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Molecular characterization and expression of arginine vasotocin V1a2 receptor in Atlantic croaker brain: potential mechanisms of its downregulation by PCB77

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Abstract

The arginine vasotocin (AVT)-V1a receptor mediates critical reproductive behaviors of the nonapeptide vasotocin in the teleost brain. In this study, we report the molecular characterization of the AVT-V1a2 receptor and its mRNA and protein expressions in Atlantic croaker brain after exposure to the planar polychlorinated biphenyl congener 3,3',4,4'-tetrachlorobiphenyl (PCB77). The full-length sequence of croaker AVT-V1a2 receptor cDNA is highly homologous to other teleost AVT-V1a2 receptor cDNAs. Double-labeled immunohistochemistry showed co-expression of AVT-V1a2 receptor and gonadotropin-releasing hormone-I (GnRH-I, a neuropeptide that regulates gonadotropin secretion) in hypothalamic neurons, thereby providing the anatomical basis for possible AVT modulation of croaker reproduction through alterations in GnRH-I secretion. AVT-V1a2 receptor mRNA and protein levels as well as GnRH-I mRNA levels were markedly decreased in hypothalamic tissues of croaker exposed to PCB77 (dose: 2 and 8 µg/g body weight for 4-week) compared to levels in untreated (control) fish. In contrast, hypothalamic cytochrome P450 1A (CYP1A, a monooxygenase enzyme) and interleukin-

1β (IL-1β, a cytokine indicator of inflammation and response to neuronal damage) mRNA levels, and plasma protein carbonyl (PC, an indicator of reactive oxygen species) contents, important biomarkers of neural stress, were increased in PCB77-exposed fish compared to controls. Collectively, these results suggest that the downregulation of hypothalamic AVT-V1a2 receptor and GnRH-I transcripts due to PCB77-exposure is associated with induction of CYP1A, cellular inflammation and oxidative stress in Atlantic croaker, a marine teleost that inhabits estuaries along the US Atlantic coast and the Gulf of Mexico that are often contaminated with persistent organic pollutants such as PCBs.

1 INTRODUCTION

Exposure to endocrine disrupting chemicals (EDCs) has been shown to cause a wide range of deleterious effects in terrestrial and aquatic animals during their susceptible periods of life.[1,2] There is growing evidence that exposure to EDCs contributes to neurological deficits due to declines in neuroenzyme, neuropeptide and neurotransmitter levels in vertebrate brains.[3-5]

Polychlorinated biphenyls (PCBs) were first identified in wildlife and biological samples in 1966.[6,7] PCBs are ubiquitous and persistent environmental contaminants and have been linked to carcinogenicity, impairments of growth, reproduction and development in terrestrial and aquatic organisms.[8-10] Although the manufacture of PCBs was prohibited in the United States in the late 1970s, and in several countries in Asia and most of Europe in the 1980s, the persistence of PCBs in terrestrial and aquatic

environments, and their bioaccumulation, biomagnification and toxicity remain an ecological risk to the present day in wildlife and aquatic organisms.[11-16]

PCB congeners are usually divided into two groups based on their chemical structures: planar (non-*ortho*-substituted) and non-planar (*ortho*-substituted). Planar PCBs are considered the most toxic and are laterally substituted with *non-ortho* Cl^- atoms, which allows hydrogen atoms to incorporate, thus creating an overall planar structure that is resistant to biodegradation.[11,17] Some of the congeners, such as PCB77, PCB153 and PCB156 are EDCs.[18] In addition, planar PCBs along with other organic pollutants and their corresponding metabolites bind to the aryl hydrocarbon receptor, a ligand-activated transcription factor, and up-regulate cytochrome P450 enzyme activity in liver and other tissues such as the brain which can increase their toxic effects.[19,20] Exposure to PCB congeners has been shown to cause developmental neurotoxicity as well as disruption of neuroendocrine functions in the vertebrate brain resulting in reproductive impairment.[21]

Arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin (AVP) influence various physiological and neuroendocrine functions such as social interactions, sexual behavior, reproduction, gonadotropin secretion, appetite reduction, metabolism, renal function, osmoregulation, circadian and seasonal rhythms, and stress responses.[22-30] The AVT receptor (AVTr) is generally considered ancestral to some other vertebrate nonapeptide receptors because its expression can be induced in a variety of tissues with either a retentive or diverse functionality.[31] Recently, three AVTrs (AVT-V1a, -V1b, and -V2) have been identified that differ in their molecular characteristics and

tissue distribution in tetrapods and teleost fishes.[32,33] The AVT-V1a receptor is highly expressed in regions of the teleost brain involved in the regulation of reproductive behaviors[34] and is co-localized with gonadotropin-releasing hormone-I (GnRH-I) neurons in the preoptic anterior hypothalamic area which is a primary hypothalamic region of the reproductive neuroendocrine system.[30] In addition, the AVT-V1a receptor is also highly expressed in the gill, intestine and kidney in teleosts through which AVT regulates osmoregulation[24,28] and in fish gonads[28], where AVT has been demonstrated to modulate steroid production,[27] while the AVT-V1b receptor is linked with adrenocorticotropic hormone function in the pituitary gland.[30,31,34-36] The V2 receptor, on the other hand, regulates osmoregulation via aquaporin in the kidney.[28,37] Among these three subtypes, the AVT-V1a receptor has received most attention in neuroendocrine and xenobiotic research on tetrapod and teleost species.[38,39]

The aim of this study was to clone and characterize the AVT-V1a receptor gene in the brain of Atlantic croaker, a marine teleost, and to examine its mRNA and protein expressions after PCB77 exposure. GnRH treatment has been found to modulate AVT release in teleost brain.[40,41] Therefore, the co-expression of hypothalamic AVT-V1a receptor and GnRH-I neurons was investigated to provide a potential neuroanatomical basis for any close interactions between these two neuropeptides. Finally, the effects of PCB77-exposure on hypothalamic cytochrome P450 1A (a monooxygenase enzyme), interleukin-1β (a pro-inflammatory cytokine) mRNA levels, and plasma protein carbonyl (an indicator of reactive oxygen species, ROS) contents were measured in order to assess

oxidative stress in Atlantic croaker as a potential mechanism for downregulation of AVT-V1a and GnRH-I expression and reproductive neuroendocrine functions.

2 MATERIALS AND METHODS

2.1 Reagents

Rapid amplification of cDNA ends kits (RACE System) and 3,3',4,4'-tetrachlorobiphenyl (PCB77) were purchased from Invitrogen (Carlsbad, CA, USA) and Chem Service (West Chester, PA, USA), respectively. Molecular biology grade water and reagents were obtained from Agilent Technologies (La Jolla, CA, USA), Argent Chemical (Redmond, WA, USA) and Promega (Madison, WI, USA). All other analytical grade chemicals were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and MilliporeSigma (St. Louis, MO, USA) unless noted otherwise. The GnRH-I antibody was a generous gift from Dr. Koichi Okuzawa and Dr. Hirohiko Kawaga at the National Research Institute, Japan. Rabbit polyclonal anti-actin and goat polyclonal to rabbit IgG horseradish peroxidase (HRP)-linked antibodies were purchased from Novus Biological (Littleton, CO, USA) and Southern Biotech (Birmingham, AL, USA), respectively.

2.2 Experimental animals

Young-of-the-year croaker (total length: 10-11 cm; body weight, BW: 12-18 g) were captured using shrimp trawls by local commercial fishermen in Redfish Bay in Texas (USA) during late summer according to the approved wildlife species capture rules and regulations by Texas Parks and Wildlife Department (permit no. SPR-0790-184). Fish were transported to laboratory, treated with Paracide-F, stocked in indoor recirculating

sea water tanks (4,727 L capacity with biofilters, salinity 30-32 ppt), and maintained in ambient natural fall temperature (22±°C) and photoperiod conditions (light/dark cycle 11:13 h). Fish were fed a mixed diet (chopped frozen shrimp and commercial trout pellets, 3% BW) once a day. All fish were allowed to acclimate for at least 1 month prior to PCB77 exposure experiments, when they had begun gonadal development.

2.3 Ethics Statement

Experimental fish were handled according to National Institutes of Health (Bethesda, MD, USA) guidelines for handling and care of animals (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf). PCB77 exposure experiments were conducted following ethical rules and regulations, and a protocol approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin (IACUC protocol no. 08080401).

2.4 Experimental protocols

Fish (30 mixed-sex fish/tank) were stocked in six recirculating seawater experimental tanks (2,025 L capacity) maintained under temperature-controlled laboratory conditions. Two tanks were designated as controls and fed a control diet (3% BW/day, no PCB77 added to chopped shrimp and commercial trout pellets), another two tanks were fed the prepared food laced with a low dose of PCB77 (2 µg PCB77 with chopped shrimp and commercial trout pellets/g BW/day), and the third set of two tanks were fed higher sublethal dose of PCB77 (8 µg/g BW/day). The concentrations of PCB77 used in this study were selected on the basis of environmental levels of PCBs and previous research

publications in croaker and other teleost fishes.[42-47] A YSI handheld multi-parameter instrument (YSI Incorporated, Yellow Springs, OH, USA) was used to measure dissolved oxygen, water temperature and pH during the experimental periods. At the end of the exposure period, fish were treated with quinaldine sulphate (20 mg/L; MilliporeSigma) for approximately 1-2 min, blood samples collected for measurement of protein carbonyl, and sacrificed following ethical rules approved by IACUC (protocol no. 08080401) at the University of Texas at Austin. Whole brains were carefully removed, quickly frozen in liquid nitrogen and stored at -80°C until used for RNA extraction, measurement of mRNA levels and protein expression. For immunohistochemical analysis, brain samples were fixed in 4% buffered paraformaldehyde solution (pH 7.4) overnight at 4°C.

2.5 Reverse transcription-polymerase chain reaction (RT-PCR), cloning and sequencing

Molecular techniques used for RNA extraction, RT-PCR amplification, plasmid DNA purification, ligation, cloning and sequencing were performed as described by Rahman and Thomas.[48,49] Briefly, RNA was extracted from croaker brain using TRI reagent (MilliporeSigma) and treated with DNase-I following manufacturer's protocol (Promega) to eliminate genomic DNA. The integrity of total RNA was determined via electrophoresis on ethidium-bromide stained agarose gel (1%) and quantified with NanoDrop™ (Thermo Scientific). First-strand cDNA was synthesized from total RNA (1 µg) using SuperScript III enzyme Oligo(dT) primer as described in manufacturer's protocol (Invitrogen). Partial cDNA of AVT-V1a2 receptor was obtained by PCR amplification using PCR master mix (GoTaq® Green MasterMix, Promega) and partial

sequence primers (Table 1). The amplified PCR products were separated using an ethidium-bromide stained agarose gel in Tris/Borate/EDTA buffer, purified and ligated into pGEM®-T Easy Vector, and then transformed into JM109 competent cells (Promega). Plasmid DNA was purified and sequenced by Sanger sequencing in both directions using T7 and Sp6 primers. The full-length nucleotide sequence of croaker AVT-V1a2 was obtained with 5'- and 3'-RACE amplification protocol (Invitrogen) using croaker AVT-V1a2 gene-specific primers (Table 1) and verified using NCBI database (https://www.ncbi.nlm.nih.gov/). The full-length amino acid sequences of vertebrate AVTr isoforms collected from GenBank® (http://www.ncbi.nml.nih.gov/genbank) were used for multiple alignments using MultiAlign program (http://multalin.toulouse.inra.fr/multalin/) according to Corpet.[50]

2.6 Phylogenetic analysis

Phylogenetic analyses of AVTr genes were carried out using the neighbor-joining method and consensus trees of AVTr genes were constructed using MEGA4 software.[51,52] A bootstrap analysis of 1,000 replicates was used to assess the degree of groupings on the trees.

2.7 Micro-dissection of croaker brain

The frozen brains were dissected on ice into seven parts: olfactory bulbs (OB), telencephalon (TEL), preoptic-anterior hypothalamus (POAH), pituitary (PIT), midbrain tegmentum (MT), cerebellum with optic tectum (CE-OT), and medulla oblongata (MO) with the aid of a croaker brain atlas.[48,53]

2.8 Real-time quantitative RT-PCR (qRT-PCR) analysis

RNA was extracted from micro-dissected brain parts (OB, TEL, POAH, MT, CE-OT, and MO), pituitary, eye, gill, heart, intestine, kidney, liver, muscle, ovary, spleen and testis using TRI reagent and treated with DNase I to eliminate genomic DNA (Promega). qRT-PCR analysis was performed according to Rahman and Thomas.[48,49,54] Briefly, the relative expression of croaker AVT-1aV2, GnRH-I, cytochrome P450 1A (CYP1A) and interleukin-1β (IL-1β) mRNAs was determined using total RNA. Total RNA from brain parts and other peripheral tissues was amplified for 40 cycles (95°C for 30 s, 55°C for 1 min, and 72°C for 30 s, and melting curve cycling profile at 95°C for 1 min, 50°C for 30 s, and 95°C for 30 s) of qRT-PCR with croaker AVT-1aV2, GnRH-I, IL-1β, CYP1A and 18S primers (Table 1). qRT-PCR amplification was assayed in 250 ng of RNA, 25 µl of reaction mixture containing 12.5 µl of 2x SYBR® Green-qRT-PCR master mix, 50 nM of gene specific primers, and 0.063 µl of StrataScript® RT/RNase enzyme by Mastercycler® realplex system (Eppendorf, Thermo Fisher Scientific). The relative mRNA expression levels of AVT-1aV2, GnRH-I, CYP1A and IL-1β were calculated using the $2^{-\Delta\Delta C}$T method.[55]

2.9 Preparation of membrane protein

Membrane fraction was isolated from croaker tissues as described previously by Thomas and Rahman[56] with minor modifications. Briefly, hypothalamic tissue was homogenized with HAED buffer (25 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA, pH 7.6) and protease inhibitor (Pierce, Rockford, IL, USA) in a glass homogenizer. The tissue homogenate was centrifuged at 1,000 x *g* for 15 min and

collected the supernatant containing the membrane fraction. The supernatant was centrifuged at 17,000 x *g* for 45 min and the membrane pellet was obtained. The pellet was then dissolved in HEAD buffer and stored at -80°C until AVT-1aV2 protein expression analysis using Western blot method.

2.10 Western blot analysis for AVT-1aV2 receptor and actin proteins

Membrane protein (10 µg total protein) was dissolved in a loading buffer (0.5 M Tris-HCL, 10% sodium dodecyl sulfate, 0.5% bromophenol blue, 10% glycerol) by boiling for 10 min and cooled on ice for 5 min. The solubilized protein was then electrophoresed in a 10% SDS-PAGE gel transferred onto a polyvinyl difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) and blocked in Tris-buffered saline with 5% nonfat milk (TBS-T: 50 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h at room temperature. Membranes were probed with AVT-1aV2 primary antibody (dilution 1:1,000) and 5% nonfat milk overnight at 4°C. The specificity of AVT-1aV2 primary antibody has been validated in a marine teleost brain previously by Kline et al.[34] Membranes were then washed three times with TBS-T and incubated with goat polyclonal rabbit secondary antibody (dilution 1:10,000; Novus Biologicals, Littleton, CO, USA) for 1 h at room temperature. Actin protein (~45 kDa) was used as an internal control. AVT-1aV2 and actin protein expressions were visualized by adding chemiluminescent substrate (Pierce, Rockford, IL, USA) on PVDF membrane and photographed on Hyperfilm™ (Amersham Biosciences Corporation) in dark condition. The intensity of AVT-1aV2 and actin protein expressions was estimated using ImageJ software.[57]

2.11 Single-immunoreactive (IR) staining of AVT-1aV2 receptor

The IR signal of AVT-V1a2 protein in croaker brain was amplified by diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA, USA) according to Kline et al.[34] Briefly, whole brains were fixed in 4% buffered paraformaldehyde solution (pH 7.4), dehydrated in a series of increasing percent of ethanol solutions (50, 75, 90, and 100%) and embedded in paraffin (Paraplast®, MilliporeSigma). The paraffin-embedded tissues were sectioned at 7 µm on a rotary microtome (Leica, Buffalo Grove, IL, USA) and mounted on glass slides (Superfrost™ Plus, Fisher Scientific). Sections were deparaffinized with xylene, rehydrated with a series of decreasing percent ethanol solutions (100, 95, 75 and 50%) and rinsed with phosphate-buffered saline (PBS, pH 7.4). Slides were then incubated in blocking solution (PBS containing 1% BSA, 5% normal rabbit serum, and 0.3% Triton X-100) for 1 h at room temperature. After blocking, sections were rinsed in PBS and incubated with rabbit AVT-1aV2 primary antibody (dilution 1:100) overnight at 4°C. After incubation, sections were rinsed in PBS and incubated for 1 h with HPR-linked goat anti-rabbit secondary antibody to detect AVT-V1a2 protein. The IR signal of AVT-1aV2 protein was detected using DAB substrate under dark condition. The IR signal of AVT-1aV2 protein was captured by CoolSNAP™ camera (Photometrics, Tucson, AZ). The IR signal intensity of each neuron was quantified after subtracting background signal using ImageJ software according to Collins.[58]

2.12 Double-labeled immunofluorescence staining of AVT-V1a2 receptor and GnRH-I neurons

Double-labeled immunofluorescence staining was used to detect co-expression of hypothalamic AVT-1aV2 and GnRH-I neurons, which employs AVT-1aV2 and GnRH-I primary rabbit antibodies according to Kline et al.,[30] Shindler and Roth,[59] and Kroeber et al.[60] Briefly, affinity purified rabbit anti-AVT-1aV2 primary antibody was purified by IgG column (Melon™ Gel Purification Kit, Cat. #45206, Thermo Fisher Scientific) and directly labeled with DyLight® 488 antibody according to the manufacturer's protocol. Brain sections were incubated with unlabeled rabbit GnRH-I primary antibody (dilution: 1:100) overnight at 4°C. The GnRH-I antibody has been validated in marine perciform fish brain previously.[34,61] Sections were rinsed with PBS three times and incubated with fluorescent Rhodamine Red™-X conjugated goat anti-rabbit secondary antibody (dilution 1:500; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h under dark condition. Slides were rinsed with PBS and incubated with 5% normal rabbit serum in PBS-T (PBS with 0.3% Triton X-100). Sections were then blocked with blocking solution for 1 h, rinsed with PBS three times, and incubated with anti-AVT-V1a2-DyLight 488 labeled-antibody (dilution 1:500) at 4°C overnight. Slides were rinsed with PBS and mounted in Fluoromount-G® solution (SouthernBiotech). The double-labeled immunofluorescence signals of AVT-1aV2 (green signal) and GnRH-I (red signal) were visualized using a Nikon Eclipse C2 confocal microscope (Nikon, Japan).

2.13 Histological staining

Whole brain was dissected and fixed in 4% paraformaldehyde, dehydrated in a series of ethanol dilutions, cleared two times in xylene, embedded in xylene/paraffin (1:1) ratio, and sectioned at 7 µm in a rotary microtome. Sections were deparaffinized in xylene, rehydrated in series of ethanol dilutions, rinsed with deionized water, and stained with diluted hematoxylin and eosin solutions using standard histological techniques. Slides were then mounted with Cytoseal™ XYL mounting media (Fisher Scientific), examined under a light microscope (Nikon Eclipse E600, Nikon) and histological pictures were captured using a CoolSNAP camera.

2.14 Measurement of protein carbonyl contents

Quantification of ROS is challenging due to its short half-life.[62] Protein carbonyl (PC, an indirect measure of ROS) contents were measured in plasma samples by reacting with 2,4-dinitrophenylhydrazine (DNPH) to generate dinitrophenyl-hydrazones according to the method of Reznick and Packer.[63] Briefly, 200 µl of plasma were added to 800 µl of 2.5 M HCl in the presence or absence of 800 µl of DNPH solution and incubated in the dark at room temperature for 1 h. After the incubation, proteins were precipitated by adding 1 ml of 20% trichloroacetic acid and centrifuged at 10,000 x g for 10 min at 4°C. The protein pellet was washed with 1 ml of ethanol:ethyl acetate (1:1) solution 3 times and centrifuged between washes to remove excess DNPH. Subsequently, the protein pellet was dissolved in 500 µl of 6 M guanidine hydrochloride. The PC contents were measured at the average absorbance (λ = 370 nm) with DNPH and without DNPH

samples using spectrophotometer (FLUOstar, BMG Labtech, Cary, NC, USA), and expressed as nmol/mg protein.

2.15 Statistical analyses

All data are shown as the mean+standard error of the mean (SEM). Experimental data were analyzed using Student's *t*-test for unpaired comparison or one-way analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) test for multiple comparisons. *P* value <0.05 was considered statistically significant for all tests. All statistical analyses were performed using GraphPad Prism (GraphPad Corporation, San Diego, CA, USA) and StatView (SAS Institute Inc., Cary, NC, USA) statistical software packages.

3. RESULTS

3.1 Molecular characterization of AVT-V1a2 receptor cDNA

The full-length croaker AVT-V1a2 receptor cDNA contained 1505 bp nucleotides consisting of a 112 bp 5'-untranslated region (UTR), a 1224 bp open reading frame, and a 169 bp 3'-UTR and included a polyadenylation (polyA) signal sequence (GenBank accession no. MN807288). Translation of croaker AVT-1aV2 receptor cDNA nucleotides predicted a protein of 408 amino acids composed of seven transmembrane (TM) domains, two intracellular loops and three extracellular loops (Fig. 1A). The TM domains, intracellular and extracellular loops show extensive similarity to AVT-V1s in other teleost fishes. The deduced amino acid sequence of the croaker AVT-1aV2 receptor showed high identity with those of perch AVT-V1a2 (90.9%), grouper AVT-V1a2

(90.6%), sea bream (89.1%), pupfish AVT-V1a2 (87.8%), and also with that of grouper AVT-V1a1 (74%), pupfish AVT-V1a1 (67.8%), perch AVT-V1a1 (66.7%) and fugu AVT-V1a1 (62.8%) (Fig. 1B).

3.2 Phylogenetic tree of the deduced amino acid sequences of AVT-V1a2 receptors

A phylogenetic tree of the deduced amino acid sequences of AVTr cDNAs was constructed to determine evolutionary relationships of croaker AVT-V1a2 cDNA to those of other vertebrate AVTr cDNAs. The phylogenetic tree showed that croaker AVT-V1a2 has a high bootstrap value and is more closely related to teleost AVT-V1a2 clade than those of other teleost and tetrapod AVT-V1a1, -V1b and -V2 clades (Fig. 2).

3.3 AVT-V1a2 receptor mRNA levels in different croaker tissues and discrete brain areas

Quantitative real-time PCR (qRT-PCR) results revealed that AVT-V1a2 receptor transcript had approximately 5- to 6-fold higher mRNA levels in croaker brain compared to all other tissues (eye, gill, heart, intestine, kidney, liver, muscle, ovary, spleen and testis) (Fig. 3A). No amplification of PCR products was detected in the qRT-PCR negative reactions (data not shown). qRT-PCR results showed that AVT-V1a2 receptor mRNA levels were higher in the preoptic-anterior hypothalamus (POAH) and midbrain tegmentum (MT) than in other discrete brain regions and the pituitary (Fig. 3B).

3.4 Effects of PCB77 exposure on hypothalamic AVT-V1a2 receptor mRNA levels

No sex differences were observed in hypothalamic (POAH+MT) AVT-V1a2 receptor mRNA levels (Fig. 4A). Exposure to PCB77 (low dose: 2 µg/body weight, BW; high dose: 8 µg/g BW; for 4 weeks) caused marked decreases of hypothalamic AVT-V1a2 receptor mRNA levels (64% decrease for low dose and 76% for high dose of PCB77) compared to untreated controls (Fig. 4B).

3.5 Effects of PCB77 exposure on hypothalamic AVT-V1a2 receptor protein expression

Western blot analysis using AVT-V1a2 receptor antibody showed a specific IR band of approximately 45 kDa, the predicted molecular size of the receptor, in hypothalamic tissues (Fig. 5A). Specificity of the immune staining reaction with the AVT-V1a2 antibody in croaker hypothalamus was confirmed by blocking the immunoreaction by preincubation with the AVT-V1a2 peptide antigen (Fig. 5B). Exposure to PCB77 (both low and high doses for 4 weeks) significantly reduced AVT-V1a2 receptor protein expression compared to controls (no PCB77 added in food) (Fig. 5C). The IR intensities of the AVT-V1a2 receptor protein band on Western blots were significantly decreased around 53% after low dose and 60% after high dose PCB77 exposure compared to control fish following normalization to actin protein (Fig. 5D).

3.6 Effects of PCB77 exposure on hypothalamic AVT-V1a2 receptor neuronal expression

The specificity of the immunohistochemical staining reaction with the AVT-V1a2 receptor antibody in the croaker hypothalamus was confirmed by blocking through preincubation with the specific AVT-V1a2 peptide antigen which diminished the IR signal intensity (Fig. 6A). Treatments with PCB77 (low dose: 2 μg/g BW, high dose: 8 μg/g BW) for 4 weeks caused marked decreases in both number and intensity of AVT-V1a2 receptor expressing neurons compared to control fish (Fig. 6C-G).

3.7 Interaction between the hypothalamic AVT-V1a2 receptor and GnRH-I neurons, and the effects of PCB77-exposure on cytokine and oxidative stress

To investigate the anatomical basis for possible interactions between the AVT-V1a2 receptor and GnRH-I neurons in the croaker hypothalamus, we investigated their co-expression using a double-immunofluorescence assay. Immunohistochemical results showed that the AVT-V1a2 receptor and GnRH-I neurons were co-expressed in the preoptic-anterior hypothalamus as shown in the merged images (Fig. 7A-C).

To elucidate the possible association between hypothalamic AVT-V1a2 receptor and GnRH-I downregulation with cellular oxidative stress, hypothalamic cytochrome P450 1A (CYP1A, a key enzyme that catalyzes the oxidation of organic substances[64]) and interleukin-1β (IL-1β, an inflammatory cytokine[65]) mRNA levels, and plasma protein carbonyl (PC, an indicator of ROS[66]) contents were measured in fish exposed to PCB77. Exposure to PCB77 (low dose: 2 μg/g BW; high dose: 8 μg/g BW, for 4 weeks)

significantly reduced hypothalamic GnRH-I mRNA levels compared to controls (Fig. 7E). Treatments with the low dose of PCB77 caused marked increases in hypothalamic CYP1A and IL-β mRNA levels, and plasma PC contents compared to controls which were further elevated when fish were exposed to the high dose of PCB77 (Fig. 7F-H).

4 DISCUSSION

The neuroendocrine system is extremely complex comprising many genes, peptides and receptors such as the AVT-V1a receptor, that regulate a wide variety of physiological and neurological functions. [22,32,67] In this study, we cloned and characterized the full-length cDNA of the AVT-V1a2 receptor in croaker brain. The receptor encodes a 408 amino acid protein and is predicted to be a seven transmembrane protein with multiple intracellular and extracellular loops.[22,31,32] Phylogenetic analysis revealed that the croaker AVT-V1a2 receptor is clustered with AVT receptors of other teleosts with high similarity, especially with AVT-V1a2 receptors (88-91%), and lower similarity with the AVT-V1a1 receptor in teleost and tetrapod species. These findings suggest the cDNA cloned from croaker is a member of AVT receptor family.

The pattern of AVT-V1a2 receptor mRNA expression in different tissues and discrete brain regions of croaker not treated with PCB77 was determined by qRT-PCR. Similar to findings in tetrapods (e.g. newt)[68] and teleosts (e.g. eel, catfish),[69,70] croaker AVT-V1a2 receptor expression was highly expressed in the brain with highest expression in the hypothalamus, and to a lesser extent in the gill, heart, liver, ovary and testis, with weak expression in the eye, intestine, kidney, muscle and spleen. Similarly, the AVT-V1a2 receptor is widely expressed in the tissues of the euryhaline teleost, Amargosa

pupfish, with highest expression in the hypothalamus and forebrain and much lower mRNA levels in the gill, kidney and liver.[29] Also, in gilthead sea bream, the AVT-V1a2 receptor mRNA was detected in all the tissues examined except the eye; however, semiquantitative PCR results suggest the relative AVT-V1a2 mRNA expression differs from that in croaker and pupfish, with higher expression in the gill, intestine, testis, heart, kidney, and swim bladder than in the brain.[28] Collectively, these findings suggest that in addition to its neurological functions, the AVT-V1a2 receptor regulates a wide variety of physiological functions in other tissues such as osmoregulation in teleost fishes.

An anticipated finding of this study is that, like several other marine teleosts (e.g. grouper),[30] croaker AVT-V1a2 receptor mRNA is highly expressed in the preoptic anterior hypothalamus. This is of particular interest because GnRH-I neurons which exert a strong stimulatory influence on reproduction are highly concentrated in this hypothalamic region.[71]

Moreover, consistent with the previous observation in grouper,[30] the immunohistochemistry results show the AVT-V1a2 receptor protein is co-localized with GnRH-I neurons in the anterior preoptic region of the croaker hypothalamus. The finding that the AVT-V1a2 receptor is present on GnRH-I neurons in a second perciform fish species suggests this localization is widespread in this teleost order. This co-localization provides an anatomical basis for possible AVT regulation of GnRH neuronal activity, although direct evidence that AVT regulates GnRH secretion is currently lacking and clearly warrants investigation.

The present results, to the best of our knowledge, provide the first information on the effects of PCB77 exposure on AVT-V1a receptor protein expression in a vertebrate brain. Chronic treatment in the food with PCB77 (2 or 8 μg/g BW for 4 weeks) drastically decreased AVT-V1a2 receptor protein expression and its neuronal IR staining intensity in the croaker hypothalamus in addition to decreasing mRNA levels. In contrast, hypothalamic AVT-V1a receptor mRNA levels were unchanged in gilthead seabream hypothalamic tissues after chronic *in vivo* treatment with Aroclor 1254, a PCB congener mixture, whereas the AVT protein level was increased in the pituitary.[39] In addition to the regulation of AVT-V1a2 receptor levels, PCBs and Aroclor-1254 have been shown to disrupt the synthesis of tryptophan hydroxylase (TPH, a neuroenzyme), serotonin (5-hydroxytryptamine, a neurotransmitter) and GnRH receptor levels in croaker brain.[72-74] Aroclor-1254 treatment also reduces TPH activity in the rat hypothalamus.[75] An important finding in the current study was that exposure to PCB77 caused marked downregulation of both the AVTR-1a2 receptor and GnRH-I transcripts, two major mediators of reproductive function, in the same neuron. However, a causal relationship for these altered expressions could not be determined from these results, for example whether a decrease in AVT receptor levels is associated with a decline in GnRH levels. Nevertheless, the results presented here suggest the potential role of AVT and the AVT-1a2 receptor in regulating GnRH activity and possible modulation of AVT signaling by GnRH are promising topics for future investigation.

Induction of ROS and cellular cytokines (e.g. IL-β) by PCB exposure is a common phenomenon in vertebrates.[10] Recently, we have shown that chronic treatment

with PCB77 (2 or 8 μg/g BW) or Aroclor-1254 (0.5 or 1 μg/g BW) for 4 weeks causes a marked increase in hepatic ROS contents and IL-β mRNA levels in croaker.[54] In addition, we have also shown that Aroclor-1254 treatment decreases hypothalamic GnRH-I mRNA levels and GnRH content.[72,73] Similar to the effects of Aroclor-1254, *in vitro* PCB74 treatment decreases GnRH peptide concentrations in mouse hypothalamic GT1-7 cells.[76] In the present study, we have shown that the decrease in AVT-V1a2 receptor expression in croaker hypothalamus induced by PCB77 treatment is accompanied by significantly declines in hypothalamic GnRH-I mRNA levels and marked increases in hypothalamic CYP1A and IL-β mRNA levels, and plasma PC content, suggesting a possible causal relationship between oxidative stress and decreased expression of AVT-V1a2 receptor and GnRH-I, potentially leading to impaired reproductive neuroendocrine functions in the teleost brain.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as influencing prejudicing the impartiality of the research reported.

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REFERENCES

[1] A. O. Cheek, P. M. Vonier, E. Oberdorster, B. C. Burow, J. A. McLachlan, *Environ. Health. Perspect.* **1998**, *106*, 5-10.

[2] M. J. Mills, C. Chichester, *Sci. Total Environ.* **2005**, *43*, 1-34.

[3] R. L. Cooper, R. J. Kavlock, *J. Endocrinol.* **1997**, *152*, 159-166.

[4] C. DeRosa, P. Richter, H. Pohl, D. E. Jones, *J. Toxicol. Environ. Health. B*, **1998**, *1*, 3-26.

[5] M. Kajta, A. K. Wojtowicz, *Pharmacol. Rep.* **2013**, *65(6)*, 1632-1639.

[6] L. G. Hansen, *Polychlorinated biphenyls (PCBs): mammalian and environmental toxicology. Environ Toxin Series 1. Springer-Verlag, New York, 1987, 15-48 pp.*

[7] S. Jensen, *AMBIO* **1997**, *1(4)*, 123-131.

[8] A. Holma-Suutari, P. Ruokojarvi, A. A. Komarov, D. A. Makarov, V. V. Ovcharenko, A. N. Panin, H. Kiviranta, S. Laaksonen, M. Nieminen, M. Viluksela, A. Hallikainen, *Environ. Sci. Eur.* **2016**, *28*, 5.

[9] M. Bazzanti, S. Chiavarini, C. Cremisini, P. Soldati, *Environ. Int.* **1997**, *23*, 799-813.

[10] T. B. Henry, *Critical Rev. Toxicol.* **2015**, *45*, 643-661.

[11] P. De Voogt, U. A. Brinlman, *Halogenated Biphenyls, Terphenyls, Napthalenes, Dibenzodioxins and Related Products, New York, NY: Elsevier Science, 1989, 3-45 pp.*

[12] K. Breivik, A. Sweetman, J. M. Pacyna, K. C. Jones, *Sci. Total Environ.* **2002**, *290*, 181-198.

[13] H. J. Shipley, D. Sokoly, D.W. Johnson, 2017. *Envn. Monit. Assess*. **2017**, *189(2)*, 75.

[14] M. V. Den Berg, L. Birnbaum, A. T. C. Bosveld, B. Brunstrom, P. Cook, M. Feeley, J. P. Giesy, A. Hanberg, R. Hasegawa, S. W. Kennedy, T. Kubiak, J. C. Larsen, F. X. Van Leeuween, A. K. Leim, C. Nolt, R. E. Peterson, L. Polliinger, S. Safe, D. Schlenk, D. Tillitt, M. Tysklund, M. Younes, F. Waern, T. Zacharewski, *Environ. Health Perspect.* **1998**, *106*, 775-792.

[15] D. K. Dodoo, D. K. Essumang, J. W. Jonathan, J. K. Bentum, *Environ. Res.* **2012**, *118*, 16-24.

[16] J. F. Brown, D. L. Bedard, M. J. Brennan, J. C. Carnahan, H. Feng, R. E. Wagner, *Science* **1987**, *236*, 709-712.

[17] S. Safe, *Critical Rev. Toxicol.* **1984**, *13*, 319-393.

[18] S. Tavolari, L. Bucci, V. Tomasi, T. Guarnieri, *Toxicology* 2006, *218*, 67-74.

[19] J. J. Stegeman, J. J. Lech, *Environ. Health Perspect.* **1991**, *90*, 101-109.

[20] H. M. H. Goldstone, J. J. Stegeman, *J. Mol. Evol.* **2006**, *62*, 708-717.

[21] N. Quinete, T. Schettgen, J. Bertram, T. Kraus, *Environ. Sci. Pollut. Res. Int.* **2014**, *21(20)*, 11951-11972.

[22] R. J. Balment, W. Lu E. Weybourne, J. M. Warne, *Gen. Comp. Endocrinol.* **2006**, *147*, 9-16.

[23] H. P. Nair, L. J. Young, *Physiology (Bethesda)* **2006**, *21*, 146-152.

[24] J. M. Warne, K. E. Harding, R. J. Balment, *Comp. Biochem. Physiol.* **2002**, *132B*, 231-237.

[25] J. Godwin, R. Thompson. *Horm. Behav.* **2012,** *61*, 769-772.

[26] M. Gesto, J. L. Soengas, A. Rodríguez-Illamola, J. M. Mígez. *J. Neuroendocrinol*. **2014**, *26*, 89-99.

[27] M. R. Ramallo, M.Grober, M. M. Cánepa, L. Morandini, M. Pandolfi, *Gen. Comp. Endocrinol*. **2012,** *179,* 221-231.

[28] J. A. Martos-Sitcha, J. Fuentes, J. M. Mancera, G. Martínez-Rodríguez, *Gen. Comp. Endocrinol.* **2014**, *197*, 5-17.

[29] S. C. Lema, E. H. Washburn, M. E. Crowley, P. G. Carvalho, J. N. Egelston, S. D. McCormick. *Am. J. Physiol. Integr. Comp. Physiol.* **2019**, *316*, R735-R750.

[30] R. J. Kline, G. J. Holt, I. A. Khan, *Gen. Comp. Endocrinol.* **2016**, *225*, 33-44.

[31] S. Mahlmann, W. Meyerhof, H. Hausmann, J. Heierhorst, C. Schonrock, H. Zwiers, K. Lederis, D. Richter. *Proc. Natl. Acad. Sci. USA.* **1994**, *91*, 1342-1345.

[32] M. Birnbaumer, *Trends Endocrinol. Metab.* **2000**, *11*, 406-410.

[33] I. Hasunuma, T. Sakai, T. Nakada, F. Toyoda, H. Namiki, S. Kikuyama, *Gen. Comp. Endocrinol.* **2007**, *151*, 252-258.

[34] R. J. Kline, L. A. O'Connell, H. A. Hofmann, G. J. Holt, I. A. Khan, *J. Chem. Neoroanatom.* **2011**, *42*, 72-88.

[35] D. A. Baeyens, L.E. Cornett, *Comp. Biochem. Physiol.* **2006**, *143B*, 12-19.

[36] S. W. Kang, S. Jayanthi, G. Nagaranjan, T. K. S. Kumer, W. J. Kuenzei, J. *Biomol. Struc. Dyn.* **2018**, *17*, 1-15.

[37] M. Hayashi, S. Sasaki, H. Tsuganezawa, T. Monkawa, W. Kitajima, K. Konishi, K. Fushima, F. Marumo, T. Saruta, *J. Clin. Invest.* **1994**, *94*, 1778-1783.

[38] S. Mohan, R. C. Moffett, K. G. Thomas, N. Irwin, P. R. Flatt, *Biochimie* **2019**, *158*, 191-198.

[39] A. K. Skrzynska, G. Martínez-Rodríguez, M. Gozdowska, E. Kulczykowska, J. M. Mancera, J. A. Martos-Sitcha, *Aquat. Toxicol.* **2019**, *212*, 98-109.

[40] C. M. Foran, A. H. Bass, *Gen. Comp. Endocrinol.* **1999**, *116*, 141-152.

[41] V. Singh, K. P. Joy, *Gen. Comp. Endocrinol.* **2009**, *162*, 172-178.

[42] I. A. Khan, P. Thomas, *Mar. Environ. Res.* **2006**, *62*, 25-28.

[43] I. A. Khan, P. Thomas. *Biol. Reprod.* **2001**, *64*, 955-964.

[44] J. Liu, L. Q. Pan, L. Zhang, J.J. Miao, J. Wang, *Fish Shellfish Immunol.* **2009**, *26*, 422-428.

[45] A. D. Deshpande, B. W. Dockum, T. Cleary, C. Farrington, D. Wieczorek, *Mar. Pollut. Bull.* **2013**, *72*, 146-164.

[46] F. Wu, Y. Zheng, J. Gao, S. Chen, Z. Wang, *Ecotoxicol. Environ. Safety* **2014**, *110*, 254-260

[47] M. S. Rahman, P. Thomas, *Environ. Toxicol.* **2018**, *33*, 411-421.

[48] M. S. Rahman, P. Thomas, *Neuroscience* **2009**, *158*, 751-765.

[49] M. S. Rahman, P. Thomas, *PLoS ONE.* **2012**, *7*, e40825.

[50] F. Corpet, *Nucl. Acids Res.* **1988**, *16*, 10881-10890.

[51] N. Saitou, M. Nei. *Mol. Biol. Evol.* **1987**, *4*, 406-425.

[52] K. Tamura, J. Dudley, M. Nei, S. Kumar, *Mol. Biol. Evol.* **2007**, *24*, 1596-1599.

[53] I. A. Khan, P. Thomas, *Gen. Comp. Endocrinol.* **1993**, *91*, 167–180.

[54] M. S. Rahman, P. Thomas, *Environ. Toxicol.* **2019**, *2*, 160-171.

[55] K. J. Livak, T. D. Schmittgen, *Methods* **2001**, *25*, 402-408.

[56] P. Thomas, M. S. Rahman, *Environ. Sci. Technol.* **2009**, *43*, 4175-4180.

[57] C. A. Schneider, W. S. Rasband, K. W. Elicein, *Nat. Methods* **2012**, *9*, 671-675.

[58] T. J. Collins, 2007. BioTechniques **2007**, *43*, S25-S30.

[59] K. S. Shindler, K. A. Roth, *J. Histochem. Cytochem.* **1996**, *44*, 1331-1335.

[60] S. Kroeber, C. Schomerus, H.-W. Korf, *Histochem. Cell Biol.* **1998**, *109*, 309-317.

[61] B. Senthilkumaran, K. Okuzawa, K. Gen, T. Ookura, H. Kagawa, *J. Neuroendocrinol.* **1999**, *11*, 181-186.

[62] A. Sanz. 2016. *Biochem. Biophys. Acta – Bioenergetics* **2016**, *1857*, 1116-1126.

[63] A. Z. Reznick, L. Packer, *Methods Enzymol.* **1994**, *233*, 357-363.

[64] W. D. Nebert, F. J. Gonzalez, *Ann. Rev. Biochem.* **1987**, *56*, 945-993.

[65] S. J. Hewett, N. A. Jackman, R. J. Claycomb, *Eur. J. Neurodegener.* **2012**, *1(2)*, 195-211.

[66] I. Dalle-Donne, R. Rossi, D. Giustarini, A. Milzani, R. Colombo, *Clinica Chimica Acta*, **2003**, *329*, 23-38.

[67] I. Hasunuma, F. Toyada, R. Okada, K. Yamamoto, Y. Kadona, S. Kikuyama, *Int. Rev. Cell Mol. Biol.* **2013**, *304*, 191-225.

[68] I. Hasunuma, T. Sakai, T. Nakada, F. Toyoda, H. Namiki, S. Kikuyama. *Gen. Comp. Endocrinol.* **2007**, *151*, 252-258.

[69] Q. Cao, F. Liang, D. Wang, X. Zhang, C. Lorin-Nebel, J. Gu, S. Y, *Gene* **2018**, *677*, 49-56.

[70] A. Rawat, R. Chaube, K. P. Joy, *Fish Physiol. Biochem.* **2015**, *41*, 509-532.

[71] J. S. Mohamid, P. Thomas, I. A. Khan**.** *J. Comp.Neurol.* **2005**, *488*, 384-395.

[72] M. S. Rahman, I. A. Khan, P. Thomas, *J. Toxicol. Environ. Health B*, **2011**, *14*, 473-494.

[73] I. A. Khan, P. Thomas, *Biol Reprod.* **2001**, *64*, 955-964.

[74] I. A. Khan, S. Mathews, K. Okuzawa, H. Kagawa, P. Thomas. *Comp. Biochem. Physiol.* **2001**, *129B*, 251-259.

[75] I. A. Khan, P. Thomas, *Arch. Toxicol.* **2004**, *78*, 316-320.

[76] S. M. Dickerson, E. Guevara, M. J. Woller, A. C. Gore, *Toxicol. Appl. Pharmacol.* **2009**, *237*, 237-245.

Figure Legends

Figure 1 Alignment of the null-length amino acid sequences of Atlantic croaker (croaker, GenBank accession no: MN807288) AVT-V1a2 receptor compared with published sequences for fugu (AVT-V1a1: AAK17004), grouper (AVT-V1a1: AEI4996, AVT-V1a2: ADO33897), perch (AVT-V1a1: XP_018550346, AVT-V1a2:XP_018531445) pupfish (AVT-V1a1: ACX85728, AVT-V1a2: ACX85729) and sea bream (AVT-V1a2: KC195974). Dashes indicate gaps introduced to facilitate alignment. The alignment was generated using the MultiAlign (http://multalin.toulouse.inra.fr/multalin/) according to Corpet.[50] Predicted transmembrane (TM I-VII) domains, intracellular loop (ICL I-III) and extracellular loop (ECL I-III) from MEMSAT3 are also identified. (B) Percent identities of the deduced amino acid sequence of croaker AVT-V1a2 receptor protein with those of AVT-V1a1 and AVT-V1a2 receptors from other teleost fishes.

Figure 1

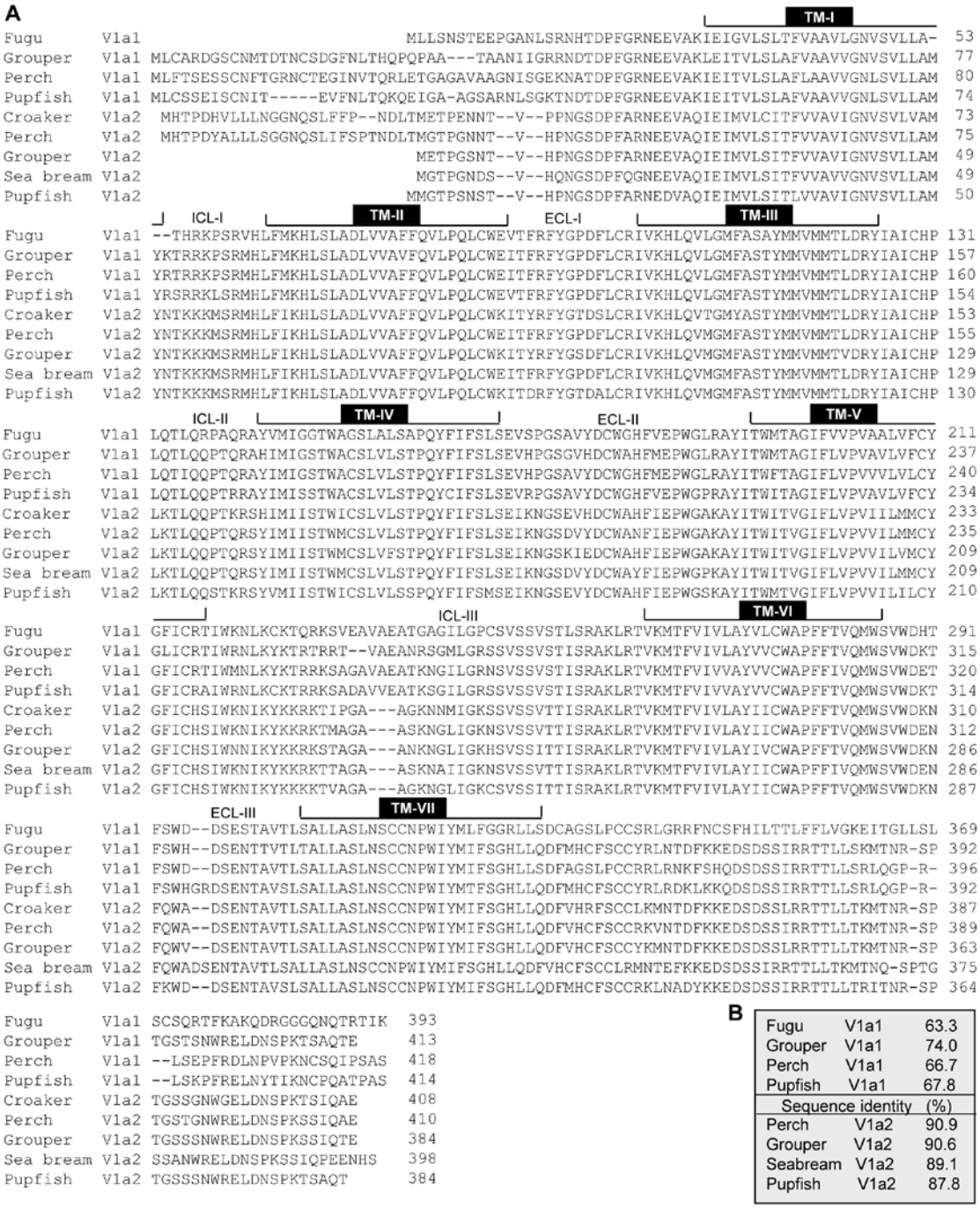

Figure 2 Molecular phylogeny of AVT-V1a1, -V1a2, -V1b and -V2 receptor proteins. GenBank accession numbers for white sucker (V1a: CAA53958), zebrafish (V1a: NP_001288043), lungfish (V1a: BAG66063), marine frog (V1a: BAF48112), bull frog (V1a: AY277924), Japanese fire belly (FB) newt (V1a: BAF38754), chicken (V1a: EU124684), opossum (V1a: XM-001372679), prairie vole (V1a: AF069304), mouse (V1a: NM_053019), human (V1a: NM_000706), cow (1Va: BC1333384), rough-skinned newt (V1B: EF567079), chicken (V1b: NM_001031486), opossum (V1b: XM_001372679), rat (V1b: NM_017205), human (V1b KJ890748), FB newt (V3/V1b: AB28453), mouse (v2: NM_019404), human (V2: NM_000054), Japanese fire belly (FB) newt (V2 AB274038), frog (V2: AB073979), marine frog (V2: AB274030), sea bream (V2: KC960488). See Fig. 1 legend for additional GenBank accession numbers.

Figure 2

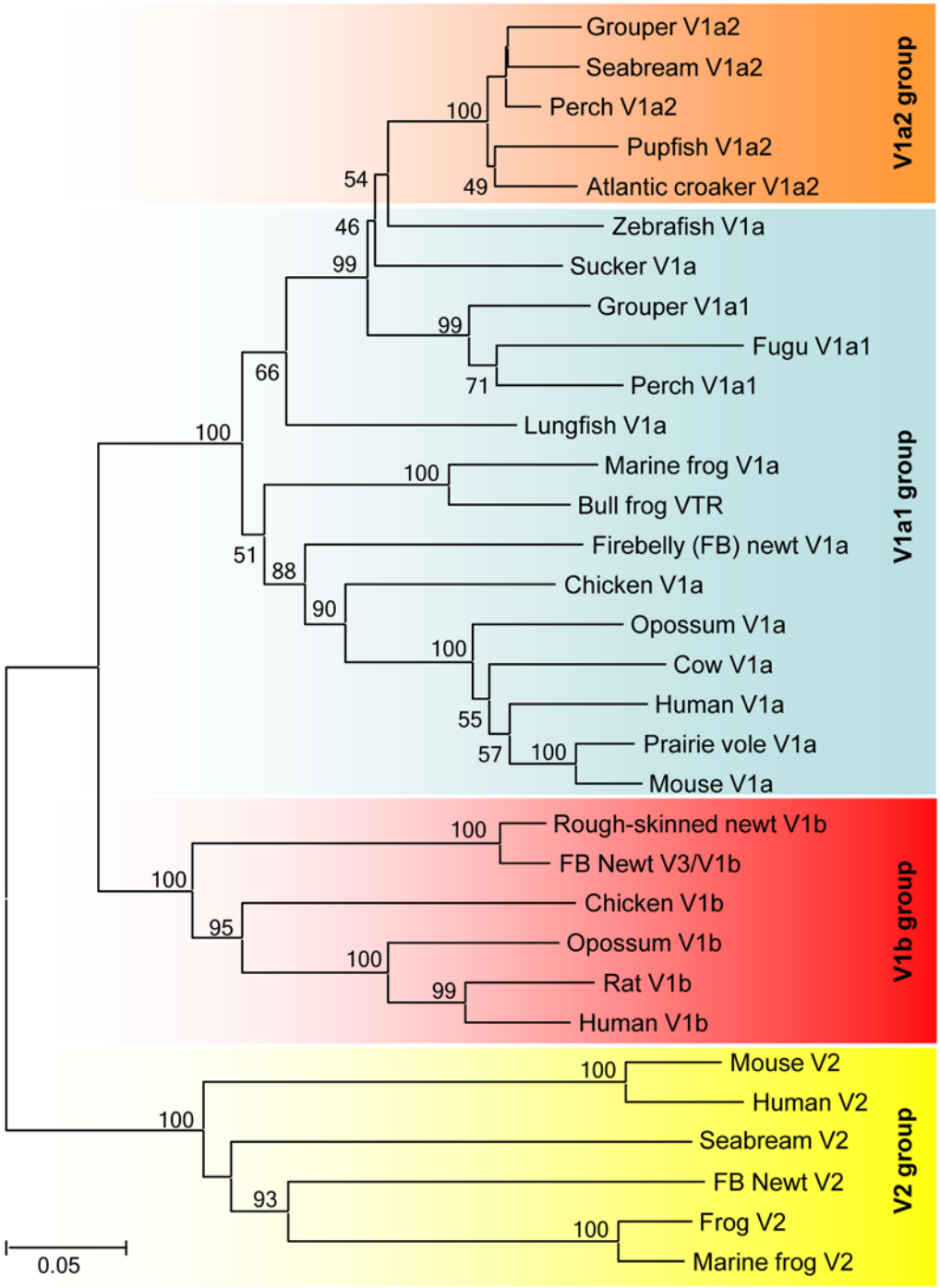

Figure 3 AVT-V1a2 receptor mRNA levels in croaker tissues. (A) Total RNA (100 ng) from each tissue was used for quantitative real-time PCR (qRT-PCR) reaction in 40 cycles to detect AVT-V1a2 receptor mRNA. Negative reactions were used to ensure that the AVT-V1a2 receptor was not amplified from genomic DNA contamination. BR, brain; EY, eye; GI, gill; HE, heart; IN, intestine; KI, kidney; LI, liver; MU, muscle; OV, ovary; SP, spleen; TE, testis. (B) AVT-V1a2 receptor mRNA levels in different parts of croaker brain and pituitary. OB, olfactory bulb; TEL, telencephalon; POAH, preoptic-anterior hypothalamic area; MT, midbrain tegmentum; CE+OT, cerebellum plus optic tectum; MO, medulla oblongata; PIT, pituitary.

Figure 3

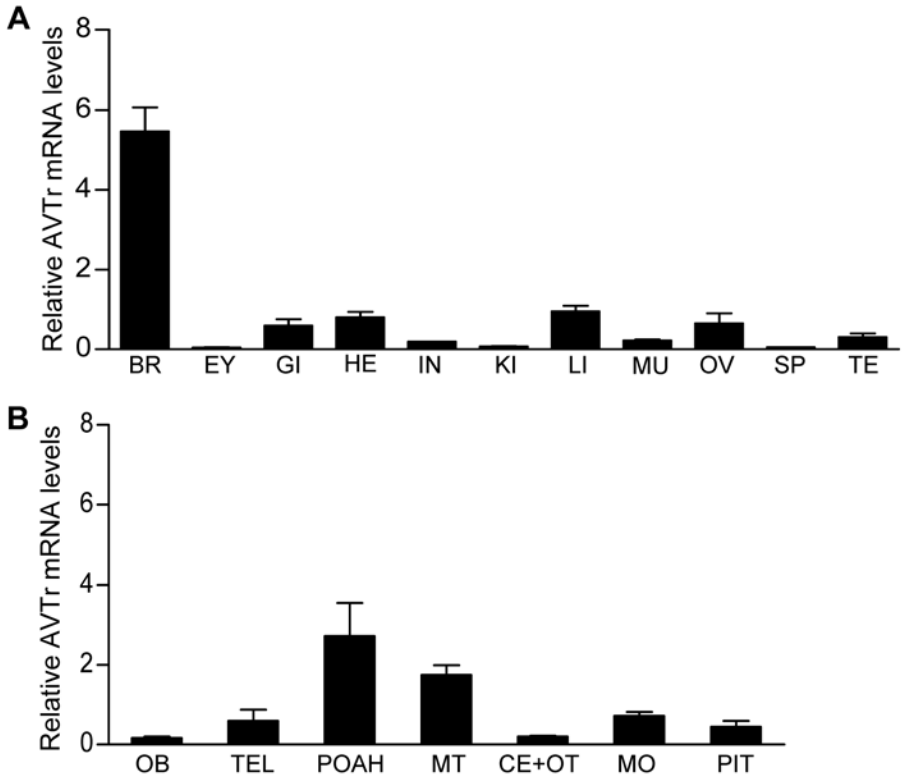

Figure 4 Effect of 4 weeks of exposure to PCB77 on AVT-V1a2 receptor mRNA levels in croaker hypothalamus determined by real-time qRT-PCR. (A) AVT-V1a2 receptor mRNA levels in male and female croaker hypothalamus. Each bar represents mean ± SE (N = 4). (B) Effects of 4 weeks exposure to PCB77 (PCB, LD: low dose, 2 µg/g body weight; HD: high dose, 8 µg/g body weight for 4 weeks) on AVT-V1a2 receptor mRNA levels. (C) C_t values of 18S in croaker hypothalamus. Each bar represents mean ± SE (N = 7-8, results of mRNA levels from both sexes were combined because they were not significantly different). Different letters indicate significant differences (Fisher's PLSD test $P<0.05$). Asterisk indicates significant difference (Student's *t*-test, $*P<0.05$). CTL, control.

Figure 4

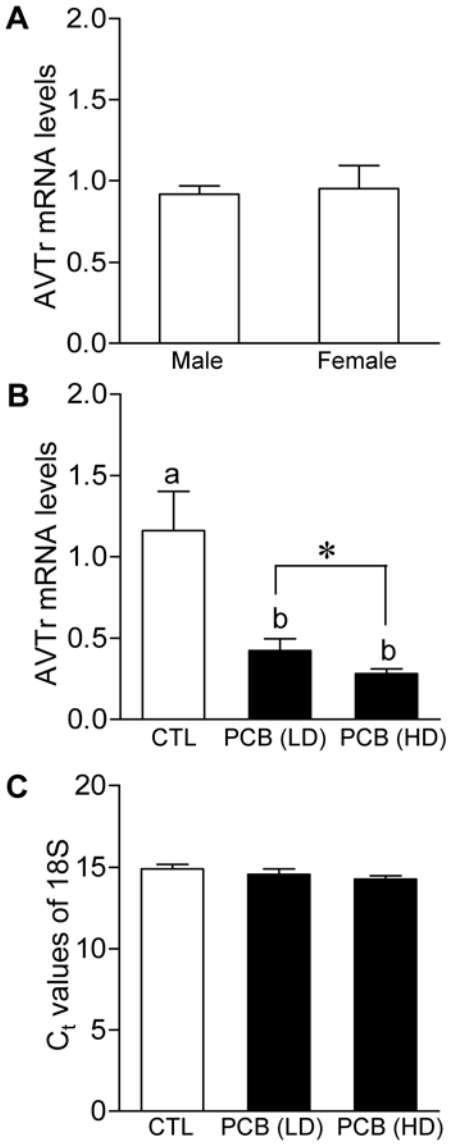

Figure 5 Effect of 4 weeks of exposure of PCB77 on AVT-V1a2 receptor protein expression and levels in croaker hypothalamus determined by Western blot analysis. (A) Immunoblot analysis of isolated AVT-V1a2 receptor protein expression in male and female croaker hypothalamus using a polyclonal AVT-V1a2 receptor antibody, and (B) immunoreaction blocked by co-incubation of antibody with peptide antigen showing the absence of protein signal. (C, D) Effects of 4 weeks PCB77 (LD: low dose, 2 µg/g body weight; HD: high dose, 8 µg/g body weight for 4 weeks) exposure on AVT-V1a2 receptor protein expression and relative protein levels in croaker hypothalamus. Each bar represents mean ± SE (N = 7-8, results of protein levels from both sexes were combined because they were not significantly different). Different letters indicate significant differences (Fisher's PLSD test $P<0.05$). CTL, control. PM, protein marker; kDa, kilodalton, M, male, F, female.

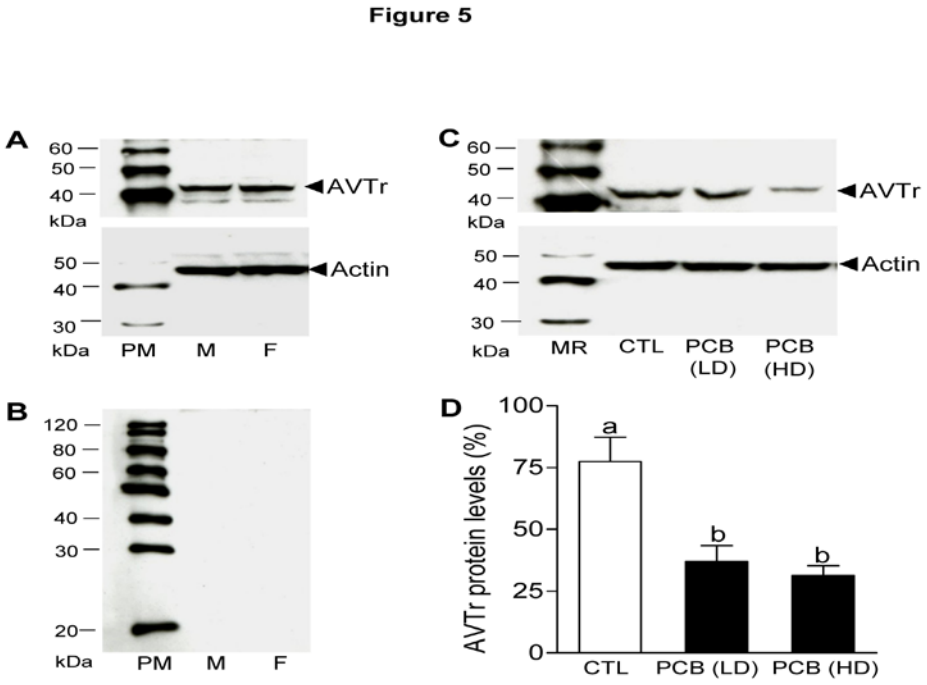

Figure 6 Effects of 4 weeks exposure of PCB77 on AVT-V1a2 receptor neuronal expression in croaker hypothalamus assessed by immunohistochemistry. (A) Immunohistochemical expression of AVT-V1a2 in croaker hypothalamus. Brain section blocked by co-incubation of AVT-V1a2 receptor antibody with peptide antigen showing the absence of signal. (B) Histological analysis of croaker hypothalamus by light microscope. (C-E) Representative immunohistochemistry micrographs of AVT-V1a2 receptor neurons in hypothalamic sections (A: control; B: low dose (LD), 2 µg/g body weight; C: high dose (HD), 8 µg/g body weight of PCB77 exposure). Magnification 40x. (F, G) Effects of 4 weeks of PCB77 (PCB) exposure on AVT-V1a2 neuron counts (F) and intensity (G). Each value represents the mean ± SE (N = 45-50 neurons). Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$).

Figure 6

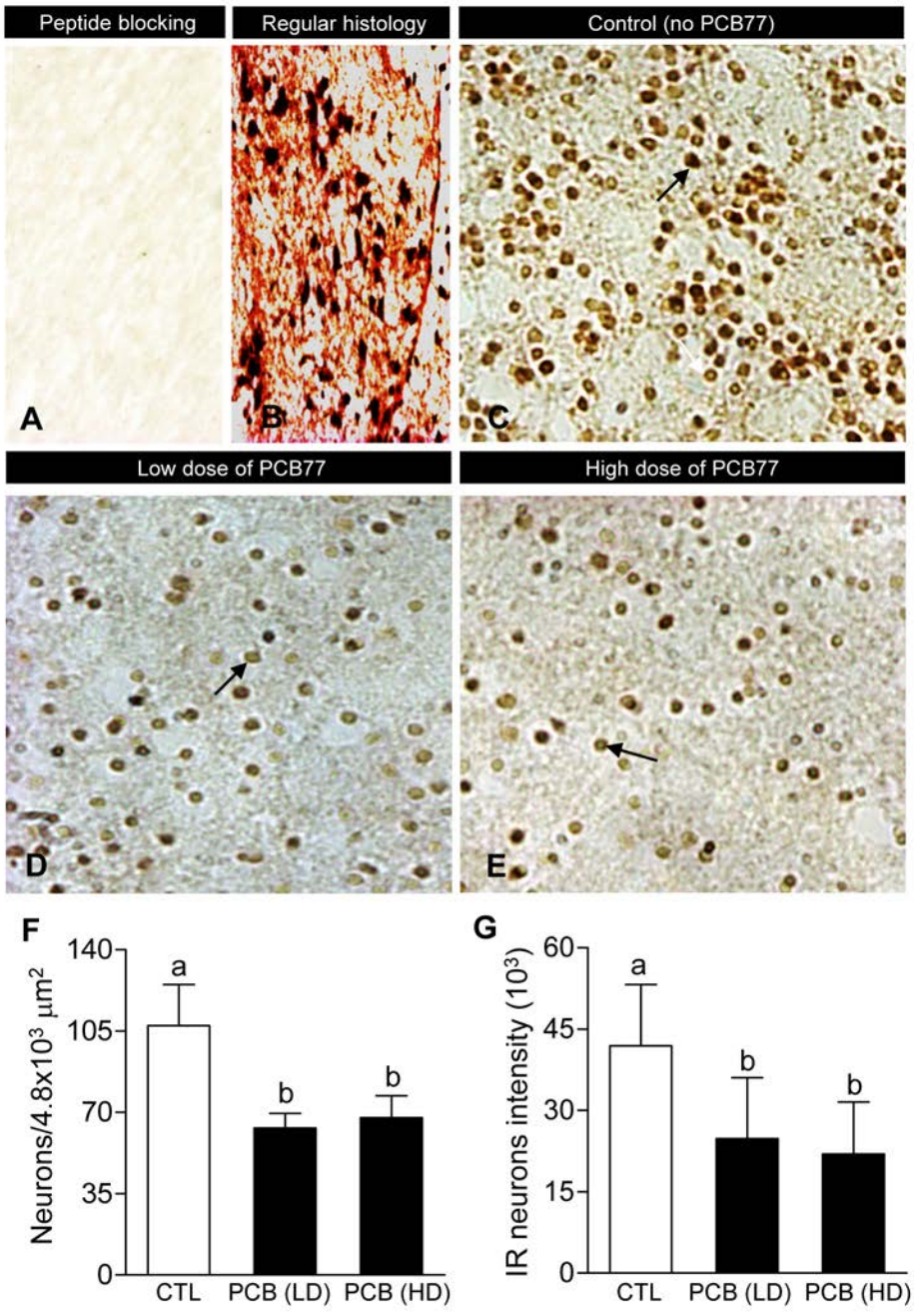

Figure 7 Interaction between the hypothalamic AVT-V1a2 receptor and GnRH-I neurons, and the effects of PCB77 exposure on cytochrome P450 and cytokine expression and oxidative stress. (A-C) Immunohistochemical co-expression of AVT-V1a2 receptor and GnRH-I neurons in croaker hypothalamic neurons determined by double-immunofluorescence staining. Arrows indicate AVT-V1a2 receptor (A) and GnRH-I (B) neurons, and their co-expression (C). (D) Histological analysis of croaker hypothalamus by light microscope. Scale bar = 25 μm. (E-H) Effects of PCB77 (PCB: low dose (LD), 2 μg/g body weight; high dose (HD), 8 μg/g body weight) on gonadotropin-releasing hormone (GnRH-I), cytochrome P450 1A (CYP1A) and interlukin-1β (IL-β) mRNA levels in hypothalamus, and protein carbonyl (PC) contents in plasma of croaker. Each value represents the mean ± SE (N = 8 for mRNA levels, and N = 15-16 for PC contents). Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). Asterisk indicates significant difference (Student's *t*-test, $*P<0.05$). CTL, control; prot., protein.

Figure 7

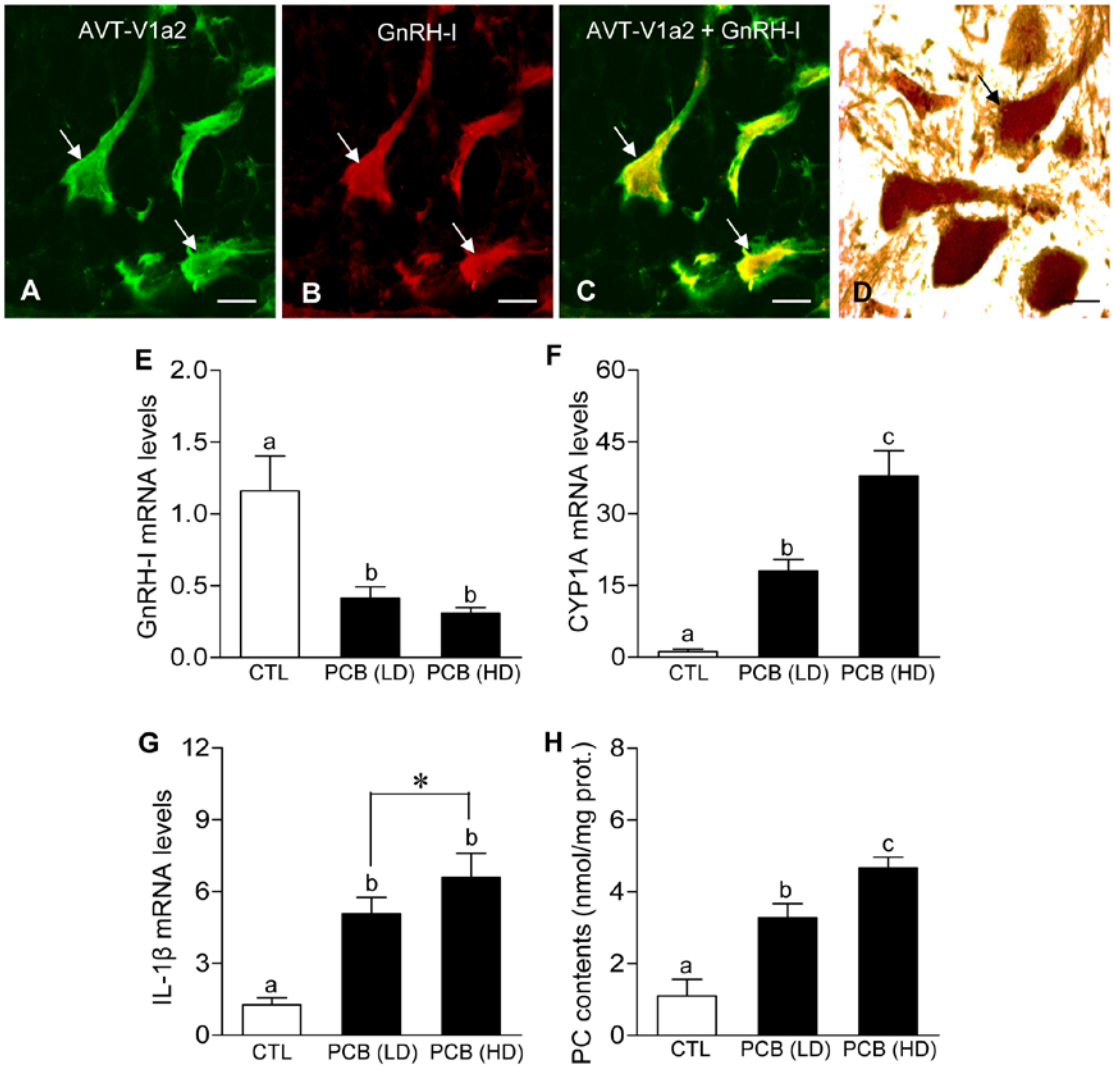

Table 1. Oligonucleotide primers used in this study.

Primers	Sequence
PSP-F	5'-GCCAAGGCGTACATCACCTGGA-3'
PSP-R	5'-TGCAGCAGCTGTTGAGACTGGC-3'
GSP 5'-1	5'-GAGGAAGATGCCCACTGTTATCCAG-3'
GSP 5'-2	5'-CCCACTGTTATCCAGGTGATGTAC-3'
GSP 3'-1	5'-GATAACAGTGGGCATCTTCCTCGT-3'
GSP 3'-2	5'- TTGGGAAGAGTTCAGTCAGCAGTG-3'
qRT-PCR-F	5'-GCGGGCAAGAACAATATGAT-3'
qRT-PCR-R	5'-TCAGCCCACTGGAAGTTTTT-3'
IL-1β-F	5'-CGTGACCGACAGTGAGAAGA-3'
IL-1β-R	5'-TCCCATCCTTATGGCAAGAG-3'
GnRH-I-F	5'-GCACTGGTCGTATGGACTGA-3'
GnRH-I-R	5'-TCTCCCGATCTGTGACTCC-3'
CYP1A-F	5'-TCAACGATGGCAAGAGTCTG-3'
CYP1A-R	5'-TACTCTGGGGTTGTGCCTTC-3'
18S-F	5'-AGAAACGGCTACCACATCCA-3'
18S-R	5'-TCCCGAGATCCAACTACGAG-3'

Abbreviations: PSP, partial sequence primer; GSP, gene-specific primer; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; F, forward; R, reverse. GenBank accession no: IL-1β: JQ622219, GnRH-I: AY324668, and 18S: AY866435.