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Effects of Ovariectomy and Estrogen Replacement on Expression of Brain Vasotocin Receptor Subtype Genes in the Catfish *Heteropneustes fossilis*

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Abstract

Ovariectomy and estradiol-17β (E_2) replacement are classical approaches to study E_2 feedback relationships on the brain-pituitary-neuroendocrine axis. Earlier studies in the catfish have shown that ovariectomy decreased brain and plasma vasotocin (VT) levels; a low dose of E_2 replacement restored, but high doses further inhibited the VT levels. The feedback effects of E_2 on VT secretion were similar to that on gonadotropin secretion. Previously, we have studied the expression of VT receptor gene subtypes (*v1a1, v1a2* and *v2a*) in the brain, which showed seasonal sex dimorphic variations. But studies on the effect of E_2 on VT receptor subtype gene expression are lacking in teleosts. We used ovariectomized-E_2 replaced catfish model to demonstrate E_2 effects on brain VT receptor gene expression. The experiment was conducted in the gonad active phase (prespawning phase). Ovariectomy (Ovx) for 3 weeks decreased the expression of *v1a1* and *v1a2* but not of *v2a.* A low dose of E_2 (0.1 µg/g BW) replacement reversed the effect of ovariectomy and restored the expression of *v1a1* and *v1a2.* A high dose of E_2 (0.5 µg/g BW) replacement did not alter the transcript level of *v1a1* compared to the Ovx-vehicle group but increased the *v1a2* expression compared to the Ovx control group, lower than that of the sham control group. But the low and high doses of E_2 replacement did not alter the *v2a* receptor transcript level. In conclusion, E_2 modulates only the expression of *V1a* type receptor gene paralogs. VT may elicit the estrogen-dependent reproductive and behavioral effects through the *V1a* type receptors.

Keywords: Catfish; Ovariectomy; E_2 replacement; Brain VT receptor subtypes; Gene expression

Introduction

Neurohypophyseal nonapeptide hormones (vasopressin- VP, oxytocin OT and vasotocin- VT) perform multiple regulatory functions related to osmoregulation, metabolism, stress, cardiovascular activity, circadian rhythm, reproduction and behavior in different vertebrates [1-6]. These effects are mediated through membrane-bound G-protein coupled receptors. Previously, three types of VP / VT receptors namely *V1a, V1b* and *V2* subtypes have been reported in mammals, birds, amphibians and teleosts [7-12]. Oxytocin / Mestocin acts through a single receptor type (OTR / MTR), which in teleosts have two isoforms, Isotocin receptor- type 1 and 2 (ITR1 and ITR2) [13-16].

Though gonadal steroid hormones have been reported to modulate the secretion of the neurohypophysial nonapeptides in vertebrates, there are few studies that report the effect of steroid hormones on nonapeptide receptor activity in sub mammalian vertebrates [14, 17-29]. Such investigations are almost non-existent among teleosts. It was shown that gonadectomy reduced the concentrations of putative receptors for VT in the brain of amphibians [7]. A possible reason for limited studies may be the non-availability of sensitive assay methods. Rawat et al. [30] cloned and characterized three VT receptor genes in the catfish: two *V1a* type and one *V2* type. This has enabled us to make RNA probes for the analysis of receptor gene expression in various tissues.

The catfish *Heteropneustes fossilis* has been extensively used to study the reproductive role of VT [21]. Apart from brain, VT is also synthesized in the ovary and the VT levels in brain, plasma and ovary were shown to vary according to the reproductive phases [31]. Subsequently, VT was demonstrated to influence ovarian steroidogenesis, oocyte maturation, oocyte hydration, prostaglandin synthesis and ovulation [32-35]. Steroid hormones including estradiol-17β (E_2) were shown to alter the VT levels [25, 26]. E_2 is the principal estrogen in teleosts and induces vitellogenin synthesis in the liver and growth of oocytes. Ovariectomy and E_2 replacement are conventionally used to study the feedback effect of E_2 on the brain-pituitary- neuroendocrine axis [36, 37].

The objective of the present study was to demonstrate estrogen regulation of expression of VT receptor sub type genes. For this, we employed ovariectomy and E_2 replacement strategy, which is established for the catfish previously. The data show differential effects of the treatments on VT receptor gene expression, which has been reported apparently for the first time in this study.

Materials and Methods

Animal collection and acclimatization

Adult female *Heteropneustes fossilis* (50-55 g) were collected from local fish markets in Varanasi in the second week of May, which was late vitellogenic phase or prespawning phase: (9.05 ± 0.1% gonado-somatic index, GSI). They were maintained in the laboratory for a week under natural photoperiod (13.0 L: 11.0 D) 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), veriquest SYBR green qPCR master mix (Affymetrix) and DNase I (Ambion) were purchased through local suppliers. Agarose, tris base, glacial acetic acid, EDTA-Na2, proteinase K and other chemicals were of molecular grade and purchased from E-Merck, Mumbai, India. The primers used were synthesized by Integrated DNA Technology (IDT), India. Diethyl pyrocarbonate (DEPC), MS-222 and estradiol-17β (E_2) were purchased from Sigma Chemical Company, St. Louis, USA.

Experiments

Ovariectomy and E_2 replacement

After acclimatization, five female fish were sacrificed and the brains collected in RNA later to make an initial control (IC) group. The operations were conducted according to the procedure of Senthilkumaran and Joy [36]. Female catfish were anesthetized by spraying 0.01% of MS-222 over the gills. A 4 cm long midventral 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 (16000 IU/I) for 3-5 days to prevent skin infection. Thirty anesthetized fish were ovariectomized (ovx) and an equal number was sham operated and maintained for 3 weeks. At the end of the three weeks, 5 fish each from the ovx and sham operated groups were killed by decapitation and brains were collected in RNA later (ovx and sham ovx groups). For estrogen replacement, the 3-week ovx and sham operated fish were administered intraperitoneally with vehicle, 0.1 and 0.5 µg/g body weight (BW) E_2 for three consecutive days, making the groups of ovx + vehicle, ovx + 0.1 µg/g BW E_2, ovx + 0.5 µg/g BW E_2, sham+ vehicle, sham + 0.1 µg/g BW E_2 and sham + 0.5 µg/g BW E_2. E_2 was dissolved in a small volume of ethanol and then diluted with propylene glycol to prepare a stock solution. At the end of the three days, the fish were sacrificed by decapitation and the brains were collected in RNA later. Tissues in RNA later were stored in -20°C until further processing.

qPCR assay for VT receptor gene expression

Total RNA was extracted from the tissues (100 mg) stored in RNA later by a single step method of RNA isolation. RNA purity was checked by calculating A260 / A280 ratio. Samples having a ratio above 2.0 were only used. Absence of genomic DNA contamination in the RNA preparation was confirmed by using non-reverse transcribed samples as templates. In addition, the absence of DNA in total RNA was ensured by treating with DNAse I before proceeding for the first strand cDNA synthesis. Five µg of 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. Gene-specific primers were designed for *v1a1*, *v1a2* and *v2a* from the respective sequences (Table 1). Primers for β-actin were used for the internal control. The specificity of each primer pair was confirmed by dissociation curve analysis. The qPCR assays were performed in triplicate for different samples using the specific primers and VeriQuest TM SYBR Green qPCR master mix with ROX (Affymetrix, Inc. Cleveland, Ohio USA) in a ABI Prism 7500 thermal cycler (Applied Biosystems, Foster, CA, USA) at 95°C (15 s), 60°C (1 min) for 40 cycles.

Table 1: Primer used for quantitative PCR.

Adaptation			Sequence (5'-3')
qPCR primer	*v1a1*	Forward	CCAAACTCCGCACCGTCAA
		Reverse	ATGCGGATAGGGTCACTGCT (150 bp)
	v1a2	Forward	TAGTGTGCTGGGCACCGTT
		Reverse	GATCCAGGGGTTGCAGCAG (140 bp)
	v2a	Forward	CAGCGTGAGCACCATCTCC
		Reverse	ATGCGGATAGGGTCACTGCT (173 bp)
DNA Control	β-actin	Forward	TGGCCGTGACCTGACTGAC
		Reverse	CCTGCTCAAAGTCAAGAGCGAC(157 bp)

Each sample was run in a final volume of 20 µL containing 1 µL of cDNA, 10 pM of each primer, and 10 µL of SYBR Green PCR master mix. Specificity of amplicons was verified by melting curve analysis (60 to 95°C) after 40 PCR cycles.

As controls, the assays were performed without templates. No amplification was observed in the control studies. Cycle threshold (Ct) values were obtained from the exponential phase of the PCR amplification and target gene (*v1a1, v1a2* and *v2a*) expression was normalized against β-actin gene expression to generate a Ct value. Comparative Ct (ΔΔCT) was used to quantify the target gene abundance [38].

Statistical analysis

The data were expressed as mean ± SEM of five replicates. Statistical analysis was carried out using two way ANOVA ($p < 0.001$), followed by Tukey's test ($p < 0.05$).

Results

The 3 week ovx and E_2 replacement produced overall significant changes in brain *v1a1* transcript levels (two way ANOVA, $p < 0.001$; $F_{ovx} = 167.89$; $F_{E2} = 34.97$; $F_{interaction} = 56.98$) (Figure 1). There was no significant change in the *v1a1* transcripts in the sham operated group as compared with the initial control group. The 3-week ovx decreased the *v1a1*transcripts significantly from the initial control and sham control groups. The low dose (0.1 µg/g BW) of E_2 increased and restored the *v1a1* transcript levels in the ovx group as compared to the ovx + vehicle group. On the other hand, the high dose (0.5 µg/g BW) of E_2 did not alter the *v1a1* transcript levels in the ovx group. The E_2 administration did not produce any effect on gene expression in the sham control groups.

The 3 week ovx and E_2 replacement caused overall significant changes in brain *v1a2* transcript levels (two way ANOVA, $p < 0.001$; $F_{ovx} = 198.13$, $F_{E2} = 24.12$, $F_{interaction} = 167.85$) (Figure 2). There was no significant change in the *v1a2* transcripts in the sham operated group as compared with the initial control group. The 3-week ovx decreased the *v1a2* transcripts significantly from the initial control and sham control groups. The low dose of E_2 increased and restored the *v1a2* transcript levels in the ovx group as compared to the ovx + vehicle group. On the other hand, the high dose of E_2 significantly increased the *v1a2* transcript levels compared to the ovx groups but did not restore the levels compared to the initial control and sham control groups. The E_2 administration did not produce any effect on the gene expression in the sham control groups.

The 3 week ovx and E_2 replacement did not alter the expression of *v2a* receptor (Figure 3).

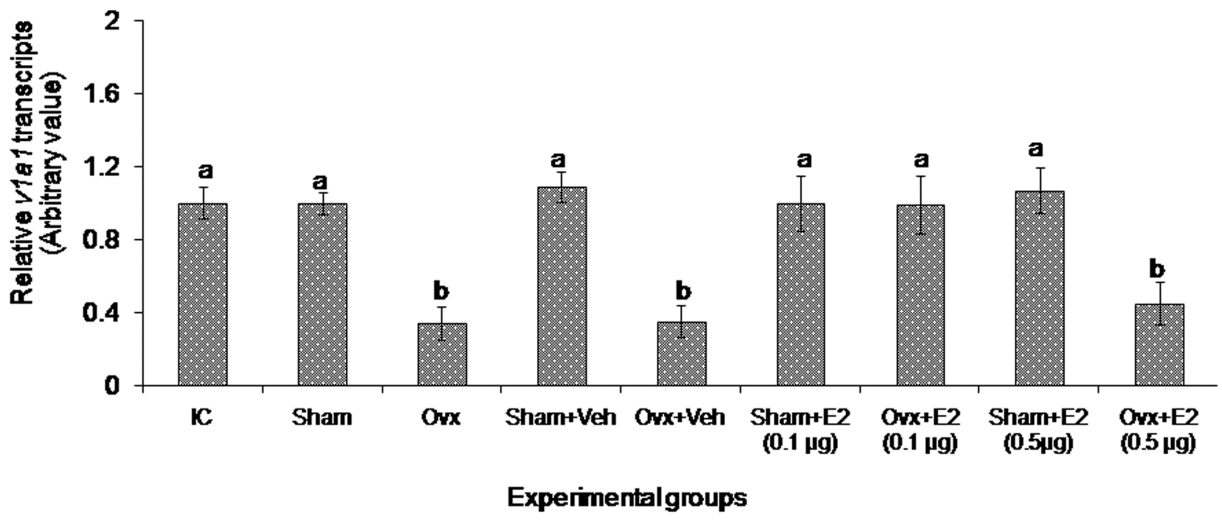

Figure 1: Expression of brain *v1a1* after 3 week ovariectomy and E_2 replacement. Data are means ± SEM (n = 5). Groups bearing different letters are significantly different. IC: Initial Control, Ovx: Ovariectomy, Veh: Vehicle Control.

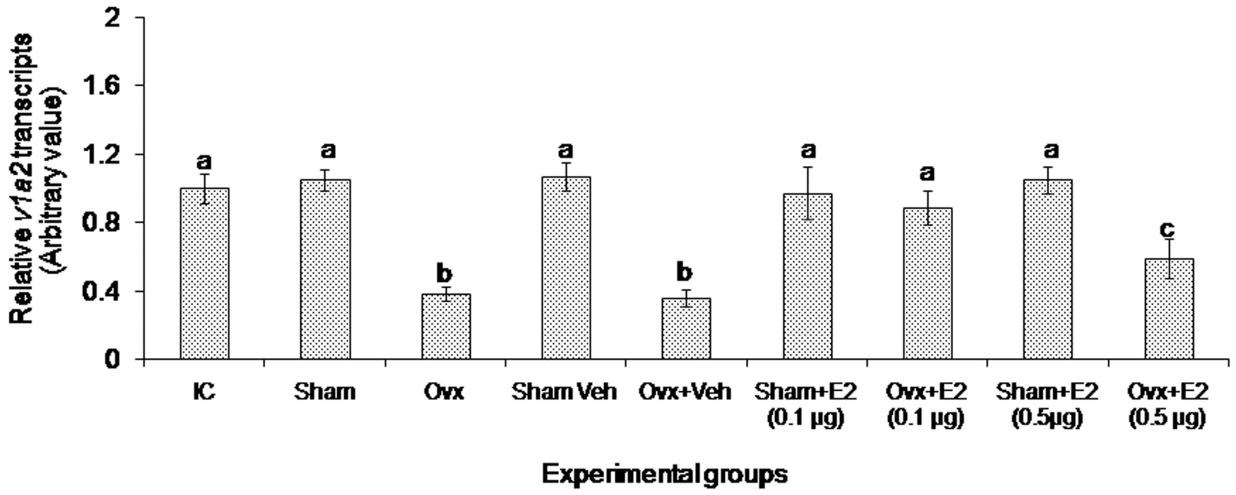

Figure 2: Expression of brain *v1a2* after 3 week ovariectomy and E_2 replacement. Data are means ± SEM (n = 5). Groups bearing different letters are significantly different. IC: Initial Control, Ovx: Ovariectomy, Veh: Vehicle Control.

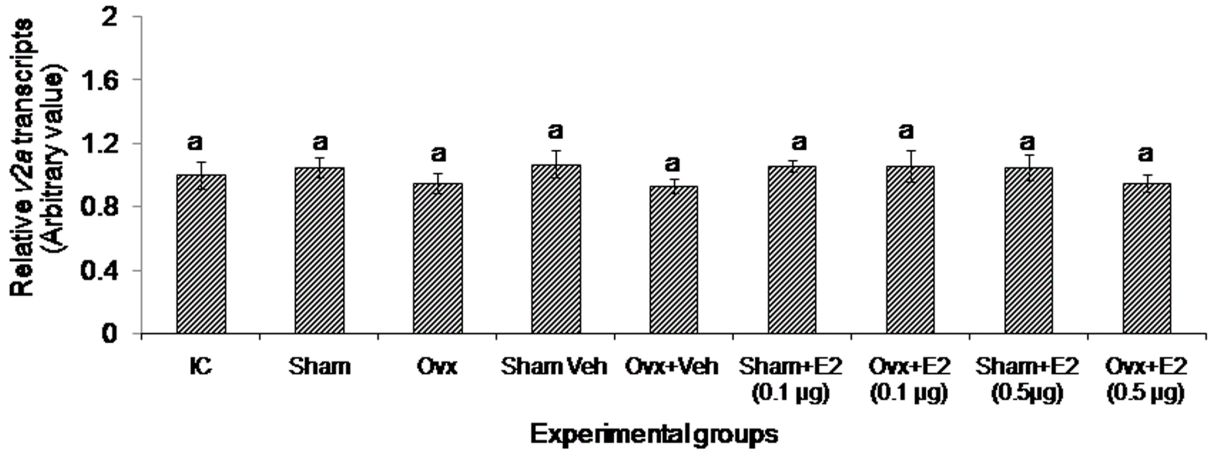

Figure 3: Expression of brain *v2a* after 3 week ovariectomy and E_2 replacement. Data are means ± SEM (n = 5). Groups bearing the same letters are significantly different. IC: Initial Control, Ovx: Ovariectomy, Veh: Vehicle Control.

Discussion

The present study appears to be the first in fishes on the E_2 modulation of brain VT receptor genes in a fish species. The data show that both ovx and E_2 replacement have elicited differential effects on the VT receptor transcript levels. The *V1a* type receptor genes (*v1a1* and *v1a2)* responded to both the treatments while neither treatment had any significant effect on the *V2* receptor gene expression. Ovariectomy and E_2 supplementation are conventional methods used to study E_2 feedback effects on gonadotropin secretion [39]. In the catfish, ovx led to a duration-dependent decrease in plasma E_2 levels and an increase in plasma gonadotropin levels [36, 37]. The 3 week ovx led to about 3-fold reduction in the transcript levels of *v1a1* and *v1a2*, apparently due to a fall in the E_2 level. Thus, ovarian E_2 exerts a positive feedback effect on *V1a* type receptor gene expression. The E_2 supplementation study gave further insight into the nature of the feedback. The E_2 treatment produced a dose-dependent effect on the expression of both *v1a1* and *v1a2*. The low dose of E_2 (0.1 µg) restored the transcript levels to that of the control groups, but the high E_2 dose (0.5 µg) did not alter the ovx effect on the *v1a1* expression and only slightly elevated the *v1a2* expression. Apparently, E_2 elicited stimulatory or inhibitory responses on the gene expression depending on the E_2 titer. The exogenous E_2 administration did not produce any effect in the sham control groups, which may suggest that the steroid treatment may not have any effect on basal transcriptional activity in the presence of endogenous E_2. There is a parallel

trend in the biphasic effects of E_2 on gonadotropin secretion on one hand and VT on the other [25, 26, 36, 37].

There are limited studies on the effect of gonadectomy and steroid treatment on VT receptor activity in vertebrates. Gonadectomy significantly reduced putative receptor concentrations in the amygdala of newt and bullfrog [27, 40]. Estradiol treatment of gonadectomized male and female bullfrogs not only restored receptor concentrations but those levels surpassed the sham levels. Treatment of photorefractory Japanese quail (*Coturnix coturnix japonica*) with estrogen increased shell gland activity (mass and length of mucosal folds) and levels of both VT and V3 (*VT3*, oxytocic-like) receptor mRNA, whereas treatment of photosensitive birds with the estrogen antagonist tamoxifen decreased shell gland activity and levels of both VT and *VT3* receptor mRNA [28, 41]. In mammals, a positive functional relationship exists between estrogen and uterine OT/OT mRNA/OT receptor [42, 43]. Oxytocin receptor (OTR) is also modulated by E_2 in receptor binding studies using 125I-ornithine vasotocin in MCF7 cells, and the upstream palindromic estrogen-response element (ERE) in the rat OR promoter imparts E_2 induced OR gene transcription [44]. Tribollet et al. [45] reported that binding of OT to the uterus was estrogen-dependent, and castration and inhibition of aromatase activity reduced, while estradiol and testosterone increased OT binding, particularly in regions of the brain in male and female rats. In the same animals, VP binding in the brain was not affected under those conditions. In sheep, estrogen increases transcription of the OT receptor gene through interaction of estrogen receptor α with a GC rich SP1 enhancer element [43].

Since the *V1a* type receptor mediates the reproductive function of VT, E_2 modulates only the reproductive function of VT and the antiduretic / antidiuretic actions of VT mediated via the *V2A* receptor are non-responsive to the estrogen status. Since VT has the ring structure of OT and the tail structure of VP, the receptor subtypes through specific signaling mechanisms delineate the reproductive and osmoregulatory functions of the peptide. The estrogen modulation of *V1a* type receptor in the catfish, the oxytocic VT3 receptor type in birds and the OT receptor type in mammals indicates a phylogenetically conserved basic mechanism of regulation, which is distinct from the regulation of the *V2* type receptor gene expression. The distinction is further evident from the fact that these receptor types (*V1a*, V3/*V1b* and OT) are coupled to the phospholipase C (PLC)/protein kinase C (PKC) signaling pathway while the *V2A* type receptor is linked to the cAMP-PKA pathway [13-15]. The receptor subtypes and the specific signalling pathways determine the specific functions of VT, given its multiple tissue targets.

In the catfish brain, the *v1a1* and *v1a2* transcripts are highly expressed throughout the reproductive cycle and the *v2a* transcripts are less expressed as shown in the semi-quantitative assay [30]. In the breeding phase (preparatory, prespawning and spawning phases), the E_2 level fluctuates due to its feedback activity with gonadotropin secretion [36]. The fluctuations in the transcript levels of *v1a1* and *v1a2* in the brain [30] can be associated with the E_2 oscillations, suggesting the existence of a negative feedback control. After spawning, the ovary enters the quiescent phase when the E_2 levels are low. The negative feedback mechanism may result in elevated transcript levels especially in the spawning phase. The seasonal pattern of expression of the *V1a* type receptor gene expression and the response to ovariectomy and E_2 treatments suggest that VT engages the *v1a1* and *v1a2* receptors for the reproductive and behavioral effects. Although we did not see any significant difference in the *V1a* type and *V2a* type transcript levels in the qPCR assay in initial control and sham control groups, the *v2a* expression in the brain may be controlled by other mechanisms, which are to be investigated in future studies. The *v2a* gene expression is low throughout and not influenced by the E_2 oscillations. The present data support this contention. It is not clear at present as to what role neuroestrogens may have on the VT receptor subtype gene expression. It is hypothesized that the neuroestrogens may be related to differential expression of the receptor genes. In the pupfish, *v1a1* and *v1a2* are most abundant in the midbrain (optic tectum and hypothalamus) and cerebellum in both sexes [11]. In the protogynous bluehead wrasse, the *v1a1* transcript is more abundant in telencephalon, hypothalamus, optic tectum, cerebellum and medulla oblongata regions of both male (initial phase and terminal phase) and female fish, while the *v1a2* transcript is more abundant in the hypothalamus and cerebellum [12]. VT receptor protein/gene (*V1a2/v1a2)* has been shown to be distributed widely in the brain of rock hind using immunohistochemical / *in situ* hybridization technique [46]. These studies indicate that the *V1a* type receptors are distributed in areas concerned with pituitary regulation, reproductive behavior, olfaction, learning and sensory functions. In the catfish, *in situ* hybridization study of the three VT receptor subtype genes shows that the *v1a* paralogs are distributed throughout the brain including the pituitary in high abundance while the *v2a* type is confined to the anterior dorsal ependymal / subependymal lining of the telencephalon and the pituitary in low levels (our unpublished results). The *v2a* transcript levels are low throughout the reproductive cycle in the catfish and pupfish [11, 30]. But in medaka and gilthead sea bream, the expression of *v2a* is high [47, 48]. In the pupfish, acute exposure to hypersalinity (17 ppt or 34 ppt) increased the *v1a1* and *v1a2* transcript levels but reduced *v2a* levels in the hypothalamus [11]. In the gilthead sea bream, hyper and hypo osmotic conditions enhanced hypothalamic *v2a* levels after 7 days of the treatments [48]. The variations in the gene expression in these species may reflect the habitat (freshwater versus marine environment) and habits (breeding pattern, morphe and behavior) and a generalization is difficult to make due to lack of comparative studies. Alternatively, different fishes may use different receptor gene paralogs to mediate VT actions.

Although we did not analyze the VT receptor subtype gene expression separately, the transcript abundance is generally low in the pituitary compared to the brain regions [30]. The transcript abundance of *v1a1* and *v1a2* is the highest in the rostral pars distalis of the pituitary while *v2a* has relatively low levels (unpublished *in situ* hybridization results). In the pupfish, the expression of *v1a1* and *v2* is high in the pituitary,

but that of *v1a2* is low [11]. In the gilthead sea bream, *v2* transcripts are highly expressed in the pituitary with low *v1a2* levels [48]. The pituitary is the major target of VT fibers from which the hormone is released into circulation [31]. Additionally, the VT fibers directly innervate the adenohypophysial cells suggesting VT modulation of the hormone secretion. Fryer and Leung, reported corticotropin (ACTH) releasing activity of VT and IT [49]. Pierson et al. [50] reported that the VT stimulation of ACTH was through the mediation of the V1 type receptor. VT has been shown to stimulate gonadotropin release in *Poecilia latipinna* [51]. In *Cichlasoma dimerus*, a species with alternative phenotypes, VT stimulated LH (dose-related effects) and FSH secretion in pituitary incubates, and androgen release from testicular fragments [52]. In the catfish, the differential response of the VT receptors to estrogen levels suggests VT control of gonadotropic activity via the *V1a* type receptor. In conclusion, estrogens may modulate reproductive and behavioural responses partly through VT actions via the *V1a* type receptors.

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