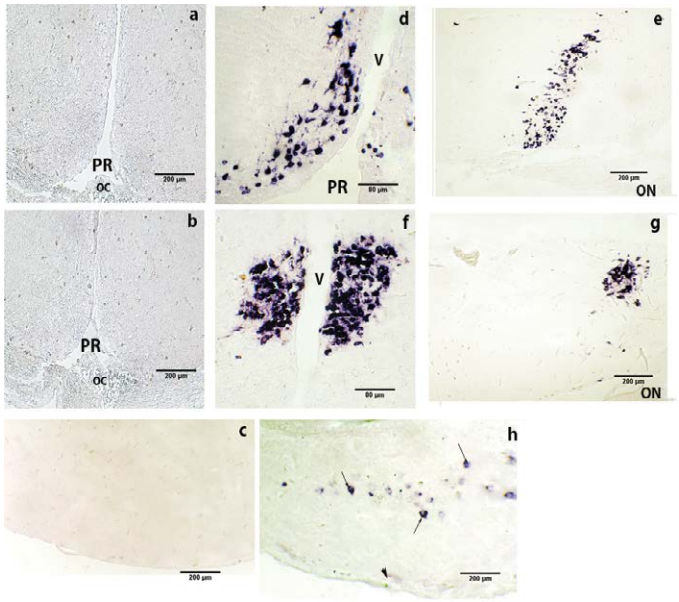


**Figure 2** *In situ* hybridization of pro-VT and pro-IT in brain sections showing the distribution of VT and IT neurons. (a) A section through the NPO probed with the pro-VT sense RNA, showing no signal. (b) A section through the NPO probed with pro-IT sense RNA, showing no signal. (c) A saggital section showing the hypothalamus probed with pro-VT sense probe, showing no signal. (d) A section through the anterior part of the NPO probed with the pro-VT antisense RNA. (e) A saggital section probed with the pro-VT antisense RNA showing the distribution of VT neurons in the NPO. (f) A section through the caudal part of the NPO probed with the IT antisense RNA. (g) A saggital section probed with the pro-IT antisense RNA, showing distribution of IT neurons in the NPO. (h) A saggital section of the hypothalamus showing small VT neurons in the anterior NLT (arrows). Arrow heads show moderate signals in the neurosecretory tract. PR- preoptic recess, V- ventricle, OC- optic chiasm, ON- optic nerve.

## Localization of pro-VT and pro-IT transcripts in the ovary

In the ovary, the hybridization with the sense probes did not yield any specific signal **(Figures 4a and 4b),** serving as a negative control. Pro-VT and pro-IT transcripts were localized in the follicular envelope of the post-vitellogenic oocytes, but not in the immature follicles **(Figures 4c and 4d).** The granulosa cells of the follicular layer showed intense reaction. Because of the scattered nature of the thecal cells, it was difficult to pin-point signals in the thecal cells at the resolution of the light microscope **(Figures 4e and 4f).**

## *In vitro* effects of exemestane and E2 supplementation on gene expression

There was an overall significant effect of the treatments with exemestane and E2 on the pro-VT gene transcription in the POA (**Figure 5a**; one way ANOVA, $p < 0.001$; $F=396.29$). The incubation of the POA with exemestane for 8 h did not alter the pro-VT transcript level. However, the co-incubation with low (1 nM) and high (10 nM) concentrations of E2 increased the transcript levels, the higher dose produced about a 3-fold increase.

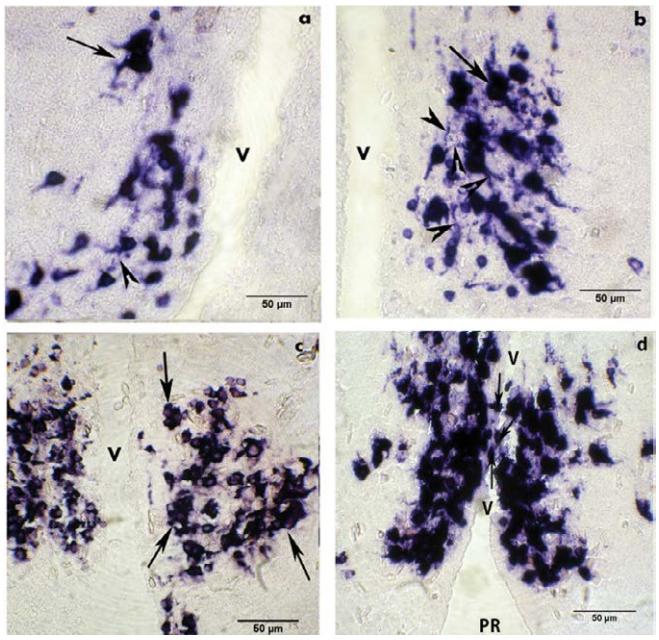


**Figure 3** Transverse sections of the brain through the brain of the catfish probed with the VT antisense probe (a) and (b), and IT antisense probe (c) and (d). The VT neurons are clubbed (arrows) and make neurite contacts (arrowheads). The IT neurons are heavily clustered than VT neurons (arrows) in (c). (d) IT neurons lying in the ependyma (arrows). V- ventricle, PR- preoptic recess.

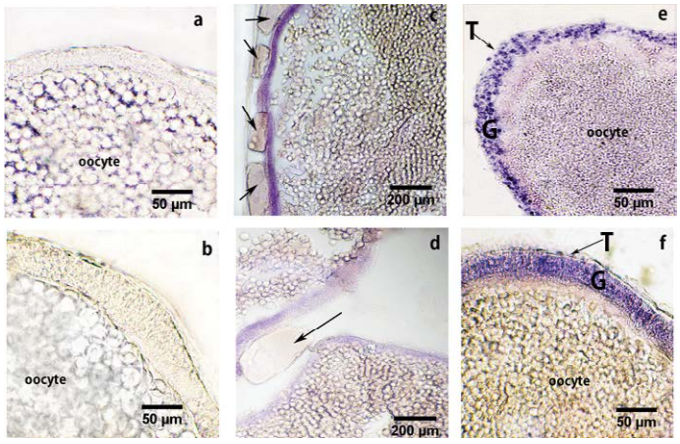


**Figure 4** Sections of postvitellogenic ovarian follicles showing localization of pro-VT and pro-IT transcripts in the follicular layer. Control sections probed with pro-VT sense RNA (a) and pro-IT sense RNA (b). Sections probed with pro-VT antisense RNA (c) and pro-IT antisense RNA (d). Note the localization in the follicular layer of the postvitellogenic follicle but not in the immature follicle (arrows). Higher magnifications of sections probed with pro-VT antisense RNA (e) and pro-IT antisense RNA (d). Note the intense reaction in the granulosa cells. G-granulosa, T- theca.

The treatment with exemestane and E2 elicited overall significant effects on pro-IT precursor transcription in the POA **(Figure 5b**;

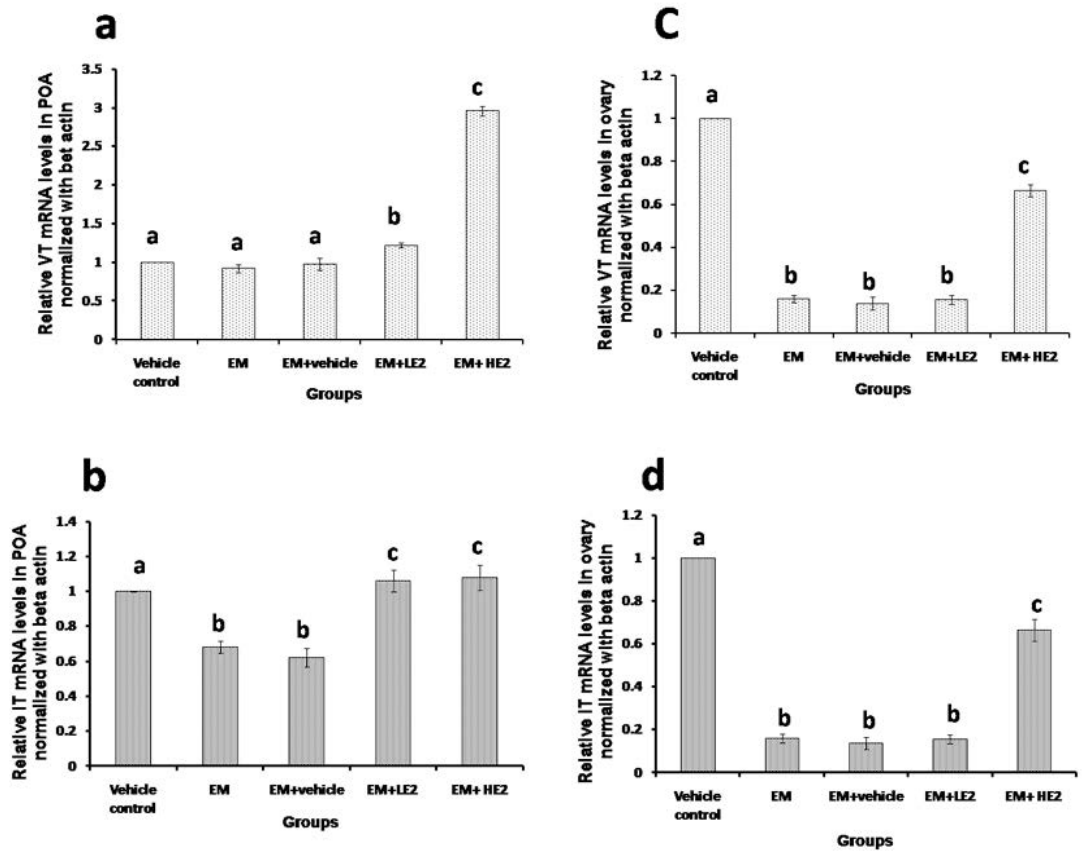


**Figure 5** Relative expression of nonapeptide precursor genes after incubation with exemestane (EM) alone or in combination with E2. (a) pro-VT expression in the preoptic area (POA), (b) pro-IT expression in the POA, (c) pro-VT expression in the ovary and (d) pro-IT expression in the ovary. The error bars represent mean ± SEM (n=5). Groups with significantly different expression levels are denoted with different alphabets. LE2 - low E2 dose, HE2 - high E2 dose.

one way ANOVA, $p < 0.001$; $F = 4.66$). The pro-IT transcript levels in the POA decreased after the exemestane treatment. The E2 supplementation restored as well as further increased the transcript levels significantly over that of the exemestane and control groups.

The incubations with exemestane and E2 produced overall significant effects **(Figure 5c and 5d;** one way ANOVA, $p < 0.00$) on the pro-VT ($F = 1077.85$) and pro-IT ($F = 170.85$) gene expression in the ovary. Exemestane drastically reduced the pro-VT and pro-IT gene expression (about 4-folds). The low dose of E2 supplementation did not restore the expression but the high dose could partially reverse the effect of exemstane but the levels were significantly lower than the basal levels.

## Discussion

### Expression of pro-VT and pro-IT in the brain: differential distribution and neuronal organization

This study reports the relative distribution of pro-VT and pro-IT neurons in the catfish brain. The VT neurons are concentrated in the antero-ventral part of the NPO-ppc to the caudo-dorsal part of the NPO-pmc. This pattern is similar to the distribution of immunoreactive VT neurons in the two subdivisions of the NPO, as reported previously [12]. In the catfish, the IT neurons were identified for the first time in this study. The IT neurons are present dorsally from the anterior to the posterior end. In the NPO of the related catfish C. batrachus, two clusters of immunoreactive IT neurons, one in the NPO-supraopticus located just above the optic chiasm and the other in the NPO-paraventricularis at the level of the third ventricle, were described [43]. In H. fossilis, we could not find the two distinct immunoreactive clusters and the pro-IT distribution may correspond to that of the NPO-paraventricularis of C. batrachus. The distribution pattern of VT and IT neurons varies with teleosts. In sea bream [44], rainbow trout [45] and zebrafish [46], the VT and IT neurons have differential spatial distribution within the NPO like H. fossilis. On the other hand in goldfish, medaka, European plaice, green molly and false clown anemonefish, VT and IT neurons are evenly distributed in the NPO [47-50].

In addition to the NPO, a few neurons in the anterior division of the NLT in the ventral hypothalamus showed pro-VT-positive signal. However, no immunoreactive VT neurons were described in the NLT region previously [12]. The presence of VT/IT neurons in centers other than NPO has been reported in plainfin midshipman [51,52]. In medaka, VT neuron populations were

described additionally in NPT (nucleus posterior tuberis), aNVT (anterior part of nucleus ventral tuberis), NAT (nucleus anterior tuberis) and pNVT (posterior part of nucleus ventral tuberis) in the hypothalamus, some of which are sexually dimorphic [50]. In larval zebrafish, *in situ* hybridization showed two domains of pro-VT gene expression, the dorsal preoptic area and the ventral hypothalamus. However, VT-positive neurons were not detected immunohistochemically in the hypothalamus of both adult and larval zebrafish neurons [46]. Since this study in zebrafish indicates that presence of transcript may not necessarily mean the presence of peptide, in the catfish too there is a need to analyze the functional implication of VT transcript expression in the NLT at the peptide level.

In the NPO, the VT neurons displayed a ventro-dorsal gradient in their size. The gradient in the neuron size has been attributed to physiological regeneration by endoreplication to meet increased demands during periods of heightened activity like spawning and migration [53,54]. The large neurons present in the dorsal side may be the ones that have undergone endoreplication that leads to an increase in cell and nuclear size. Desai and Akhunji [55] reported binucleated neurons and neurons having horseshoe-shaped nuclei in the NPO of the teleost Pampus argenteus, which indicates that a genome amplifying mechanism may be operative in the magnocellular neurons. These neurons may cater to very high secretory demands. In the present study, a gradient in neuron size was observed only for the VT neurons, and not for the IT neurons, and may indicate that the gene amplifying mechanism may be operative only in the VT neurons. Apart from a device to fulfill increased demands, endoreplication may also provide the advantage that a single large neuron would have, over several smaller ones, the advantage in warding off the need of a complex coordinating system [56].

The neuron-specific clusters that were observed for IT neurons also indicate that mechanisms leading to a high degree of coordination in neurons producing the same peptide are highly favored in the NPO. Saito et al. (2004) have reported neuron-specific clusters in the rainbow trout NPO. Cumming et al. [57] reported direct soma to soma appositions without intervening glial sheath in the goldfish NPO cells. Such contacts may facilitate communications by locally changing the membrane potential or release of the peptides [58]. The IT neuron clustering in the NPO of the catfish may be a physiological adaptation for neuron- to-neuron communications leading to a high degree of coordination. Apart from perikaryal clustering, the NPO neurons make contacts through their processes. In the catfish such contacts are visibly high among VT neurons as compared to the IT neurons. In the rainbow trout, close appositions of VT proximal processes were described [45]. Cumming et al. [57] reported axo-dendritic synapses with VT-immunoreactive axon terminals in pars magnocellularis, facilitating a communication between the VT neurons. Such contacts among the IT neurons are less frequent in the rainbow trout [45], which is similar to the case in the catfish. The localization study in the brain shows that both VT and IT neurons have a discrete distribution in the NPO with distinct spatial and neuronal organization, implying distinct physiological functions and regulations.

## Expression of pro-VT and pro-IT in the ovary

Both pro-VT and pro-IT transcripts are distributed in the follicular layer of the oocytes. The pro-VT transcript localization in the ovary supports an earlier observation of VT immunoreactivity in the theca and granulosa cells of the ovarian follicles [12] and the study confirms that VT is synthesized de novo in the ovary. The study also reports the localization of pro- IT for the first time in the follicular layer. Further, both pro-VT and pro-IT transcripts were observed only in the fully grown post-vitellogenic follicles and not in the immature follicles. The study corroborates an earlier study by qRT-PCR in which the pro-VT and pro-IT expression was elicited by the isolated follicular envelope, and not in the denuded oocytes. The transcript levels in the ovary was shown to increase with gonadal recrudescence, reaching the peak in the spawning phase [24] when the catfish ovary contains the highest percentage of post-vitellogenic follicles. The non-neuronal expression of pro-VT and pro-IT in the ovary implies de novo production of the hormones in the ovary where they may act as paracrine/autocrine factors. Earlier studies from our laboratory have shown that VT has important physiological roles such as stimulation of steroidogenesis, prostaglandin secretion, oocyte hydration, final oocyte maturation (FOM) and ovulation [13-16,35]. Further, the expression of VT receptor genes (v1a1, v1a2 and v2A) was demonstrated in the catfish ovary [19]. Taken together these studies show that the catfish ovary has a functional VT system where it acts as a paracrine/autocrine factor. Although IT was found to be less potent in these functions [13-14], the presence of pro-IT in the follicular envelope like pro-VT indicate a paracrine role of IT too, but needs further study. In rainbow trout, IT gene expression was higher than VT expression during oocyte maturation [23]. The physiological relevance of extra-neural expression of nonapeptides may necessitates additional regulatory mechanisms that can cater exclusively to ovary-specific requirements. Therefore, the estrogen regulation of the nonapeptide precursor genes in the brain and ovary was separately investigated.

## Estrogen regulation of expression of pro-VT and pro-IT in the brain and ovary

In teleost ovary, estradio-17β (E2) is the principal estrogen and is mainly concerned with the induction of hepatic vitellogenin synthesis and feedback regulation of gonadotropins. Estrogens are also produced in the brain and are concerned with brain functions such as sexual differentiation, neuronal proliferation and growth, behaviour, etc. [32]. In the catfish, both brain- and ovary-specific aromatase genes and E2 were demonstrated [33,35]. Therefore, in the present study we employed a different strategy to differentiate the effects of E2 derived from the ovary and brain on the nonapeptide gene expression in the NPO-containing POA and ovarian follicles in vitro. The earlier studies using ovariectomy and E2 replacement model could show only the role of ovary-derived estrogen on nonapeptide secretion. Indeed, ovariectomy decreased, and E2 replacement reversed the effect of ovariectomy depending on the steroid dose [30,59]. The POA is a major site of aromatase activity and neuroestrogen production [34]. Exemestane is a known inhibitor of Cyp19a1a

(ovarian form). The concentration used in this experiment was about 100 times more than the IC50 value for cyp19a1a. It is very likely that the brain isoform Cyp19a1b may also be inhibited at this concentration causing a significant reduction in the total pool of estrogen in the brain and ovary. From the data, it is evident that the POA experimental model has responded to the exemestane and exogenous E2 treatments. The exemstane treatment did not alter the pro-VT gene expression but inhibited the expression of pro-IT. In other words, pro-VT expression may not be influenced by the POA-derived estrogen but the pro-IT expression seems to be modulated by the endogenous E2. The results suggest that VT and IT synthesized in distinct neurons in the NPO are differently controlled by the locally produced E2. In the catfish, it is not known, if VT and IT neurons synthesize neuroestrogens though the POA is aromatase- rich [34]. On the other hand, it is likely that estrogen receptor subtypes (ER-α, ER-βa or ER-βb) are distributed in different divisions of the NPO containing the VT and IT neurons [36,37]. In bluehead wrasse, aromatase-immunoreactive fibers (radial glial cells) are closely associated with VT-immunoreactive neurons throughout the POA, indicating the potential for functional interactions [60]. In the catfish, radial glial cells in the telencephalon and preoptic recess showed strong signals for brain type aromatase gene [35]. Both pro-VT and pro-IT expression increased in co-incubations of exemstane and E2. While the VT expression responded strongly to the high E2 dose (10 nM), the magnitude of IT expression was low but both doses responded equally. The results suggest that the VT and IT neurons respond to the exogenously supplemented/circulating peripheral estrogens and the IT neurons may be highly sensitive than the VT neurons. The previous ovariectomy and E2 replacement studies in the catfish reiterate the results. The differential dose effects of E2 and the lack of response of the VT expression to the exemstane treatment may be due to differential distribution and sensitivity of the ERs. The IT neurons may be rich in ERs or more sensitive to estrogen regulation than the VT expression. Further work is needed for a direct evidence of nonapeptide gene expression and ER interactions.

In the ovary, exemestane drastically reduced the pro-VT and pro-IT gene expression, implying that the ovarian E2 modulates the basal nonapeptide gene expression. The low dose of E2 did not restore the expression but the high dose did. It is possible that the 1 nM dose may not be sufficient to reverse the effect of exemstane and increase the gene expression. Our previous study reports that E2 exerts biphasic effects (stimulatory or inhibitory) depending on the dose, duration and reproductive phase [30]. In the preparatory phase, low doses (1 ng/mL and 10 ng/mL) were stimulatory and high dose (100 ng/mL) was inhibitory but in the prespawning phase, these doses were all inhibitory in a dose-dependent manner. Exemstane is strongly effective to decrease the gene expression in the ovary than the POA. This may be due to the fact E2 acts in a paracrine/autocrine manner in the ovarian follicles. Both E2 and nonapeptide genes are localized in the granulosa cells [35]. In the POA, E2 may act through the receptors directly or indirectly through other neural inputs like catecholamines [18,33]. The POA (NPO) nonapeptide systems are exposed to multiple control mechanisms since the hormones are secreted into the blood for the regulation of multiple physiological functions such as osmoregulation, stress, metabolism, reproduction, behaviour, circadian rhythms, and cardiovascular functions [3]. Therefore, the expression of nonapeptide genes in the NPO is regulated and integrated by various peripheral inputs. This is in contrast to the regulation of the nonapeptide genes in the ovary, which execute only the ovary-specific functions.

In conclusion, the present study demonstrates the spatial distribution of the nonapeptide precursor transcripts in the brain and ovary, and the differences in the extent of regulatory influence of E2 on the expression of the nonapeptide precursor genes in both the sites. This study forms a basis for future studies involving estrogen receptors and promoter activity of the genes.

## Acknowledgment

The research work was supported by a research grant of Department of Science and Technology, New Delhi (Grant No. SA/SO/AS-43/2009) to KPJ and RC. The first author is grateful to the Council of Scientific and Industrial Research, New Delhi for award of Junior and Senior Research Fellowships.

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