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Serotonin Reuptake Inhibitor Citalopram Inhibits GnRH Synthesis and Spermatogenesis in the Male Zebrafish[1]

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ABSTRACT

Selective serotonin reuptake inhibitors (SSRIs) are widely used antidepressants for the treatment of depression. However, SSRIs cause sexual side effects such as anorgasmia, erectile dysfunction, and diminished libido that are thought to be mediated through the serotonin (5-hydroxytryptamine, 5-HT) system. In vertebrates, gonadotropin-releasing hormone (GnRH) neurons play an important role in the control of reproduction. To elucidate the neuroendocrine mechanisms of SSRI-induced reproductive failure, we examined the neuronal association between 5-HT and GnRH (GnRH2 and GnRH3) systems in the male zebrafish. Double-label immunofluorescence and confocal laser microscopy followed by three-dimensional construction analysis showed close associations between 5-HT fibers with GnRH3 fibers and preoptic-GnRH3 cell bodies, but there was no association with GnRH2 cell bodies and fibers. Quantitative real-time PCR showed that short-term treatment (2 wk) with low to medium doses (4 and 40 μg/L, respectively) of citalopram significantly decreased mRNA levels of *gnrh3,* gonadotropins (*lhb* and *fshb*) and 5-HT-related genes (*tph2* and *sert*) in the male zebrafish. In addition, short-term citalopram treatment significantly decreased the fluorescence density of 5-HT and GnRH3 fibers compared with controls. Short-term treatment with low, medium, and high (100 μg/L) citalopram doses had no effects on the profiles of different stages of spermatogenesis, while long-term (1 mo) citalopram treatment with medium and high doses significantly inhibited the different stages of spermatogenesis. These results show morphological and functional associations between the 5-HT and the hypophysiotropic GnHR3 system, which involve SSRI-induced reproductive failures.

5-HT, gonadotropins, reproduction, SSRI, testes

INTRODUCTION

Depression is a common neuropsychiatric disorder for which selective serotonin reuptake inhibitors (SSRIs) are widely used as treatment [1]. An antidepressant effect of SSRI is produced through inhibiting uptake of serotonin (5-hydroxytryptamine, 5-HT) by blocking its transporter (SERT) [2]. Among the SSRIs, citalopram is one of the most selective inhibitors of 5-HT uptake because of its low affinity and selectivity for other neurotransmitter receptors besides 5-HT [3]. However, citalopram like other SSRIs also induces sexual dysfunction such as decreased libido, orgasm and erectile dysfunction [4, 5], and decrease in the sperm concentration, motility, and morphology [6]. Similar effects have also been demonstrated in nonmammalian species, in particular, in teleosts species [7–9]. For example, fluoxetine, a SSRI, significantly reduces egg production and plasma estrogen levels and gene expression levels of ovarian gonadotropin receptors in female fish [10, 11]. However, the neuroendocrine mechanism of SSRI-induced sexual dysfunction is not fully understood.

Reproductive and sexual functions, including sexual behavior and arousal, are mainly controlled by pulsatile secretion of gonadotropin-releasing hormone (GnRH) in the hypothalamus [12]. GnRH acts on pituitary gonadotropes to regulate the synthesis and release of LH and FSH [13, 14]. These gonadotropins control gonadal development and maturation, stimulating steroidogenesis and spermatogenesis in male testes and folliculogenesis and oogenesis in female ovaries [15, 16]. Several studies have shown the involvement of 5-HT in the regulation of GnRH neurons. In rats, 5-HT axons terminate on GnRH neurons [17] and 5-HT regulates GnRH gene expression and GnRH secretion [18, 19]. In immortalized GnRH neural cells (GT1-7), 5-HT directly stimulates GnRH release via 5-HT receptor types [20, 21]. These observations indicate that GnRH neurons are regulated by presynaptic 5-HT action. SSRIs produce robust increases in extracellular 5-HT by depleting brain stores of 5-HT as well as suppressing 5-HT synthesis [22]. Therefore, GnRH neuronal activity could be interfered with by SSRIs. However, the influence of SSRIs on the GnRH system and potential involvement of GnRH in SSRIs-induced sexual dysfunction remains unknown.

In the present study, we used sexually mature male zebrafish (*Danio rerio*) as a model. Many teleost species possess at least two or three GnRH types (GnRH1, GnRH2, and GnRH3) or multiple GnRH neuronal populations in the brain [23, 24]. Zebrafish has two GnRH types (GnRH2 and GnRH3) [25], and GnRH2 neurons are localized in the midbrain tegmentum while GnRH3 neurons are present in the preoptic area (POA) of the hypothalamus with fibers ending in the pituitary gland as well as a GnRH3 population in the olfactory bulb-terminal nerve [25]. Functional studies have further confirmed the hypophysiotropic role of GnRH3 in the POA-hypothalamus in the

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zebrafish [26]. Furthermore, the organization of the 5-HT system—including SERT, tryptophan hydroxylase (TPH), a catalytic enzyme responsible for the synthesis of 5-HT, and monoamine oxidase, the enzyme for degradation of 5-HT—is highly conserved in the zebrafish [27, 28]. In the brain of zebrafish, three TPH gene subtypes (*tph1a, tph1b,* and *tph2*) and two SERT gene types (*slc6a4a* and *slc6a4b*) are expressed [27]. Among them, *tph2* and *slc6a4a* are expressed in the raphe nuclei and are responsible for serotonergic modulation of forebrain [27].

In the present study, we first examined the neural associations between 5-HT and two GnRH forms in the brain and pituitary using double immunofluorescence by confocal microscopy and three-dimensional (3D) image analysis. We then examined the effect of citalopram on immunoreactivities of GnRH2, GnRH3, and 5-HT by quantification of fiber density. We also examined the effect of citalopram on the expression of GnRH types (*gnrh2* and *gnrh3*), 5-HT-related genes (*tph2* and *slc6a4a*), and gonadotropins (*fshb* and *lhb*) in the brain and pituitary. Finally, citalopram-induced reproductive failures in male zebrafish were determined by morphological analysis of different stages of spermatogenesis.

MATERIALS AND METHODS

Animals

Sexually mature male zebrafish (>6-mo-old) were maintained in fresh water aquaria at 27°C ± 0.5°C under a controlled natural light regimen (14L:10D). Fish were fed with adult zebrafish diet (Zeigler/Aquatic Habitat) twice a day. The fish were maintained and handled in accordance with the guidelines of the Animal Ethics Committee of Monash University (approval number: MARP/2011/025).

SSRI (Citalopram) Treatment

Fish were treated with citalopram (Sigma) by immersion in water containing citalopram. The fish were given two different treatment periods, that is, short-term (2 wk) and long-term (4 wk), with three different concentrations of citalopram, that is, low (4 μg/L), medium (40 μg/L), and high (100 μg/L) doses, in 6 L water, (n = 20/group). Because there is no study examining the effect of citalopram in any teleost model, the three doses selected were determined based on the concentration of other SSRIs, in particular fluoxetine, that was previously used in in vivo assays in fish species (0.1~54 μg/L) [10, 29, 30] and the median lethal dose values of citalopram for Japanese medaka (9.1 mg/L) [31]. During the treatment, the water containing the citalopram and the control group (without citalopram) was changed every day. Both the control and treatment groups were handled simultaneously in order to avoid any experimental errors or outcomes. After 2 and 4 wk of treatment (at Day 15 and Day 29), the brain and pituitary samples (n = 10) were dissected for gene expression and morphological analysis, and the testes (n = 10) were used for morphological analysis. For morphological analysis of 5-HT and GnRH immunoreactivities, we used the brain and pituitary samples of the 2-wk treatment group. In the gene expression study, citalopram treatment for 2 wk at low (4 μg/L) and medium (40 μg/L) doses showed similar results. Therefore, for the long-term (1 mo) citalopram treatment, we only measured gene expression in fish treated with medium (40 μg/L) and high (100 μg/L) doses of citalopram because the aim was to examine testicular morphology as evidence for reproductive failure in testes.

Double Immunofluorescence of 5-HT and GnRH Types

To examine the neuronal associations between 5-HT and GnRH types, double immunofluorescence was performed. Fish were anesthetized by immersion in 0.01% solution of 3-aminobenzonic acid ethyl ester (MS-222; Sigma) before they were killed by decapitation. The brains (n = 6 samples each, for 5-HT-GnRH2 and 5-HT-GnRH3) were dissected, fixed in buffered 4% paraformaldehyde (Sigma) solution in phosphate buffer at 4°C for 6 h, and cryoprotected in 20% sucrose overnight at 4°C. For the pituitary, the whole head (n = 3) was dissected and transferred into 0.5 M ethylenediaminetetra-acetic acid solution and incubated for 1 wk at 4°C for decalcification. The decalcified head samples were transferred into 20% sucrose overnight at 4°C. The cryoprotected tissues were embedded in optimal cutting temperature compound (Surgipath, Leica Microsystems) and kept at −80°C until used. Sagittal sections (30 μm) were cut by using a cryostat and were thaw-mounted onto 3-aminopropylsilane-coated glass slides (Fisher Scientific). The immunostaining procedure used antibodies targeting 5-HT and GnRH in sequence by first incubating the tissue sections with the 5-HT antibody followed by the GnRH antibody. To detect GnRH types, two antibodies were used: a rabbit polyclonal antibody against GnRH2 (aCII6, dilution of 1:2000; a gift from Dr. Okuzawa, National Institute of Aquaculture, Japan) and a mouse monoclonal antibody against human GnRH (LRH-13, dilution of 1:3000; a gift from Prof Wakabayashi, Gunma University, Japan) for GnRH3. Specificity of the GnRH antibodies in the zebrafish has been reported previously showing that the LRH-13 antibody detects only GnRH3 and the CII6 antibody has cross-reactivity to GnRH2 and GnRH3 [25, 32]. To further confirm the specificities of the GnRH antibodies, LRH13 and aCII6 antibodies were preabsorbed with GnRH2 and GnRH3 decapeptides (10 μg/μl) followed by immunofluorescence labeling in the brain of zebrafish (Supplemental Fig. S1; Supplemental Data are available online at www.biolreprod.org).

For double immunofluorescence, the sections were incubated with a polyclonal rabbit antibody against 5-HT (20080; Immunostar) at a dilution of 1:2000 in blocking buffer containing 2% normal goat serum, 0.5% Triton-X100, and 0.01 M phosphate buffered saline (PBS, pH 7.5) for 48 h at 4°C. The sections were then incubated for 30 min with Alexa Fluor 488-labeled donkey anti-rabbit immunoglobulin G (Life Technologies). The sections immunoreacted with the 5-HT antibody were further incubated with GnRH antibody (LRH-13 for GnRH3 and aCII6 for GnRH2) followed by incubation with Alexa Fluor 594-labeled goat anti-mouse or donkey anti-rabbit immunoglobulin G (Life Technologies). After the staining, the sections were cover slipped with Vectashield (Vector Laboratories Inc.), viewed, and images were captured under a fluorescent microscope (90i; Nikon) that was attached to a digital camera (DXM 1200c; Nikon). The brightness and contrast adjustments were made using Adobe Photoshop CS5.1 (Adobe Systems).

The close associations between 5-HT and GnRH neuronal fibers were further analyzed by a confocal laser microscope (C1s; Nikon). The images were captured at 60× magnification with laser wavelength of 488 and 543 nm and were superimposed by confocal imaging software (NIS elements AR, version 4; Nikon). For high magnification, images were captured with a 60× water immersion objective (numerical aperture = 1.2) with an additional 9.9× optical zoom to give a final magnification of 594×, which yielded a voxel size of 0.08 μm. The close association was quantified by calculating the limit of the image by optical resolution using the wavelength formula $\lambda /2 \times$ (numerical aperture) and finally determining the pixel size of each cube that existed between the two labeled processes to confirm whether there was any blank pixel present in between them [33] (Supplemental Fig. S2). For the 3D construction, visualization, and conversion of the confocal image, we used confocal imaging software (NIS Element AR, version 4; Nikon). From the confocal software, under the 3D constructor option, the volume measurement parameter box was displayed, which helps to create the volume rendering of the binary layer of the Z-stack confocal image by defining the threshold value for the image. Once the value was defined, the software automatically loaded the 3D of the confocal image.

The 5-HT-GnRH3 fiber association/mean per nuclei were counted using imaging software (NIS element D, version 3; Nikon). The fiber association was estimated by considering three sections per sample from the respective brain region. The sizes of grids for each brain area that was measured were determined based on average size of the respective brain nuclei according to the zebrafish brain atlas [34] (Supplemental Table S1). Measurements taken from the predetermined field of each brain were pooled together, averaged, and expressed as the mean ± SD. Statistical analysis included univariate analysis followed by a least significant difference post hoc test.

Gene Expression Analysis

The effect of citalopram on mRNA levels of GnRH (*gnrh2* and *gnrh3*), 5-HT related genes (*tph2* and *slc6a4a*) in the brain, and gonadotropin-beta subunits (*lhb* and *fshb*) in the pituitary was examined by real-time PCR. Total RNA was extracted from the whole brain and pituitary (n = 10/control and treated group) using Trizol (Invitrogen) according to the manufacturer's instruction. RNA pellets were reconstituted in 20 μl of RNase-free water. First-strand cDNA was synthesized from 1 μg (for whole brain) or 0.05–0.1 μg (for pituitary) of total RNA using the high-capacity reverse transcription kit (Applied Biosystems) following the manufacturer's instruction. The cDNA was then subjected to real-time PCR using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The PCR reaction mixture (10 μl) contains 1× power SYBR green PCR mix (Applied Biosystems), 0.2 μM each of forward and reverse primer, and 1 μl of sample cDNA. Distilled water was used as a negative control (nontemplate control). The reaction program consisted of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by a dissociation stage. The threshold cycle (Ct) of each gene was determined and normalized to β-actin (*bactin1*) mRNA levels. The data were then analyzed according to the relative gene expression calculated by ddCt method. The primer sequences, PCR product size, and GenBank accession numbers of the genes are listed in Supplemental Table S2.

Effect of Citalopram on Fiber Density and Association of 5-HT with GnRH2 and GnRH3 Fibers

The effect of short-term citalopram treatment on 5-HT and two GnRH forms neural fiber density and the number of their associations were examined by image analysis (n = 5 for each 5-HT-GnRH2 and 5-HT-GnRH3). Double immunofluorescence was performed as described above. For specific identification of GnRH2, the brain sections were double-labeled with aCII6 (labeled with Alexa Fluor 488) and LRH-13 (labeled with Alexa Fluor 594) antibodies. Because aCII6 cross-reacts with both GnRH2 and GnRH3, the double-labeled GnRH fibers seen in yellow color were considered GnRH3 fibers, which were then subtracted to define green fluorescent-labeled fibers as genuine GnRH2 fibers (Supplemental Fig. S3). The densities of 5-HT and GnRH immunoreactive fibers were measured in different brain areas covering the forebrain, midbrain, and hindbrain regions by applying an approximate size of grid manually as described above. The area occupied with immunoreactive fibers was measured based on pixel density using imaging software (NIS element D, version 3; Nikon). The average pixel density fixed was 130 for the background tissue, and darker stained objects such as fibers had lower density levels. Threshold was set as 25% of maximum for the density of fibers and was used to define the boundaries of the image to be filled with a color overlay.

Gene Expression of 5-HT Receptors in the Testes

To confirm the expression of 5-HT receptor types (5-Htr1aa, 5-Htr1ab, 5-Htr1bd, 5-Htr2a, and 5-Htr7) in the testes, RT-PCR was performed. The testes were dissected from anesthetized male zebrafish (n = 5), and the total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instruction. First-strand cDNA was synthesized as described above. The primer sequences, PCR product size, and GenBank accession numbers of genes are listed in Supplemental Table S2. RT-PCR was performed in PCR reaction mixture (10 μl) containing premixed PCR solution (IPCR; iDNA Biotechnology Ltd.), 0.5 μM each of forward and reverse primer, and 1 μl of sample cDNA. Distilled water was used as a negative control, and the cDNA of whole brain sample was used as a positive control. The reaction program consisted of 95°C for 10 min, 95°C for 30 sec, and 35 cycles of 59°C for 30 sec, 72°C for 30 sec, and 72°C for 7 min, followed by 10°C. The PCR products were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet illuminator.

Histology of the Testes

For the morphological analysis of the testes, the trunk (n = 10/group) was fixed in Bouin solution (Sigma) for 18–24 h at room temperature followed by dehydration using different grades of ethanol (70%, 95%, and 100%) and butanol. The trunk was embedded in paraffin, and serial sections (5 μm) were cut using a microtome and mounted onto 3-aminopropylsilane-coated slides (Fisher Scientific). The deparafinized and rehydrated sections were stained with hematoxylin and eosin (Surgipath, Leica Microsystems) and coverslipped with DPX mountant (Fisher Scientific). The images of sections were captured under a light microscope (50i; Nikon) that was attached to a digital camera (DS-L2; Nikon). Stages of spermatogenesis, including spermatogonium, primary spermatocytes, secondary spermatocytes, spermatid, and sperm cell, were characterized as described previously [35]. The matured sperm cell area (500 × 400 μm^2) was measured by applying a grid manually. The areas taken and the applied grid for all the different stages of spermatogenesis were kept constant in order to reduce the bias of the analysis. The area occupied was counted using imaging software (NIS element D software, version 3; Nikon). Estimation of the matured sperm count was done by considering five sections per sample from the respective testes. Measurements taken from the predetermined field of each testis were pooled together and mean ± SD were calculated. Statistical analysis of the data included one-way ANOVA followed by the Dunnett post hoc test.

RESULTS

Association Between 5-HT and GnRH (GnRH2 and GnRH3) Neurons

Double-label immunofluorescence showed close association between 5-HT fibers with preoptic-GnRH3 neurons (Fig. 1A), but not with GnRH3 neurons in the olfactory bulb (Fig. 1B) and terminal nerve (Fig. 1C). There was no close association between 5-HT fibers with GnRH2 neurons in the midbrain (Fig. 1D). The close association between 5-HT fibers (1~2 fiber/cell) with preoptic-GnRH3 cell soma (approximately 20% of GnRH3 cells) was further confirmed by 3D analysis of confocal images (Fig. 1E–H). We also noted close association between 5-HT fibers and GnRH3 fibers in several brain regions (Fig. 2A–C). Quantification of the number of close associations between 5-HT and GnRH3 fibers revealed significantly higher numbers of close appositions in the POA and the hypothalamic nuclei in comparison with other brain regions (Fig. 2C). Those close associations between 5-HT and GnRH3 fibers (Fig. 3A–D) were further confirmed with 3D-deconvolution analysis (Fig. 3E–H). In the pituitary, there were GnRH3 fibers, but no 5-HT fibers were detected (Supplemental Fig. S4).

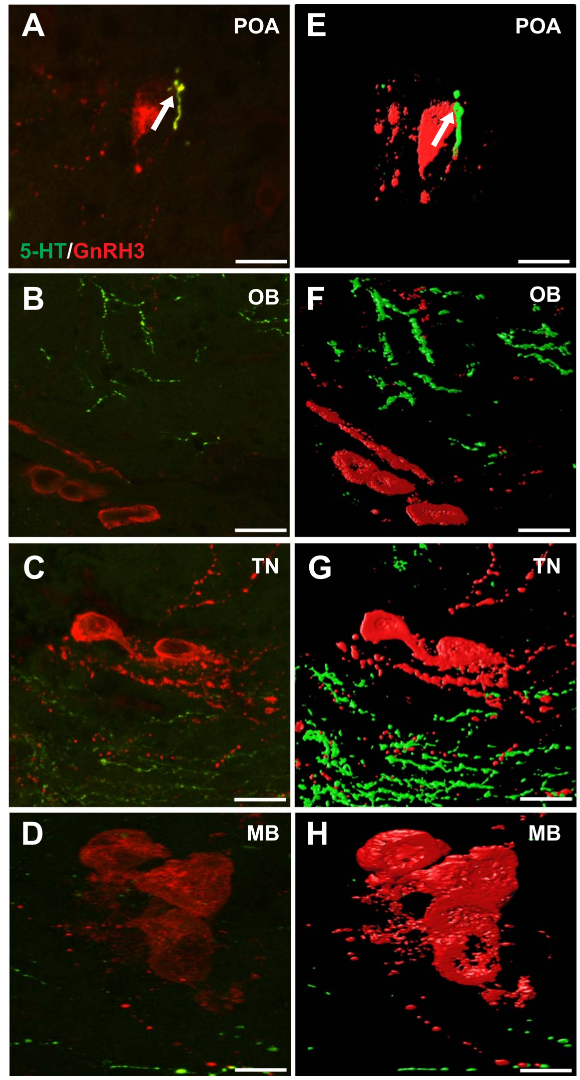

FIG. 1. Neuronal associations between 5-HT and GnRH neuronal types (GnRH2 and GnRH3) in the brain of adult male zebrafish. Confocal images of double immunofluorescence (**A**–**D**) and their respective 3D structures (**E**–**H**) for 5-HT fibers (green) and GnRH3 neurons (red) in the preoptic area (POA, **A** and **E**), olfactory bulb (OB, **B** and **F**), and terminal nerve (TN, **C** and **G**), and GnRH2 neurons (red) in the midbrain tegmentum (MB, **D** and **H**). Arrows (**A** and **E**) indicate the close apposition of 5-HT fibers to GnRH3 neurons in the POA. Bars = 20 μm.

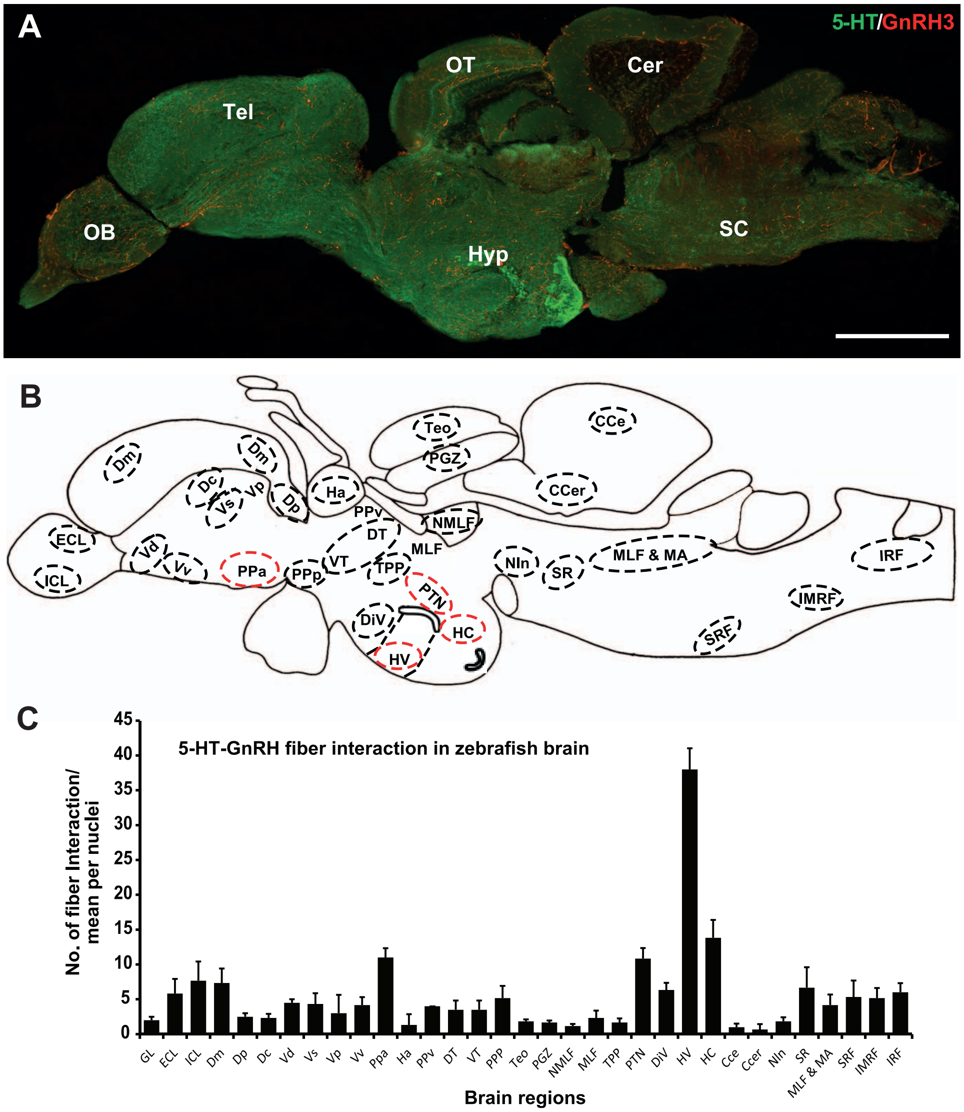

FIG. 2. Photomicrograph of sagittal section showing the distribution of 5-HT and GnRH3 fibers in the zebrafish brain (**A**). Schematic diagram showing the brain area (circled with dotted line) where number of close associations between 5-HT and GnRH3 fibers were counted (**B**). The areas circled with red lines were chosen for further confocal imaging analysis in Figure 3. Graphs (**C**) represent the numbers of close associations between 5-HT and GnRH3 fibers in the brain areas. OB, olfactory bulb; Tel, telencephalon; OT, optic tectum; Hyp, hypothalamus; Cer, cerebellum; SC, spinal cord. For definitions of abbreviations in **B** and **C**, see Supplemental Table S2. Bar = 500 μm.

*Effect of Citalopram on the Expression Levels of GnRH Types (*gnrh2 *and* gnrh3*) and Gonadotropins (*lhb *and* fshb*), and 5-HT-Related Genes (*tph2 *and* sert*)*

There was no effect of short-term (2 wk) citalopram treatment on *gnrh2* mRNA levels at low and medium doses (Fig. 4A), while *gnrh3* mRNA levels were significantly ($P < 0.05$) decreased compared with controls (Fig. 4B). Citalopram treatment at the high dose significantly increased *gnrh2* ($P < 0.001$) and *gnrh3* ($P < 0.05$) mRNA levels (Fig. 4, A and B). In the pituitary, citalopram treatment significantly decreased *lhb* and *fshb* mRNA levels at low and medium doses (Fig. 4, C and D), while citalopram treatment at the high dose significantly increased *lhb* mRNA levels ($P < 0.01$), and decreased *fshb* mRNA levels ($P < 0.01$) (Fig. 4, C and D). Gene expression levels of *tph2* ($P < 0.01$) and *sert* ($P < 0.001$) were significantly suppressed at low, medium, and high doses compared with controls (Fig. 4, E and F).

Long-term (1 mo) citalopram treatment at medium and high doses had no effect on *gnrh2* mRNA levels (Fig. 4G), while *gnrh3* mRNA levels were significantly ($P < 0.01$) decreased compared with controls (Fig. 4H). In the pituitary, citalopram treatment significantly ($P < 0.01$) decreased *lhb* and *fshb* mRNA levels at medium and high doses (Fig. 4, I and J). There was no effect of citalopram treatment on mRNA levels of 5-HT-related genes (*tph2* and *sert*) (Fig. 4, K and L).

Effect of Citalopram on 5-HT and GnRH Fibers

In short-term citalopram-treated zebrafish, the fiber density of 5-HT (Fig. 5A–F) and GnRH3 fibers (Fig. 5G–L) were significantly reduced in several brain regions, including the POA (Fig. 5, A–C and G–I) and the hypothalamus (the posterior tuberal nucleus and ventral and caudal hypothalamus) (Fig. 5, D–F and J–L). There was no difference in the fiber density of GnRH2 fibers between the short-term citalopram-treated group and the control group (Supplemental Fig. S3). Short-term citalopram treatment significantly reduced the

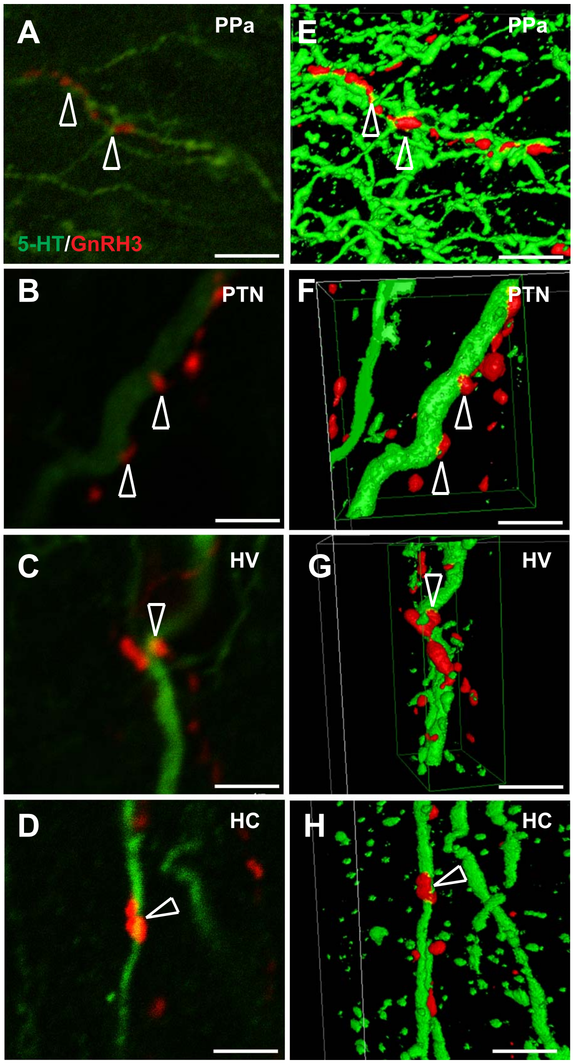

FIG. 3. Confocal images (**A**–**D**) and their 3D converted images (**E**–**H**) showing close association of 5-HT (green) and GnRH3 (red) immunoreactive fibers in the anterior part of the parvocellular preoptic nucleus (PPa), posterior tuberal nucleus (PTN), and the caudal (HC) and ventral zone of periventricular hypothalamus (HV) in the brain of adult male zebrafish. Arrowheads indicate close associations of 5-HT and GnRH fibers seen in yellow. Bars = 3 μm.

number of close associations of 5-HT and GnRH3 fibers in the POA, posterior tuberal nucleus, ventral and caudal hypothalamus, medial zone of dorsal telencephalic area, and superior raphe when compared to controls (Fig. 6A–J).

5-HT Receptor Expression in the Testes

RT-PCR results showed expression of three subtypes (*5-htrabd, 5-htr1bd,* and *5-htr2a*) in the testes (Supplemental Fig. S5A).

Effect of Citalopram on Spermatogenesis

After short-term citalopram treatment with low, medium, and high doses, there was no difference in the profiles of spermatogenic stages in the testes when compared to controls (Fig. 7A–G). Long-term medium- and high-dose citalopram treatments displayed a drastic decrease in the developmental stages of spermatogenesis ($P < 0.05$ and $P < 0.01$; Fig. 7H–K) as well as in the matured sperm cell count ($P < 0.001$ and $P < 0.01$; Fig. 7L). In addition, in the testes for the high-dose citalopram-treated group, there were more testicular interstitial cell prominences compared with the control group (Fig. 7I).

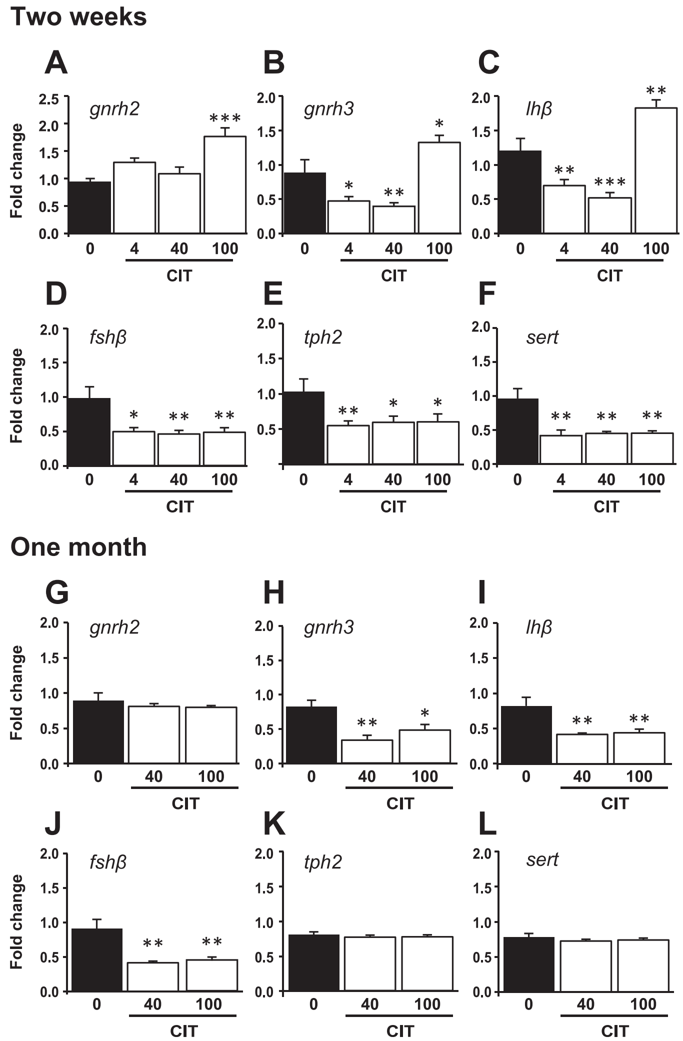

FIG. 4. Graphs represent effect of 2 wk and 1 mo treatment of citalopram (CIT) on gene expression levels of GnRH (*gnrh2* and *gnrh3*), gonadotropins (*lhβ* and *fshβ*), and 5-HT-related genes (*tph2* and *sert*). The relative abundances of mRNA were normalized to the amount of β-actin mRNA levels using the comparative threshold cycle method. **A**–**F**) Two weeks of CIT treatment at low (4 μg/L), and medium (40 μg/L) (open columns) doses significantly reduced mRNA expression of GnRH3 (**B**), gonadotropins (**C** and **D**), and 5-HT-related genes (**E** and **F**) in comparison with control (0 μg/L, closed columns). CIT treatment with high dose (100 μg/L) significantly increased *gnrh2, gnrh3,* and *lhβ* mRNA levels (open columns) (**A**–**C**). **G**–**L**) One month of CIT treatment at medium (40 μg/L) and high (100 μg/L) doses significantly reduced *gnrh3* (**H**) and gonadotropins (**I** and **J**) mRNA levels in comparison with control. There was no effect of CIT treatment on mRNA levels of *gnrh2* (**G**) and 5-HT-related genes (**K** and **L**). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control.

DISCUSSION

Association Between 5-HT and GnRH Neuron Types

Using confocal microscopy with double immunofluorescence, close association between 5-HT fibers and preoptic-GnRH3 neurons were observed in the brains of male zebrafish. Similar findings have been reported in mammals and teleosts [17, 36]. In addition, close associations between 5-HT and preoptic-GnRH3 neurons correspond with the stimulatory

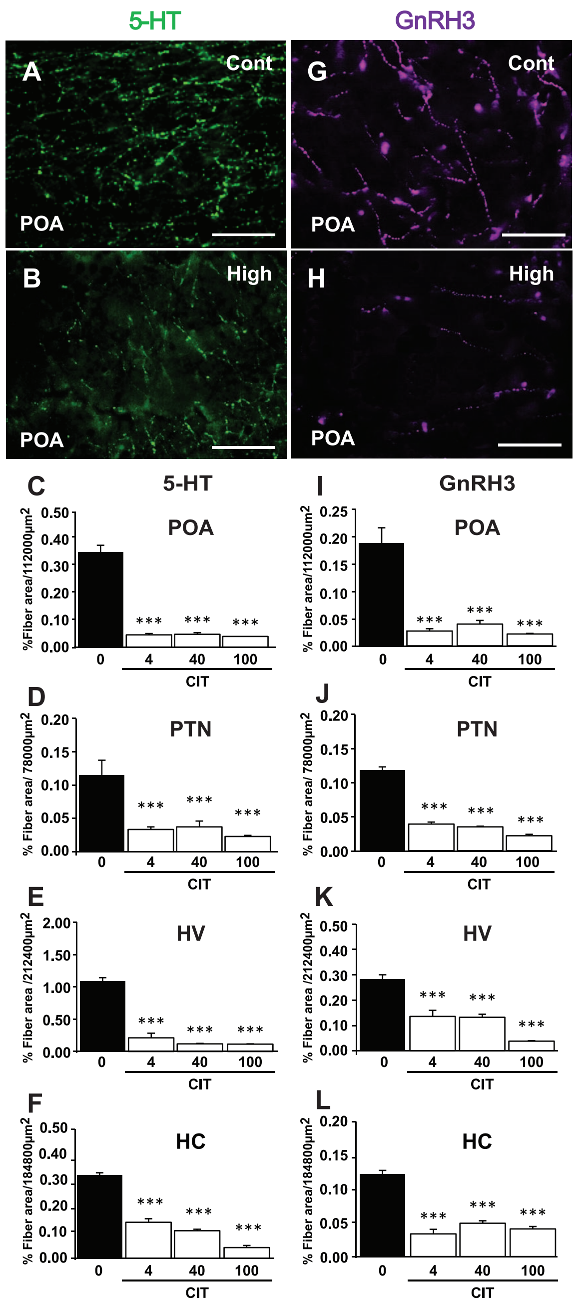

FIG. 5. Effect of citalopram (CIT) exposure on the density of 5-HT (**A–F**) and GnRH3 (**G–L**) immunoreactive fibers in the POA region. Photomicrographs of 5-HT (**A** and **B**) and GnRH3 (**G** and **H**) fibers in the control (Cont; **A** and **G**) and high concentration (100 µg/L) of CIT-treated (**B** and **H**) samples. Graphs represent the influence of CIT (control, 0 µg/L: closed bars; 4, 40, and 100 µg/L: open bars) treatment on density of 5-HT (**C–F**) and GnRH3 fibers (**I–L**) in the POA region and hypothalamus (posterior tuberal nucleus, PTN; ventral zone of periventricular hypothalamus, HV; and caudal zone of periventricular hypothalamus, HC). ***$P < 0.001$ versus control. Bars = 50 µm.

effect of 5-HT on GnRH release in goldfish [37]. However, in the present study, very few preoptic-GnRH3 cell somas were associated with 5-HT fibers. Similarly in rats, 5-HT synapses on GnRH dendrites are fewer than those of other amine neurotransmitters [17]. Therefore, the synaptic action of 5-HT on GnRH neural cells could be very minor when compared to other neurotransmitters. There were no fiber associations between 5-HT and GnRH2, which is similar to the observation in the Atlantic croaker [36]. We noted predominant close associations between 5-HT and GnRH3 neurons at the axon-axon level rather than at the axon-cell soma level. However, it remains to be further verified whether their associations are synaptic. Thus far, there are no reports demonstrating axon-axon association between 5-HT and GnRH neuron in other vertebrates as well as a study elucidating their physiological significance. The association between 5-HT fibers and GnRH3 fibers were mainly observed in the POA and hypothalamic regions. However, the origin of those GnRH3 fibers, either from the hypophysiotropic preoptic-GnRH3 (POA-GnRH3) population or from the olfactory-terminal nerve population of GnRH3 (OB-TN GnRH3) neurons or both, is unknown. This is very important in helping to elucidate the potential involvement of the associations between 5-HT fibers and GnRH3 fibers in the control of reproduction, an area that remains to be investigated.

Our immunohistochemical study failed to show any 5-HT immunoreactivity in the pituitary of male zebrafish. This contradicts previous observations in other fish species that demonstrate the presence of 5-HT fibers, cell bodies, or 5-HT receptors in the pituitary [38, 39]. There are in vivo as well as in vitro studies demonstrating direct action of 5-HT on pituitary gonadotrophs in various teleost species [40–42]. These contradictions could be because of undetectable levels of 5-HT in the male zebrafish pituitary due to reproductive status as reported in some teleosts [39, 43] or sexual differences, which remains to be further confirmed.

Effect of Citalopram on 5-HT, GnRH, and Gonadotropins

Short-term citalopram treatment (low and medium doses) significantly reduced 5-HT fiber density and expression of 5-HT-related genes, indicating the reduction of 5-HT synthesis as seen in mammals [22, 44]. On the other hand, long-term citalopram treatment had no effect on 5-HT-related gene expression. Several studies demonstrating the effect of chronic SSRI treatment on 5-HT synthesis in mammals show contradictory results [22, 45], which could be due to differences in methodologies. Nevertheless, it is clear that SSRIs produce robust increases in extracellular 5-HT and deplete brain stores of 5-HT [22]. Because sexual dysfunction still persists after discontinuation of SSRI treatment [46, 47], it remains to be examined at the neurotransmission level whether long-term treatment as well as withdrawal of SSRIs have compensatory or adaptive effect on 5-HT, which was beyond the scope of this study.

In the fish treated with citalopram, GnRH3 fiber density and GnRH3 mRNA expression levels were significantly reduced. In addition, gonadotropin mRNA levels were significantly decreased in fish treated with low and medium doses of citalopram. In rats, 60 days of fluoxetine treatment reduced circulating LH and FSH levels [48, 49]. Similarly in male goldfish, 2 wk of fluoxetine treatment reduced *lhβ* mRNA levels in the pituitary but had no effect on circulating LH levels [50]. It has been shown that 5-HT stimulates release of GnRH [19, 20, 51, 52], while 5-HT also exerts a negative tonic influence on the biosynthesis of GnRH [18]. These results suggest that citalopram treatment induced an increase of extracellular 5-HT, which suppressed GnRH3 synthesis, whereby reducing gonadotropin synthesis. Additionally, it is also possible that citalopram directly act on gonadotrophs in the pituitary, which remains to be confirmed by examining of effect of citalopram on GnRH neural activities. Short-term high-dose citalopram treatment showed an upregulation of GnRH2, GnRH3, and LH gene expression. Although the

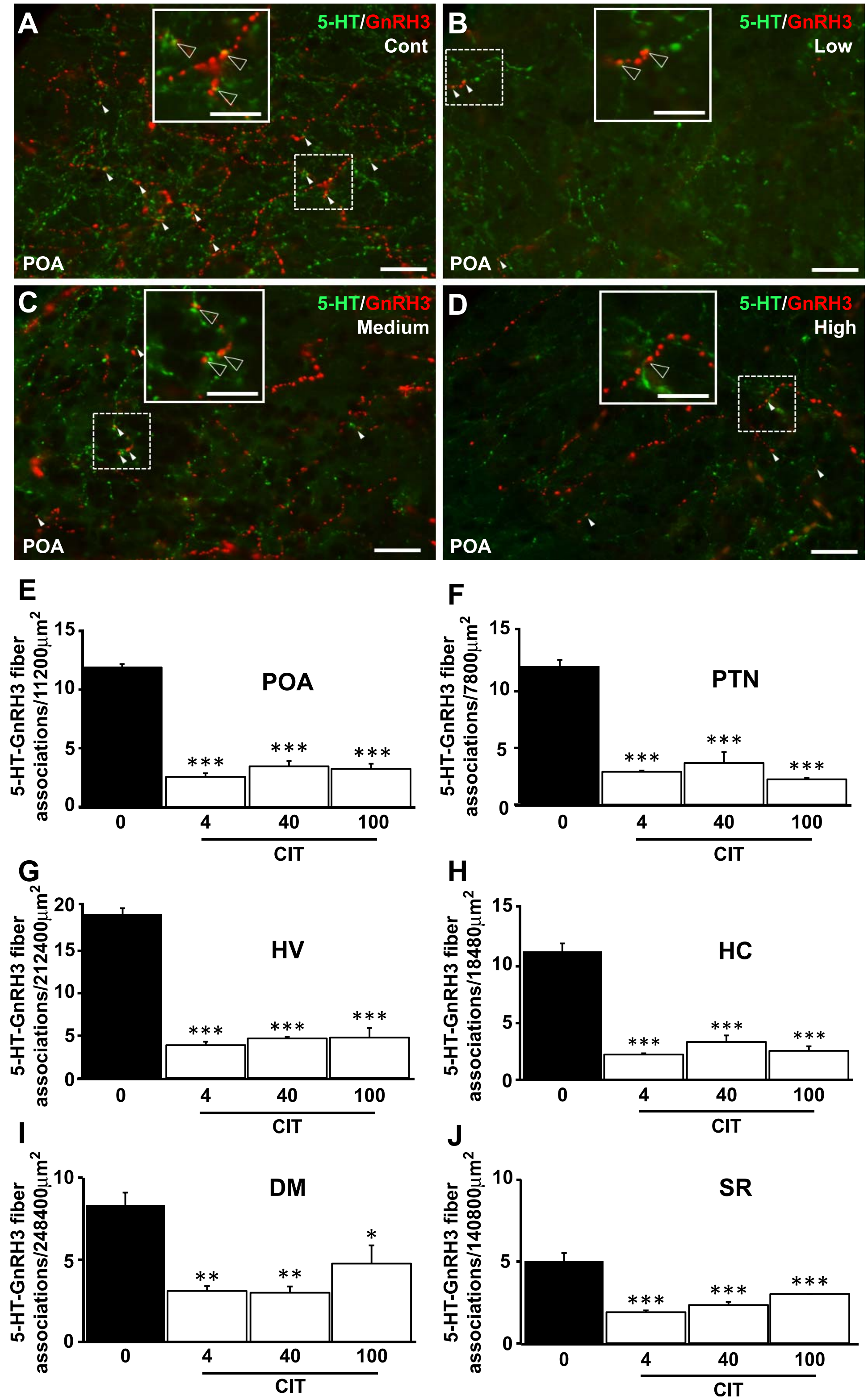

FIG. 6. Microphotographs showing the effect of 2 wk of citalopram (CIT) treatment at low (4 μg/L), medium (40 μg/L), and high (100 μg/L) doses on number of close associations of 5-HT (green) and GnRH3 (red) fibers in the preoptic area (POA; **A**–**D**). Arrowheads indicate close associations between 5-HT and GnRH3 fibers (yellow). Graphs (**E**–**J**) represent the effect of CIT (4, 40, and 100 μg/L, 2 wk; open columns) or water (0 μg/L, closed columns) on the number of fiber associations between 5-HT and GnRH3 in the POA (**E**), the hypothalamic nuclei (posterior tuberal nucleus, PTN; ventral zone of periventricular hypothalamus, HV; and caudal zone of periventricular hypothalamus, HC; **F**–**H**), the medial zone of dorsal telencephalic area (Dm, **I**) and superior raphe (SR, **J**). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control. Bars = 100 μm; insets: 50 μm (with ×2 zoom). Cont, control.

potential mechanism underlying upregulation of those genes is still unclear, SSRIs are known to act via multiple pathways [53]. For example, in fish, SSRIs are suggested to interact with and inhibit some P450 isozymes that are responsible for steroid metabolism [7]. High doses of SSRI may alter those steroids, which may have a significant influence on the reproductive

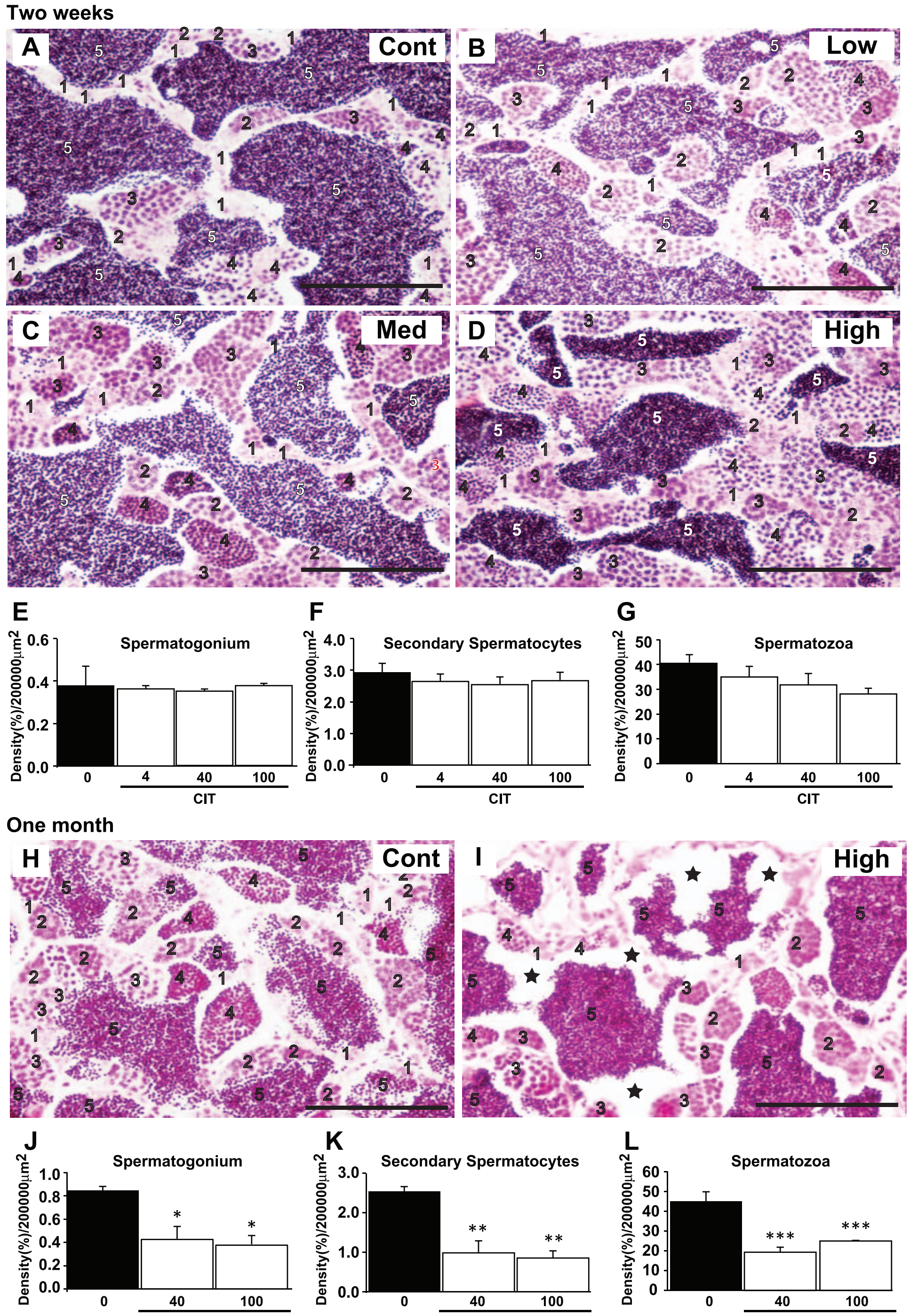

FIG. 7. Morphological analysis of the effect of citalopram (CIT) treatment on different stages of spermatogenesis in the testis of male zebrafish after 2 wk (**A**–**D**) and 1 mo (**H**–**I**) of treatment. **A**–**D**) Microphotographs showing the testis of male zebrafish treated with CIT (low: 4 μg/L; medium: 40 μg/L; and high: 100 μg/L) for 2 wk. **E**–**G**) Graphs represent the cell density (%) of spermatogonium (**E**), secondary spermatocytes (**F**), and spermatozoa (**G**) in the fish treated with CIT (open columns) and control (closed column). **H** and **I**) Microphotographs showing the testis of male zebrafish treated with high dose of CIT (100 μg/L, **I**) or water (**H**) for 1 mo. **J**–**L**) Graphs represent the effect of CIT (medium: 40 μg/L, and high: 100 μg/L; open columns) and water (0 μg/L, closed columns) on the cell density (%) of spermatogonium (**J**), secondary spermatocytes (**K**), and spermatozoa (**L**). Stars indicate the testis interstitial space seen in fish treated with high concentration of CIT (**I**). Numbers in photomicrographs (**A**–**D**, **H**, and **I**) represent different stages of spermatogenic cells: 1, spermatogonium; 2, primary spermatocytes; 3, secondary spermatocytes; 4, spermatid; and 5, sperm cells (spermatozoa). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. Bars = 100 μm. Cont, control.

neuroendocrine system. In mice, there are no such effects on GnRH and gonadotropins when they were treated with other SSRIs, including sertraline, paroxetine, and escitalopram (S-enantiomer of citalopram, which is more effective and specific than citalopram) [54]. Furthermore, in male mice, there was no effect citalopram treatment on the expression of GnRH (neuronal numbers and mRNA levels) [4]. These observations indicate that different SSRI drugs may differentially act on reproductive neuroendocrine signaling because of their different specificities on 5-HT system.

Effect of Citalopram on Gonadal Morphology

There was no profound effect of citalopram treatment on the testes morphology and gametogenesis despite significant reduction of GnRH3, LH, and FSH synthesis. Similarly, in other teleosts, fluoxetine treatment exhibits inhibition of *fshβ* mRNA in the pituitary but has no major effect on all stages of spermatogenesis [30, 50]. However, in the present study, long-term citalopram treatment caused deleterious effect on spermatogenesis, which corresponds with reduction of milt volume in the goldfish exposed with fluoxetine [50]. Additionally, we also notice occurrence of interstitial space in the testes of long-term treated fish, which is very similar to the interstitial cell prominence seen in male fathead minnows treated with high concentration of fluoxetine [55]. This phenotypic alteration in the testicular morphology could be considered as morphological evidence for reproductive failure in male zebrafish. Similar to our study, in the testes of male fathead minnows, significant interstitial cell hypertrophy was only apparent in the fish treated with high (28 ng/L) concentration of fluoxetine after 21 days of exposure [56]. These results suggest that citalopram-induced sexual dysfunction at the level of gonads only can be seen when the fish were exposed with high dose and for longer period, although hormonal, behavioral alteration and milt release by SSRIs can be produced in lower doses with shorter periods in other teleosts [11, 50, 56].

Although it remains unknown how citalopram induces a deleterious effect on spermatogenesis, we speculate two potential possibilities. SSRIs might be capable to directly act at the level of the gonads in teleosts because 5-HT receptors (present study) and the SERT gene is expressed in the testes [29]. In addition, in the zebrafish, fluoxetine treatment reduces the expression of *lhr, fshr,* and aromatase gene in the ovaries [10]. Alternatively, the major target of SSRIs could be the brain. Several studies in fish have demonstrated high concentrations of SSRIs in the brain [7, 55]. Further, we have shown a significant change in GnRH and gonadotropins levels in the brain and pituitary following citalopram exposure, which could alter testicular morphology and spermatogenesis.

Because 5-HT is an evolutionarily conserved neurotransmitter, it is highly possible that SSRIs could display similar pharmacological activates in fish as in humans and other mammals [7]. However, it is important to note that most antidepressant drugs are specifically designed for mammals and not for nonmammalian species, including fish; therefore SSRIs may interact with the hypothalamic-pituitary-gonadal axis via multiple known or unknown signal transduction pathways in fish that are either absent or differently regulated in mammals [7, 57]. This could be the reason for the contradiction between our results and those in male mice that show no effect of citalopram treatment on GnRH (neuronal numbers and mRNA levels) [4].

This study shows significant close association between 5-HT fibers and GnRH3 fibers, but not GnRH3 cell soma, in the brains of male zebrafish. In the pituitary, there was no close association between 5-HT and GnRH3. These observations suggest the serotonergic action on the GnRH3 system via fiber-fiber interactions. Short-term citalopram treatment reduced the density of 5-HT and GnRH3 fibers as well as expression of 5-HT-related genes and GnRH3 indicating a reduction of 5-HT and GnRH3 synthesis. Furthermore, citalopram treatment also reduced gene expression of gonadotropins in the pituitary. In the testes, there was no effect of short-term citalopram treatment on the testicular morphology, but long-term treatment significantly altered testicular morphology, including increasing testicular interstitial cell prominence and decreasing spermatogenesis. These results suggest that chronic citalopram treatment has a significant influence on the hypophysiotropic GnRH3 system, which leads to testicular failure in the male zebrafish. However, because 5-HT receptors and SERT are expressed in the testes, it is also possible that citalopram could act directly on the testes, which remains to be examined.

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