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# Vasoactive intestinal peptide indirectly elicits pituitary LH secretion independent of GnRH in female zebrafish

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**Abstract**

Vasoactive intestinal peptide (Vip) regulates luteinizing hormone (LH) release through the direct regulation of gonadotropin-releasing hormone (GnRH) neurons at the level of the brain in female rodents. However, little is known regarding the roles of Vip in teleost reproduction. Although GnRH is critical for fertility through the regulation of LH secretion in vertebrates, the exact role of the hypophysiotropic GnRH (GnRH3) in zebrafish is unclear since GnRH3 *null* fish are reproductively fertile. This phenomenon raises the possibility of a redundant regulatory pathway(s) for LH secretion in zebrafish. Here, we demonstrate that VipA (homologues of mammalian Vip) both inhibits and induces LH secretion in zebrafish. Despite the observation that VipA axons may reach the pituitary proximal pars distalis including LH cells, pituitary incubation with VipA in vitro, and intraperitoneal injection of VipA, did not induce LH secretion and *lhβ* mRNA expression in sexually mature females, respectively. On the other hand, intracerebroventricular administration of VipA augmented plasma LH levels in both wild type and *gnrh3*$^{-/-}$ females at 1 hour post-treatment, with no observed changes in pituitary GnRH2 and GnRH3 contents and *gnrh3* mRNA levels in the brains. While VipA's manner of inhibition of LH secretion has yet to be explored, the stimulation seems to occur via a different pathway than GnRH3, dopamine, and $E_2$ in regulating LH secretion. The results indicate that VipA induces LH release possibly by acting with or through a non-GnRH factor(s), providing proof for the existence of functional redundancy of LH release in sexually mature female zebrafish.

Keywords

Vip, LH, GnRH, zebrafish, reproduction

## Introduction

Gonadotropin-releasing hormone (GnRH) is known to be the ultimate regulatory element of the hypothalamus-pituitary-gonadal (HPG) axis, which controls reproduction in vertebrates. GnRH stimulates the synthesis and release of the gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), from gonadotropes in the pituitary. Natural mutation in the *GnRH* gene in mammalian species results in reduced serum FSH and LH levels leading to hypogonadotropic hypogonadism, in which all individuals are infertile (1,2). Similar to all studied vertebrates, when the hypophysiotropic GnRH is eliminated in medaka (*gnrh1*$^{-/-}$; *Oryzias latipes*), a fish with three GnRH isoforms, final oocyte maturation and ovulation are compromised in females presumably due to insufficient LH production (3). However, in zebrafish (*Danio rerio*), a vertebrate model possessing two GnRH isoforms (GnRH2 and GnRH3), recent studies have shown that the lack of the hypophysiotropic GnRH isoform, GnRH3, does not affect reproductive fertility (4,5). Moreover, GnRH-less zebrafish (*gnrh2*$^{-/-}$; *gnrh3*$^{-/-}$) also display normal reproductive performance and success (6). These findings have reopened a question as to how the hypothalamus communicates with the pituitary to drive reproduction, sparking a debate regarding the roles of GnRH in reproduction of this species. The lack of a mutant phenotype, e.g., normal fertility in *gnrh3*$^{-/-}$ zebrafish (4,5), may be explained by functional redundancy, in which the hypophysiotropic GnRH, GnRH3, is not a sole or a central controller of reproduction in zebrafish, particularly pertaining to regulating LH release from the pituitary. Multiple molecules have recently been implicated in the regulation of pituitary gland coordination of the perfectly timed and accurate secretion of pituitary hormones (7). Hence, unlike GnRH1-dependent mechanisms in other species, functional redundancy may exist along the HPG axis and support the successful LH secretion in zebrafish. Although multiple factors were shown to induce LH release in teleosts (8), the exact mechanism and whether they can substitute for the GnRH secretagogue action have yet to be demonstrated.

Vasoactive intestinal peptide (Vip) is a 28 amino acid neuropeptide originally isolated from porcine gastrointestinal wall (9) that is widely distributed in the central and peripheral nervous systems (10), exhibiting a wide-ranging profile of biological activities. Its neurons in the

suprachiasmatic nucleus (SCN) innervate GnRH neurons in the median preoptic area (POA) in mice (11). Exogenous Vip increases the electrical activity of GnRH neurons during proestrus, indicating that Vip mediates an excitatory signal from the circadian clock to time the GnRH and LH surge (12,13). Its genetic loss causes disruption of reproduction, estrous cycle, and circadian rhythms in female mice (14). Vip is also found within the external zone of the median eminence with a high concentration in hypophyseal portal blood of rats (15–17), suggesting that it directly acts on endocrine cells in the anterior pituitary. However, the effect of Vip on GnRH and LH secretion in teleosts remains unclear, and little has been reported on the functions of Vip in fish reproduction. Our initial neuroanatomical analysis of VipA (homologues of mammalian Vip in zebrafish) showed that VipA cells are localized in the POA and hypothalamus of sexually mature female zebrafish. In addition, VipA neuronal fibers may directly innervate pituitary endocrine cells in the proximal pars distalis (PPD) including some gonadotropes, suggesting that VipA, similar to the case in mammals, may regulate pituitary hormones, including LH secretion in zebrafish.

Our goal was to examine potential redundant pathways that regulate pituitary LH synthesis and secretion in zebrafish. Here, we examined the roles of VipA in zebrafish reproduction and evaluated whether VipA is a potential redundant inducer of LH secretion in zebrafish. To determine whether VipA functions as a potential reproductive compensator in the absence of the hypophysiotropic GnRH3 in *gnrh3*$^{-/-}$ zebrafish, we compared the number of *vipa*-expressing cells in the preoptic area between wild type (WT) and *gnrh3*$^{-/-}$ mature females. We then examined whether VipA is a hypophysiotropic neuropeptide that directly regulates LH secretion at the pituitary level via in vitro whole pituitary explants, as well as in vivo intraperitoneal (IP) injection to determine whether VipA induced LH β subunit (*lhβ*) mRNA levels. To examine whether VipA indirectly induces LH release through the hypophysiotropic GnRH signal, we also performed in vivo intracerebroventricular (ICV) administration of VipA and determined the effect on plasma LH levels, brain *gnrh3* mRNA levels and pituitary GnRH3 protein content. Additionally, we eliminated VipA in the brain using a specific Vip antibody and determined the effect on plasma LH levels. Finally, we generated *vipa*$^{-/-}$ and *vipa*$^{-/-}$; *gnrh3*$^{-/-}$ zebrafish models and assessed their reproductive characteristics. Together, our data

illustrates that, while VipA is not a critical regulator for LH release in zebrafish, it indirectly induces LH release by acting with or through a non-GnRH brain factor(s). We were able to provide clear proof for the existence of a functional redundant pathway(s) for LH secretion in zebrafish that does not rely on GnRH functions.

## Material and methods

### Animals

All zebrafish were maintained in a 28°C recirculating system under a 14-hour light, 10-hour dark photoperiod. Tübingen WT was used for the experiments including the generation of knockout fish. Before tissue sampling, adult zebrafish were deeply anesthetized in tricaine methanesulfonate (MS-222; Sigma-Aldrich) and then rapidly decapitated. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine (approval # 0519010).

### in situ hybridization

Digoxigenin (DIG)-labeled cRNA riboprobes were prepared from full-length zebrafish *vipa* cDNA (GenBank accession # NM_001114553.3) using SP6 or T7 RNA polymerase (New England Biolabs) with a DIG RNA Labeling Kit (Roche). Brains were excised via dissection from sexually mature females at 4 months of age and fixed with 4% paraformaldehyde (PFA) in PBS at 4°C overnight. The brains were transferred into 30% sucrose in 0.1M phosphate buffer (PB) at 4°C until they sunk to the bottom of glass vials. After mounting with OCT compound (Sakura Fineteck, Inc.), the brains were sectioned coronally at 13 µm thickness using a cryostat (Sakura Fineteck, Inc.), transferred to positively charged slides (Denville UltraClear; Denville Scientific), and stored at -80°C until processing. in situ hybridization (ISH) was conducted as described in a previous study (6) with some modifications. Briefly, after incubation with 1 µg/mL proteinase K (New England Biolabs) in

PBS at 37°C for 10 minutes, the sections were post-fixed with 4% PFA in PBS at room temperature for 20 minutes. The sections were prehybridized at 62°C for 2 hours followed by a hybridization with the 500 ng/mL generated *vipa* anti-sense or sense DIG-labeled riboprobe overnight at 62°C. After the incubation with TNB blocking buffer [100 mM Tris-HCl, pH 7.5; 0.15 M NaCl; 0.5% TSA Blocking Reagent (Perkin Elmer)], slides were incubated with anti-DIG Fab fragment conjugated to alkaline phosphatase (Roche) (18). The hybridization signals were then developed with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP stock solution; Roche). After mounting the slides, they were analyzed using Zeiss Axioplan microscope (Carl Zeiss Microscopy), and section images were photodocumented. We adhered to the zebrafish brain atlas (19) nomenclature for the zebrafish brain nucleus descriptions in this study.

Quantification of *vipa*-expressing neurons

Brains of sexually mature WT and *gnrh3*$^{-/-}$ females at 4 months of age (N=6 each) were removed and subjected to *vipa* ISH as described above. The number of *vipa*-expressing cells was counted, manually in the brain regions, the parvocellular preoptic nucleus anterior part (PPa) or postcommissural nucleus of ventral telencephalic area (Vp) of the preoptic area (POA) on the sections. We depicted the average number of *vipa*-expressing cells per 10 consecutive sections for each brain region.

Generation of polyclonal antibody against zebrafish VipA precursor protein

The cDNA of zebrafish *vipa* (GenBank accession # NM_001114553.3) corresponding to a fragment from 157 bp to 601 bp was cloned into pET-15b vector and expressed in Rosetta-gami B (DE3) pLysS *E. coli* cells (Novagen) as N-terminal His-tagged recombinant proteins. The proteins from the insoluble fraction were collected according to a standard protocol (20). Collected proteins were dissolved in 8 M urea, purified with nickel-nitrilotriacetic acid columns (Promega), followed by resolution on 16% Tris-Tricine SDS-PAGE. The protein band at the expected size of –19.5 kDa was

excised from the gel and used as an antigen to produce antiserum in rabbits (Protein Tech). The final bleed antiserum was used as the primary antibody in all zebrafish VipA immunohistochemistry (IHC) in this study.

Verification of anti-zebrafish VipA precursor polyclonal antibody

Specificity of the generated zebrafish VipA polyclonal antibody was verified by two approaches: I. Specificity via IHC using the pre-immune serum as a negative control alongside the generated VipA antiserum on mature female brain sequential sections; II. Co-localization of the signals using a combined *vipa* ISH and VipA IHC. Brain sections (20 µm thickness) for both approaches were prepared as described in the ISH procedure above. IHC was performed based on previously described protocols (4). Briefly, the sections were blocked with blocking buffer (3% normal goat serum; 3% BSA; 0.3% Triton X-100 in PBS) for 1.5 hours at room temperature followed by incubation at 4°C overnight with anti-VipA polyclonal antibody or pre-immune serum diluted 1:500 in blocking buffer. After washing in PBST (0.05% Tween 20 in PBS), the sections were incubated with Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen) (21) diluted 1:850 in blocking buffer at room temperature for 1 hour. The sections were mounted with mounting reagent [20 mM Tris-HCl, pH 8.0; 0.5% n-propyl gallate; 90% glycerol; 1 µg/mL Hoechst 33258 (Sigma-Aldrich)]. Combined ISH with IHC was conducted as follows: Post-fixation, the sections were incubated for 30 minutes with 0.3% $H_2O_2$ in methanol to quench endogenous peroxidases. Then, ISH using antisense *vipa* DIG-labeled riboprobe was performed based on the protocol described above, except for the signal development methods. The signals were developed using a Tyramide Signal Amplification (TSA) Plus kit (Perkin Elmer), according to the manufacturer's protocol, with anti-DIG Fab fragment conjugated to horseradish peroxidase (Roche) (22) diluted 1:200 in the TNB buffer. Fluorescent labeling was obtained via Cy3 tyramide from the TSA Plus kit. After washing in Tris-NaCl-Tween buffer (150 mM NaCl; 0.1% Tween 20), the sections were incubated with VipA antibody diluted 1:500 in blocking buffer at 4°C overnight followed by incubation with Alexa Fluor

647-conjugated goat anti-rabbit IgG as described above. The section images were photodocumented using a Leica SP8 confocal microscope (Leica Microsystems) and compiled using Fiji (23) and Photoshop (Adobe).

Immunohistochemistry on brains and pituitaries

IHC was performed on sagittal sections of brains from 5 months old sexually mature females as described above. For pituitary section IHC, heads were removed from 4 months old transgenic [*Tg (gnrh3*: *eGFP*; *lhβ*: *mCherry)*] sexually mature females (24,25), and fixed with 4% PFA in PBS at 4°C overnight followed by a decalcification for 5 days in 0.5 M EDTA, pH 8.0. Section preparation, IHC and imaging were performed as delineated above, except for the section thickness which was 30 µm.

Whole pituitary incubation, in vitro

The protocol followed our standard lab protocol (26) with several modifications. Whole pituitaries were excised from 4 months old sexually mature WT females (N=5 each) at 1000 h–1100 h (1–2-hour after lights on), and placed in 24-well plates with 70 µm pore size membrane inserts (Millipore) filled with 700 µL of precooled phenol red-free L-15 media (Himedia) supplemented with 0.1% BSA, 9 mM sodium bicarbonate, 20 mM HEPES, 100 units/mL penicillin and 12.5 units/mL streptomycin, adjusted to pH 7.4. After a 2-hour preincubation at 28°C with rotation at 30 rpm, pituitaries with the culture insert were transferred into fresh medium containing 100 nM VipA (HSDAIFTDNYSRFRKQMAVKKYLNSVLTG-$NH_2$; synthesized by Biomatik with >95% purity as determined by analytical HPLC) or 100 nM GnRH3 (Sigma-Aldrich, cat # L4897) with or without 100 nM 17β-estradiol ($E_2$) (Sigma-Aldrich) and incubated for 18 hours at 28°C with rotation at 30 rpm. The concentration and incubation time of $E_2$ and GnRH3 are based on previous studies showing effective stimulation (26–29). Also, different GnRH3 concentrations (10 and 100 nM) and different incubation times were tested in preliminary experiments (Supplementary Fig. 1) (30). Subsequently,

the culture media was collected from each well and transferred to 2 mL tubes, lyophilized, and resuspended with sterile water. LH concentration in the culture media was determined using carp LH ELISA (31,32) in duplicate.

VipA intraperitoneal treatment, in vivo

IP injections were conducted using a 5 µL Hamilton Neuros syringe (Hamilton Company) set with 33-gauge needle and a 2 mm penetration stopper. At 1030 h–1230 h, sexually mature WT females, 5 months of age, were deeply anesthetized in MS-222 and injected at 24-hour intervals with 4 µL/g body weight (BW) fish saline containing either: 1) No peptide (as negative control), 2) 200 ng/g BW (58.98 pmol/g BW) VipA, 3) 200 ng/g BW VipA with 10 µg/g BW dopamine D2 receptor antagonist, domperidone (DOM; Sigma-Aldrich), 4) 200 ng/g BW GnRH3 (162 pmol/g BW), 5) 200 ng/g BW GnRH3 with 10 µg/g BW DOM, 6) 10 µg/g BW DOM alone (N=5 each, except for the control for which N=4 and GnRH alone and DOM alone for which N=3). The concentrations of GnRH3 and DOM used in this study were based on a previous study (33), and the VipA concentration was set as similar to the GnRH concentration that effectively increased pituitary *lhβ* mRNA levels when IP-injected with DOM in adult zebrafish females. 18 hours after the second injection, pituitaries were excised, frozen in dry ice, and stored at -80°C until the assay.

Real-time quantitative PCR

Total RNA was extracted from the excised pituitaries using Trizol (Invitrogen) according to the manufacturer's protocol. 250 ng of total RNA was then used for 10 µL reaction of reverse transcription using QuantiNova Reverse Transcription Kit (Qiagen) containing oligo-dT and random primers. Real-time quantitative PCR (qPCR) was performed using GoTaq qPCR Master Mix (Promega) on a 7500 Fast Real-Time PCR System (Applied Biosystems), and the cycling condition was set as 95°C activation for 2 minutes, followed by 40 repeating cycles of 95°C denaturation for 3

seconds and 60°C annealing and extension for 30 seconds. Production of a single PCR amplicon for each primer pair was verified by melting curve analysis. Relative expression levels for the target gene, *lhβ*, were quantified in duplicate using the $2^{-\Delta\Delta Ct}$ method and normalized against the levels of the internal housekeeping gene, *eef1α1*. The primers used for qPCR are listed in Table 1.

Intracerebroventricular treatment, in vivo

ICV injections were performed using a 5 µL Hamilton Neuros syringe (Hamilton Company) with a 33-gauge needle and a 1.5 mm penetration stopper. At 1000 h–1100 h (1–2-hour after lights on), sexually mature WT or *gnrh3*$^{-/-}$ females, 5 months of age, were deeply anesthetized in MS-222 and injected with 0.5 µL/g BW fish saline containing either 4 pmol/g BW VipA or no peptide. The VipA concentration was determined by a previous study conducted using Vip and PACAP ICV injection in goldfish (34) and a study in which GnRH2 ICV injection at 2 pmol/g BW in female zebrafish was sufficient to increase *lhβ* mRNA expression in the pituitary (26). The injection solution also contained 0.05% Evans Blue (Sigma-Aldrich) to track the correctness of the injection placement. The sample number ranged between 10–13 per group. 1 hour and 6 hours (only for WT) after the injection, blood samples were collected with heparinized glass microcapillary needles following a described method (35). Subsequently, brains and pituitaries were removed and flash-frozen in dry ice. Brains were carefully separated to three regions (olfactory bulb, forebrain + midbrain, hindbrain) before freezing. All samples were stored at -80°C until use. The *gnrh3* mRNA expression level in the forebrain and midbrain regions were determined using qPCR as described above except that 500 ng of total RNA was used for 10 µL of the reverse transcription reaction. The primers used for *gnrh3* quantification are listed in Table 1. The blood samples (20–30 µL from each individual) were stored at 4°C overnight then centrifuged at 2,500 rpm for 15 minutes at 4°C, then plasma was collected and stored at -20°C until use. Plasma LH concentrations were measured using carp LH ELISA as described in previous studies (31,32) except for coating with 10 ng/mL carp recombinant LHβ, anti-carp LHβ diluted 1:100,000 and a standard curve range 1–2,000 pg/mL. Plasma samples for the ELISA were prepared by adding plasma assay buffer (10% fetal calf serum; 0.1% BSA: 0.05% Tween

20 in PBS) to each 10–15 µL plasma to bring the sample to a 60 µL volume. Similarly, ICV injections with no peptide or 4 pmol/g BW GnRH3 in 5 month old sexually mature WT females were performed, and plasma LH levels were measured at 1hour post-injection.

Quantification of GnRH peptide in pituitaries

GnRH3 and GnRH2 peptide content in the ICV-injected sexually mature WT female pituitaries were determined using specific ELISAs (36). For the sample preparation, each excised frozen pituitary was sonicated in 200 µL $H_2O$ for 20 seconds on ice using Sonifier 450 (Branson) with output control set at 2 and constant duty cycle. 15 µL of the 200 µL was used for the total protein quantification of each pituitary, using a CBQCA Protein Quantitation Kit (Invitrogen) following the manufacturer's protocol. Then, 185 µL of 4 N acetic acid was added to the tubes, which were vortexed and kept on ice for 15 minutes. The samples were lyophilized and resuspended with 65 µL of GnRH ELISA assay buffer (400 mM NaCl; 1 mM EDTA; 0.1% BSA; 0.001% sodium azide in 0.1 M phosphate buffer, adjusted to pH 7.4). Specific ELISA for GnRH3 was performed based on a previous study (36) with several modifications. 96-well plates were first coated with 5 µg/mL streptavidin overnight at 37°C, blocked with 2% BSA in PBS, and placed at 37°C until dry. After the plates were washed, 100 µL of 10 ng/mL biotinylated-GnRH3 (biotin-EHWSYGWLPG-$NH_2$; synthesized by Biomatik with 97% purity as determined by analytical HPLC) diluted in PBST was added to all wells except for non-specific-binding control wells and incubated for 3 days at 4°C. The standard curve consisted of GnRH3 peptide (GenScript, cat # RP10673) diluted to 10 to 20,000 pg/mL with a series of a two-fold dilution in 125 µL of the GnRH ELISA assay buffer. Equal volumes of GnRH3 antibody (antiserum 1668; kindly provided by the late Dr. Judy King) (37), previously used for GnRH3 ELISA and validated (36), was diluted 1:40,000 in the GnRH ELISA assay buffer and added to the standards and samples for a 3-day incubation at 4°C. The plates were then washed with PBST, and the samples/standards-antibody mixes were loaded to the wells in duplicate (for the standards) or singlicate (for the samples) and incubated at 37°C for 2 hours, washed

and incubated with goat anti-rabbit conjugated to HRP (Bio-Rad) (38). Color was developed using TMB 2-Component Microwell Peroxidase Substrate Kit (KPL) according to the manufacturer's protocol. Displacement curves for pituitary samples were generated by serial dilutions of the pooled samples of female zebrafish brains in the GnRH ELISA assay buffer and comparison with the corresponding standard curve (Supplementary Fig. 2) (30). The lower limit of the detection for GnRH3 was determined to be 4.6 pg/well. Intra-assay coefficient of variation (CV; 2.3%) was determined by assaying 10 replicates of the same sample within a same plate. Inter-assay CV (13.4%) was determined by assaying the same sample five times in different plates. The measured GnRH3 or GnRH2 protein content was normalized against total protein contents of each pituitary and displayed as GnRH3 or GnRH2 protein per 1 µg pituitary total protein. The protocol for GnRH2 ELISA was described elsewhere (36,39).

VipA immuno-neutralization

Anti-mouse Vip antibody (40) was used to neutralize the action of VipA. Undiluted commercially available affinity purified anti-mouse Vip antibody raised against the Vip precursor (40) was ultrafiltered using Amicon Ultra-15 Centrifugal Filter Unit 100K (Millipore) to remove sodium azide. As a control, we used an IgG-enriched preparation of pre-immune normal rabbit serum as follows: 1 mL serum was ultrafiltered using Amicon Ultra-15 Centrifugal Filter Unit 100K (Millipore) followed by IgG purification using Protein A HP SpinTrap (Cytiva). The resulting IgG were ultrafiltered again and recovered in 100 µL (×10 concentrated). Sexually mature females (N=7–11), 5 months of age, were ICV-injected (as described above) with the Vip antibody or the pre-immune IgG-enriched serum twice with 4-hour intervals (1000 h –1100 h and 1400 h–1500 h). At 2 hours and 18 hours post-injection, blood samples were collected, and plasma LH levels were determined using the above LH ELISA.

Generation of *vipa* knockout zebrafish

*vipa* single mutant (*vipa*$^{-/-}$) was generated by CRISPR/Cas9 technology. The CRISPR/Cas9 target site for the exon2 in *vipa* gene (Ensembl Stable ID: ENSDARG00000078247) was designed using an online tool, CCTop - CRISPR/Cas9 target online predictor (41). A single-guide RNA (sgRNA) was synthesized and purified following a previously described method (42). Cas9 nuclease (IDT, cat # 1081062) was diluted in Cas9 nuclease reaction buffer (20 mM HEPES; 100 mM NaCl; 5 mM $MgCl_2$; 0.1 mM EDTA; adjusted to pH 6.5). Cas9 nuclease (250 ng/µL) and sgRNA (25 ng/µL) were co-microinjected into one-cell-stage embryos. F0 founders, possessing mosaic mutations identified by PCR (forward primer: 5'-GCTGGGTTCCTTCGCATTTAT-3'; reverse primer: 5'-GGCGATTCATTCAGCTTCGC-3') on genomic DNA extracted from clipped fin samples, followed by restriction enzyme BceAI (New England Biolabs) digestion, were mated with WT fish to produce heterozygous F1 offspring. The PCR products were cloned in pGEM-T Easy Vector (Promega), and sequencing analysis was performed. The acquired sequence was compared with the WT genome and an 8-bp deletion was identified on the designed CRISPR/Cas9 target site, which resulted in elimination of VipA peptide. The F1 generation was crossed with WT to obtain a 50% heterozygous *vipa*$^{+/-}$ F2 generation. The F2 heterozygous fish were in-crossed to produce homozygous *vipa*$^{-/-}$ offspring. The verification of loss of VipA peptide on the homozygous mutants was performed by IHC using zebrafish VipA antibody as described above, except the secondary antibody used was Cy3-conjugated goat anti-rabbit IgG (1:1000; KPL) (43). To establish the *vipa* and *gnrh3* double knockout fish, *vipa*$^{-/-}$ fish were crossed with *gnrh3*$^{-/-}$ fish previously generated (4), and *vipa*$^{+/-}$; *gnrh3*$^{+/-}$ fish were obtained. *vipa*$^{+/-}$; *gnrh3*$^{+/-}$ fish were in-crossed to produce a double homozygous mutant (*vipa*$^{-/-}$; *gnrh3*$^{-/-}$). The mutation in *gnrh3* were verified by a previously established method (4). The reproductive fitness of these lines was then assessed as follows: Sexually mature 4 month old females of *vipa*$^{-/-}$ (N=5) and WT siblings (*vipa*$^{+/+}$; N=6), *vipa*$^{-/-}$; *gnrh3*$^{-/-}$ (N=5) and WT siblings (*vipa*$^{+/+}$; *gnrh3*$^{+/+}$; N=4) fish were mated with WT males. Plastic dividers, which separated a tested female and a male, were removed immediately after the lights on at 0900 h, and fish were allowed to mate for 2 hours. All spawned eggs were collected from each breeding container, and the number of fertilized and

unfertilized egg were counted to examine the fecundity and fertilization rate (number of fertilized eggs/total eggs released).

Statistical analysis

Data in bar charts represent means ± SEM. In box plots, middle lines represent median, and bottom and top lines show lower and upper quartiles, respectively. Rhombuses and circles indicate mean and individual data points, respectively. All statistical analyses were conducted using R 4.0.3 software (44). For comparison between two groups, the two-sample Student's t-test was performed. If the Shapiro-Wilk test indicated that the normality cannot be assumed for the data, the two-sample Wilcoxon rank sum test was used. For comparison among more than two groups, one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test or two-way ANOVA followed by Tukey's Honest Significant Difference test was performed. If the Shapiro-Wilk test indicated that the normality cannot be assumed for the data, the Kruskal-Wallis test followed by Dunn's *post hoc* test was performed. $P<0.05$ was considered to be statistically significant. All data with outliers were included for the statistical analyses.

**Results**

*vipa*-expressing neurons are found in the preoptic area and hypothalamus

The distribution of *vipa*-expressing neurons in the brain was first determined by ISH. *vipa*-expressing soma were localized to the parvocellular preoptic nucleus anterior part (PPa), postcommissural nucleus of ventral telencephalic area (Vp), and ventromedial thalamic nucleus (VM) of the hypothalamus in the WT zebrafish brain (Fig. 1B). Hybridization with *vipa* sense riboprobe always resulted in a lack of signal (Supplementary Fig. 3A) (30).

Number of vipa-expressing neurons are not changed in gnrh3$^{-/-}$ female brain

The number of *vipa*-expressing neurons in WT and *gnrh3*$^{-/-}$ females was determined using ISH on the brain sections. There were no significant differences in the number of *vipa*-expressing neurons in both PPa and Vp between WT and *gnrh3*$^{-/-}$ females ($P$=0.1149 for the PPa; $P$=0.5018 for the Vp), with WT females having 94±9 (mean±SEM) in the PPa and 44±8 in the Vp, and *gnrh3*$^{-/-}$ females having 139±23 in the PPa and 37±7 in the Vp (Fig. 1C).

Validation of anti-zebrafish VipA specificity

The specificity of zebrafish VipA polyclonal antibody was validated by running a negative control alongside the VipA immune serum using the pre-immune serum on sequential sections. This test always resulted in a lack of positive signal when the pre-immune serum was applied while the immune serum produced consistent staining (Supplementary Fig. 3B) (30). The combined *vipa* ISH and IHC for VipA in adult female brains resulted in stained somas in the PPa with the anti-sense *vipa* riboprobe, as well as with the zebrafish VipA antibody (Supplementary Fig. 3C) (30), substantiating the quality and specificity of the antibody.

VipA-ir axons are found in close vicinity of gonadotropes in the PPD of the pituitary

VipA immunostained sagittal brain sections revealed that VipA-ir fibers were distributed throughout the ventral hypothalamus (Fig. 1E). To determine whether VipA is a potential hypophysiotropic neuropeptide, we analyzed VipA neuronal projections in female pituitary. VipA immunostained pituitary sections attached to the brain of sexually mature *Tg (gnrh3: eGFP; lhβ: mCherry)* females revealed that VipA-ir axons possibly innervate the PPD in the pituitary, and some of them were found in close vicinity of LH cells. Additionally, some LH cells interacted with both VipA-ir and GnRH3 axons simultaneously (Fig. 1F).

VipA does not directly induce LH secretion from the pituitary, in vitro

As VipA axons were found in close vicinity of LH cells in the pituitary, we next examined whether VipA directly induces LH secretion from the pituitary. Whole female pituitaries were incubated with 100 nM VipA peptide followed by LH measurement in the culture medium. Under this condition, the LH levels of VipA-treated pituitaries were similar to those of control ($P$=0.91) (Fig. 2A). Addition of 100 nM $E_2$, however, significantly induced LH secretion when incubated with $E_2$ alone ($P$=0.04) or in combination with GnRH3 ($P$=0.04) to 1.46 or 1.39 times higher than the mean control levels, respectively, as well as over the VipA alone incubation ($P$=0.04).

VipA intraperitoneal administration does not upregulate pituitary *lhβ* mRNA level, in vivo

To understand whether VipA plays a role in *lhβ* mRNA expression as a hypophysiotropic factor, we next performed VipA IP injection in sexually mature WT females. The relative mRNA levels of LH β subunit gene (*lhβ*) in the pituitary, 18 hours post-injection, were analyzed by real-time qPCR. When the *lhβ* mRNA levels in each treatment group was compared with that of the control saline group, neither VipA or a combination of VipA and DOM, a dopamine D2 receptor antagonist, upregulated *lhβ* mRNA levels ($P$=0.94 or $P$=0.76, respectively), nor did treatments with GnRH3 alone ($P$=0.3) or DOM alone ($P$=0.37) (Fig. 2B). By contrast, the combination of GnRH3 and DOM, significantly increased the *lhβ* relative mRNA levels by 2.9 times of the control group ($P$=0.012).

VipA ICV administration stimulates LH secretion to the plasma in 1 hour, in vivo

The ability of VipA to elicit LH release in sexually mature WT female fish was revealed by ICV injection. 4 pmol/g BW VipA induced and increased plasma LH levels at 1 hour post-injection by 1.55 times the control group levels ($P$=0.01) (Fig. 3A). When the same experiment was conducted on *gnrh3*$^{-/-}$ females, plasma LH levels were also significantly increased by 2.44 times the control group levels ($P$=0.002) at 1 hour post-injection (Fig. 3A). On the contrary, there was no significant increase of plasma LH levels at 6 hours post-injection of VipA in WT females ($P$=0.45)

(Supplementary Fig. 4A) (30). Moreover, ICV injection with 4 pmol/g BW GnRH3 did not induce a significant increase of plasma LH levels compared to the control group at 1 hour post-injection (*P*=0.2277) (Fig. 3B).

VipA ICV administration does not affect GnRH levels

To study whether VipA can induce GnRH3 release into the pituitary of sexually mature WT females, we conducted ICV injection. 4 pmol/g BW VipA did not upregulate the *gnrh3* relative expression levels in the forebrain and midbrain regions at 1 hour post-injection (*P*=0.6556) (Fig. 3C). On the contrary, there was a decrease at 6 hours post-injection (*P*= 0.013) (Supplementary Fig. 4B) (30). In the pituitary, 4 pmol/g BW VipA induced no change in either GnRH3 protein content at 1 hour post-injection (*P*=0.115) (Fig. 3D) and at 6 hours post-injection (*P*=0.065) (Supplementary Fig. 4C) (30) or GnRH2 protein contents at 1 hour post-injection (*P*=0.1431) (Fig. 3E).

VipA immuno-neutralization induces an increase of plasma LH levels

To examine whether VipA signals play a role in the regulation of LH secretion, we attenuated VipA functions by ICV injection with mouse Vip antibody. The affinity purified mouse Vip antibody significantly increased plasma LH levels at 2 hours (3.7 times) and 18 hours (2.3 times) post-injection, compared to those of the control group (*P*=0.0000094 and *P*=0.0051, respectively) (Fig. 4).

*vipa*$^{-/-}$ and *vipa*$^{-/-}$; *gnrh3*$^{-/-}$ females display normal reproductive performance

To investigate whether VipA is essential for LH secretion and successful reproduction in females, we generated *vipa* homozygous knockout zebrafish (*vipa*$^{-/-}$) with 8 bp deletion in exon2 of the *vipa* gene (Fig. 5A). The induced frame shift mutations caused complete lack of VipA peptide. The lack of VipA protein was verified via IHC using the anti-zebrafish VipA. VipA-ir fibers were

detected only in the PPa and pituitary of *vipa*$^{+/+}$ (WT siblings) but not in those of *vipa*$^{-/-}$ (Fig. 5B). We then evaluated reproductive characteristics in *vipa*$^{-/-}$ females by allowing them to mate with WT males. 100% of the females successfully spawned in both groups (*vipa*$^{-/-}$: 5/5; *vipa*$^{+/+}$: 6/6), and the results showed no significant differences in the number of fertilized eggs and fertilization rate between the *vipa*$^{-/-}$ and *vipa*$^{+/+}$ pairs ($P$=0.9271 for the number of fertilized eggs; $P$=0.331 for the fertilization rate) (Fig. 5C). To determine whether VipA elicits LH release via GnRH3, which in turn induces spawning, we generated a *vipa*$^{-/-}$; *gnrh3*$^{-/-}$ double knockout and assessed the same reproductive parameters. However, similar to the *gnrh3*$^{-/-}$ and *vipa*$^{-/-}$ females, *vipa*$^{-/-}$; *gnrh3*$^{-/-}$ females were fertile with normal reproductive performance ($P$=0.5476 for the number of fertilized eggs; $P$=0.4206 for the fertilization rate) (Fig. 5D) with a 100% of the spawning success rate (*vipa*$^{-/-}$; *gnrh3*$^{-/-}$: 5/5; *vipa*$^{+/+}$; *gnrh3*$^{+/+}$: 4/4).

**Discussion**

The present study localized VipA neurons in the POA and hypothalamus of zebrafish, with axon terminals abutting pituitary endocrine cells including some gonadotropes. Although whole pituitary in vitro incubation with VipA does not affect LH secretion, and VipA IP injection in vivo does not upregulate *lhβ* mRNA expression at 18 hours post-injection, the time in which a combination of GnRH3 and DOM induces an upregulation of *lhβ* mRNA expression, this study found that VipA ICV injection elicits an immediate increase in plasma LH in sexually mature female zebrafish. The different outcome between those VipA treatments suggests that VipA indirectly induces LH release from the pituitary through a brain factor, not a pituitary factor(s), or VipA can directly exert its effect on LH cells with the help of unknown permissive factors that may have been eliminated in the in vitro study. In other words, the results suggest that VipA does not act alone to elicit LH secretion. Since VipA also induces LH release in *gnrh3*$^{-/-}$ zebrafish, it implies that VipA acts through or with a non-GnRH3 brain factor(s) to induce LH secretion in zebrafish. Thus, unlike the case in mammalian species (11,45–47), the induction of LH secretion by VipA likely occurs independently of the

hypophysiotropic GnRH. More importantly, the action of VipA is indicative of the presence of a redundant pathway to induce LH secretion in zebrafish (Fig. 6).

GnRH has been considered essential for successful reproduction in all studied vertebrates. The recent finding that the loss of the hypophysiotropic GnRH alone or together with the other GnRH isoform in zebrafish, GnRH2, result in normal reproduction capacity in zebrafish (4–6) has revived a debate as to how the brain regulates LH secretion. In contrast, natural mutation in the *GnRH* gene in mammalian species results in infertile individuals due to low gonadotropins levels (1,2). Similarly, when the hypophysiotropic GnRH is knocked-out in medaka (*gnrh1*$^{-/-}$), a fish with three GnRH isoforms, the females are unable to undergo final oocyte maturation and ovulation presumably due to insufficient LH secretion (3). One of the scenarios to be considered is that GnRH may not be the sole or a central controller of reproduction, particularly as an LH secretagogue in zebrafish. We have shown before that even the double KO line (*gnrh2*$^{-/-}$; *gnrh3*$^{-/-}$) is fertile (6), ruling out GnRH2 as a potential compensator for GnRH3 in *gnrh3*$^{-/-}$ zebrafish. It is well known that the pituitary gonadotropes are tightly controlled by multiple molecular regulators, which cooperate towards a single aim, possibly through redundant pathways (7,8). Several brain neurohormones and neurotransmitters, such as kisspeptin, GnRH, gonadotropin inhibitory hormone, dopamine, gamma aminobutyric acid, pituitary adenylate cyclase-activating polypeptide (PACAP), neuropeptide Y, secretoneurin, among others, are known to affect LH cells and release in teleosts (8,48). In pubertal female zebrafish, estrogen can directly stimulate the LHβ protein synthesis at the pituitary level (27). VipA may activate each of the above factors to elevate LH secretion. Regardless of the exact identity of the mediating/cooperating factor, to best of our knowledge, our study demonstrates for the first time the presence of functional redundant pathway(s) that regulate LH secretion, possibly apart for secretoneurin (48–50), for ensuring reproductive success at the level of the brain-pituitary of zebrafish female.

Vip is found in both the central and peripheral nervous systems of mammals (10), and is implicated in the control of the anterior pituitary (51). Pertinent to reproduction, SCN Vip axons interact with POA GnRH neuronal soma and play a key role in the control of the LH surge in female

rodents (11,45–47). However, to date, the effect of Vip on GnRH in teleosts is unclear, and even less has been reported on its functions in fish reproduction. Our goal was, therefore, to determine whether VipA in zebrafish is a redundant inducer of reproduction and explore its action on GnRH and LH in sexually mature female zebrafish. As a first step, we characterized *vipa*-expressing and VipA-ir neuronal distributions in the brain and pituitary of sexually mature zebrafish females. ISH localized *vipa*-expressing soma in the brain nuclei, expressing other important neuropeptides and steroid receptors that regulate reproduction (52–55), the Vp, PPa and VM, while VipA-ir fibers were detected throughout the ventral hypothalamus and innervated the pituitary PPD where gonadotropes, somatotoropes and thyrotropes are located (56). These findings indicated that VipA could potentially regulate LH at both the brain and the pituitary levels in zebrafish. Similar localization patterns have been previously reported using anti-porcine Vip polyclonal antibody during ontogeny of zebrafish (57) and *vipa* ISH in zebrafish larvae (58). In adult females, our study demonstrates that some LH cells may be simultaneously innervated by both VipA-ir and GnRH3 axons, suggesting that VipA directly regulates LH cells. Intriguingly, a very recent study in medaka has described a sexual dimorphic distribution of Vip neurons, in which females contain a higher number of POA Vip neurons, thus supporting the idea of Vip's sex-related role in females (59). In the rat, Vip is found within the external zone of the median eminence and is abundant in the hypophyseal portal blood (15–17). Hence, our neuroanatomical analysis of VipA implies that VipA neuronal distribution remained conserved during vertebrate evolution.

VipA can exert its effect on LH secretion through four possible pathways: Independent direct action, permissive signals-dependent direct effect, indirectly through a brain factor or through a pituitary paracrine factor. Regarding the induction of a pituitary factor, it has been reported that PACAP, which is an endogenous peptide that is structurally similar to Vip, can activate common receptors (e.g., PAC-Rs and VPAC-Rs) in vertebrates (60), and stimulates LH secretion in vitro and in vivo in goldfish (61,62). However, the effect of Vip on gonadotropes is largely unexplored in zebrafish and other teleosts. To determine whether VipA directly stimulates LH cells to induce LH release in zebrafish, we measured LH secretion from whole female pituitaries in response to VipA.

Contrary to the effect of PACAP in goldfish, VipA does not elicit LH secretion in the presence or absence of $E_2$, while GnRH3 with $E_2$ and $E_2$ alone induced a significant increase of LH secretion. The results with $E_2$ corroborate a previous finding that $E_2$ directly augments LHβ protein synthesis at the pituitary level in pubertal female zebrafish (27). Similarly, in rats, although Vip is abundant in hypophyseal portal blood (15–17), it does not stimulate LH, FSH or growth hormone secretion but stimulates prolactin (PRL) secretion, in vitro (63). In contrast, in grass carp pituitary cells, human and cod Vip (0.1–100 nM) are ineffective in altering basal levels of PRL release, PRL cell content, and *prl* mRNA expression (64). In zebrafish, VipA may act directly on other endocrine cells, such as somatotropes, to regulate the release and/or synthesis of pituitary peptide hormones. In medaka, Vip displays sexually dimorphism in the POA neurons projecting to the pituitary where females display greater expression. Additionally, female medaka in vitro pituitary incubation studies illustrate that Vip upregulates *prl* and somatolactin (*sl*) mRNA levels and downregulates follicle-stimulating hormone β subunit (*fshβ*) mRNA levels (59). Nevertheless, despite the apparent conservation in Vip neuronal distribution between rats and zebrafish, VipA seems to have species-specific roles as a hypophysiotropic factor. Thus, the hypophysiotropic roles of Vip need to be explored in a variety of species. In addition, mapping the distribution of Vip receptors, such as VPAC-Rs, in the pituitary will help to detect its target cells and advance our understanding of the roles of VipA as a hypophysiotropic neuropeptide at the pituitary level in zebrafish and in other species. With regard to VipA action on LH secretion, the results from the pituitary in vitro experiments indicate that VipA does not act independently on LH cells and requires for its action either a mediating or a permissive factor, which is likely not a pituitary paracrine factor. Interestingly, the immuno-neutralization also induced an increase of plasma LH. This unexpected result suggests that VipA has a dual function with regard to LH secretion and can both induce and inhibit LH secretion through different pathways. Therefore, elimination of VipA signal may unleash an unknown pathway resulting in LH secretion, while the addition of VipA activates a factor that also induces LH secretion. Likewise, in female rat, the inhibition of LH secretion by Vip infusion is steroid dependent, e.g., when plasma LH levels are high (65); while in middle-aged female rats, Vip infusion treatment rescues the attenuated LH surge (66). Similar to our results, the effect of Vip is independent of dopamine, and treatment with Vip

antagonist, [4Cl-*D*-Phe$^{6}$, Leu$^{17}$]VIP, alone does not affect circulating LH levels (65). Altogether, the authors conclude that in the female rat, Vip has a modulatory role but not an obligatory role. Congruently, our results demonstrate that the effect of VipA on LH secretion is modulatory also probably in female zebrafish. Hence, because zebrafish is a daily spawner with a distinct circadian reproductive gene expression pattern (67), the effect of VipA may change depending on dynamic daily circuitries and/or depending on the time of the day. The exact effect of VipA on LH secretion under varying circumstances needs to be elucidated, nevertheless the results demonstrate that VipA signaling is physiologically relevant to the regulation of LH secretion in sexually mature zebrafish females.

The mechanism by which VipA induces LH secretion is currently unknown, but some clues can be drawn from the in vitro pituitary incubation and IP injection experiments. In vitro pituitary incubation indicates that the native form of GnRH3 alone is unable to induce LH secretion in zebrafish. Together with the finding by Biran et al. 2012 that long-acting mammalian GnRH analog only slightly, though not significantly, induced LH plasma levels at 6 hours post-IP injection (31), the effect and importance of GnRH3 on LH secretion in sexually mature female zebrafish still need clarification. Moreover, in the in vivo IP injection experiment, whereas the blockage of dopamine action by DOM is needed alongside GnRH3 to upregulate *lhβ* mRNA expression, it has no effect when added to VipA. Collectively, our results suggest that VipA acts as a modulator and activates, through an unknown brain mediator, a distinct pathway in zebrafish to induce LH secretion, possibly bypassing the traditional GnRH/dopamine/$E_2$ signal transduction pathways. To confirm that the hypophysiotropic GnRH is not the mediating factor, as is the case in rodents (11,45–47), we performed ICV injection of VipA in sexually mature *gnrh3*$^{-/-}$ female zebrafish that lacked GnRH3 functions. Indeed, plasma LH levels were elevated following VipA ICV injection in both WT and *gnrh3*$^{-/-}$ fish. Congruently, ICV injection of VipA did not upregulate brain *gnrh3* mRNA level and pituitary GnRH3 contents at 1 hour post-injection in the WT females. On the question of whether VipA acts via GnRH2 in WT and *gnrh3*$^{-/-}$ zebrafish, ICV injection of VipA did not induce an increase

of GnRH2 pituitary content in WT females, indicating that the factor(s) that enables LH secretion in zebrafish females is probably not GnRH.

The observation that the elevation in blood LH is detected as soon as 1 hour post-VipA ICV injection, combined with the presence of VipA-ir axon terminals next to LH cells suggests that VipA acts on axon terminals within the pituitary to induce the release of the unknown mediator, and not through upregulation of its mRNA expression. No significant increase in GnRH3 pituitary content is seen following VipA ICV treatment, and the observation that *gnrh3* transcript levels in the brain remained unchanged supports this idea, as do the results that there is no upregulation in *gnrh3* relative mRNA levels, GnRH3 pituitary content and plasma LH levels at 6 hours post-VipA ICV injection (Supplementary Fig. 4B) (30). A possible neuronal target of VipA is tyrosine hydroxylase (TH), a rate-limiting enzyme in dopamine biosynthesis. Dopamine is a known inhibitory factor along the HPG axis in teleosts, inhibiting both basal and GnRH-stimulated LH secretion, subsequently affecting final maturation and ovulation in females (68). Treatments with dopamine antagonists, pimozide or metoclopramide (specific dopamine D2 receptor antagonists) cause increased serum LH levels in goldfish (69), tilapia (70), and carp (71). In zebrafish, TH-ir fibers innervate LH cells, and IP administration of GnRH analog with DOM increases *lhβ* mRNA levels and gonadosomatic index and stimulates vitellogenesis in sexually regressed females (33). However, at least in our in vivo IP experiment, the effect of VipA did not mimic DOM injection, suggesting that VipA does not modulate the dopaminergic pathway under these conditions.

The involvement of another factor in inducing LH secretion does not rule out the involvement of GnRH3. This means that zebrafish GnRH3 may still act as one of the players in inducing final maturation and ovulation, however it does not play a prominent role as GnRH1 does in other species. An indication that GnRH3 may have a role in female reproduction is provided by the arrested oocyte development and sterility in female zebrafish, when GnRH3 neurons were ablated at early stages at 4- or 6-day post-fertilization (72). Additionally, treatments with GnRH3 peptide, in vitro (73), and in vivo (results from GnRH3 IP injection with DOM in this study), consistently upregulated pituitary *lhβ* mRNA level over the control, whereas pituitary incubation with GnRH3 alone did not induce LH

secretion. Indeed, the number of samples in the GnRH3 ICV injection study is relatively low (N=5–7), which warrants careful interpretation of the results. Whether GnRH3 directly elicits the LH release still needs to be determined.

The finding that *vipa*$^{-/-}$ zebrafish females display normal reproduction suggests that VipA is not essential for the regulation of zebrafish female reproduction, specifically in the control of LH-dependent events like maturation, ovulation, and spawning. Vip *null* mice and Vip receptor 2 (*Vipr2*) *null* mice exhibit disrupted estrous cycles and/or deficits in ovulation, caused by the disruption of estrous cycle and circadian rhythms (14,74). This result infers that Vip is a critical SCN factor mediating circadian rhythms for precise GnRH surge, subsequently affecting the LH surge in the pituitary of female rodents. Although VipA neurons in the PPa of the POA may mediate circadian signals to other neurons in zebrafish, our results that *vipa*$^{-/-}$ females successfully spawned at regular timing suggest that VipA signal is possibly not a major regulator of the timing of LH surge in zebrafish females. This suggestion can be also supported by the fact that the pineal gland and other tissues, not the SCN or an anatomical brain region potentially analogous to the mammalian SCN, are more likely to serve as the timekeepers of the endocrine clock systems in teleosts (75). In fact, zebrafish appears to have a decentralized clock system, where all tissues and the majority of cells possess a direct light-entrainable circadian pacemaker (76–78), which may render the need for a circadian messenger (e.g., as found in mammals) obsolete. Additionally, we found that *vipa* and *gnrh3* double knockout (*vipa*$^{-/-}$; *gnrh3*$^{-/-}$) zebrafish females also exhibit normal reproduction. These results suggest that VipA may not be a primary compensator for reproductive success in *gnrh3*$^{-/-}$ zebrafish. In support of this idea, the number of *vipa*-expressing cells in the POA does not change between WT and *gnrh3*$^{-/-}$ sexually mature females. Altogether, these results suggest that the role of regulating LH secretion in female zebrafish is fulfilled by additional factors that may cooperate with GnRH3 but can also act independently.

In summary, our findings demonstrate that, while VipA is not a main regulator for LH release in zebrafish, VipA can stimulate other hypophysiotropic neuropeptides/factors, or work in tandem with a permissive factor(s) to induce LH secretion in sexually mature female zebrafish, independent of the hypophysiotropic GnRH. Although, given the lack of effects of knockout of several neuropeptides on reproductive parameters in the zebrafish, it remains to be determined whether this phenomenon is specific to this species or common to other vertebrates, we were able to provide evidence for the presence of functional redundant pathway(s) for LH secretion in zebrafish, which does not rely on GnRH functions.

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**Data Availability**

All data generated or analyzed during this study are included in this published article or in the data repositories listed in References (30) (https://doi.org/10.6084/m9.figshare.15167175; Supplementary Fig. 1–4).

**References**


1. Cattanach B, Iddon CA, Charlton H, Chiappa SA, Fink G. Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature*. 1977;269(5626):338–340.

2. Chan Y-M, Guillebon A de, Lang-Muritano M, Plummer L, Cerrato F, Tsiaras S, Gaspert A, Lavoie HB, Wu C-H, Crowley WF, Amory JK, Pitteloud N, Seminara SB. GNRH1 mutations in patients with idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci USA*. 2009;106(28):11703–11708.

3. Takahashi A, Kanda S, Abe T, Oka Y. Evolution of the hypothalamic-pituitary-gonadal axis regulation in vertebrates revealed by knockout medaka. *Endocrinology*. 2016;157(10):3994–4002.

4. Spicer O, Wong T-T, Zmora N, Zohar Y. Targeted mutagenesis of the hypophysiotropic Gnrh3 in zebrafish (*Danio rerio*) reveals no effects on reproductive performance. *PLoS One.* 2016;11(6):e0158141.

5. Liu Y, Tang H, Xie R, Li S, Liu X, Lin H, Zhang Y, Cheng CHK. Genetic evidence for multifactorial control of the reproductive axis in zebrafish. *Endocrinolgy*. 2017;158(3):604–611.

6. Marvel M, Spicer O, Wong T-T, Zmora N, Zohar Y. Knockout of the Gnrh genes in zebrafish: effects on reproduction and potential compensation by reproductive and feeding-related neuropeptides. *Biol Reprod*. 2018;99(3):565–577.

7. Pereira SS, Lobato CB, Monteiro MP. Cell signaling within endocrine glands: Thyroid, parathyroids and adrenal glands. In: Silva J, Freitas M, Fardilha M, eds. Tissue-specific cell signaling. Springer, Cham; 2020:33–61.

8. Levavi-Sivan B, Bogerd J, Mañanós EL, Gómez A, Lareyre JJ. Perspectives on fish gonadotropins and their receptors. *Gen Comp Endocrinol*. 2010;165(3):412–437.

9. Said SI, Mutt V. Isolation from porcine intestinal wall of a vasoactive octacosapeptide related to secretin and to glucagon. *Eur j biochem*. 1972;28(2):199–204.

10. Sims KB, Hoffman DL, Said SI, Zimmerman EA. Vasoactive intestinal polypeptide (VIP) in mouse and rat brain: An immunocytochemical study. *Brain Res*. 1980;186(1):165–183.

11. Ward DR, Dear FM, Ward IA, Anderson SI, Spergel DJ, Smith PA, Ebling FJP. Innervation of gonadotropin-releasing hormone neurons by peptidergic neurons conveying circadian or energy balance information in the mouse. *PloS One*. 2009;4(4):e5322.

12. Christian CA, Mobley JL, Moenter SM. Diurnal and estradiol-dependent changes in gonadotropin-releasing hormone neuron firing activity. *Proc Natl Acad Sci USA*. 2005;102(43):15682–15687.

13. Christian CA, Moenter SM. Vasoactive intestinal polypeptide can excite gonadotropin-releasing hormone neurons in a manner dependent on estradiol and gated by time of day. *Endocrinology*. 2008;149(6):3130–3136.

14. Loh DH, Kuljis DA, Azuma L, Wu Y, Truong D, Wang HB, Colwell CS. Disrupted reproduction, estrous cycle, and circadian rhythms in female mice deficient in vasoactive intestinal peptide. *J Biol Rhythm*. 2014;29(5):355–69.

15. Mezey E, Kiss JZ. Vasoactive intestinal peptide-containing neurons in the paraventricular nucleus may participate in regulating prolactin secretion. *Proc Natl Acad Sci USA*. 1985;82(1):245–247.

16. Ceccatelli S, Fahrenkrug J, Villar MJ, Hökfelt T. Vasoactive intestinal polypeptide/peptide histidine isoleucine immunoreactive neuron systems in the basal hypothalamus of the rat with special reference to the portal vasculature: An immunohistochemical and in situ hybridization study. *Neuroscience*. 1991;43(2–3):483–502.

17. Shimatsu A, Kato Y, Inoue T, Christofides ND, Bloom SR, Imura H. Peptide histidine isoleucine- and vasoactive intestinal polypeptide-like immunoreactivity coexist in rat hypophysial portal blood. *Neurosci Lett*. 1983;43(2–3):259–262.

18. RRID:AB_514497. https://scicrunch.org/resolver/RRID:AB_514497.

19. Wullimann MF, Rupp B, Reichert H. Neuroanatomy of the zebrafish brain, a topological atlas. Switzerland: Birkhäuser Basel; 1996.

20. Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K. Current protocols in molecular biology. Hoboken, NJ: John Wiley & Sons, Inc.; 2003.

21. RRID:AB_2535814. https://scicrunch.org/resolver/RRID:AB_2535814.

22. RRID:AB_514500. https://scicrunch.org/resolver/RRID:AB_514500.

23. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9(7):676–682.

24. Golan M, Biran J, Levavi-Sivan B. A novel model for development, organization, and function of gonadotropes in fish pituitary. *Front Endocrinol*. 2014;5:182.

25. Abraham E, Palevitch O, Ijiri S, Du S, Gothilf Y, Zohar Y. Early development of forebrain gonadotrophin-releasing hormone (Gnrh) neurones and the role of Gnrh as an autocrine migration factor. *J Neuroendocrinol*. 2008;20(3):394–405.

26. Marvel M, Spicer O, Wong T-T, Zmora N, Zohar Y. Knockout of Gnrh2 in zebrafish (*Danio rerio*) reveals its roles in regulating feeding behavior and oocyte quality. *Gen Comp Endocrinol*. 2019;280:15–23.

27. Li G, Tang H, Chen Y, Yin Y, Ogawa S, Liu M, Guo Y, Qi X, Liu Y, Parhar IS, Liu X, Lin H. Estrogen directly stimulates LHb expression at the pituitary level during puberty in female zebrafish. *Mol Cell Endocrinol*. 2018;461:1–11.

28. Karigo T, Kanda S, Takahashi A, Abe H, Okubo K, Oka Y. Time-of-day-dependent changes in Gnrh1 neuronal activities and gonadotropin mRNA expression in a daily spawning fish, medaka. *Endocrinology*. 2012;153(7):3394–3404.

29. Habibi HR. Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary: effects of native GnRH peptides and a synthetic GnRH antagonist. *Biol Reprod*. 1991;44(2):275–283.

30. Tanaka S, Zmora N, Levavi-Sivan B, Zohar Y. Supplemental Materials for: Vasoactive intestinal peptide indirectly elicits pituitary LH secretion independent of GnRH in female zebrafish. figshare. Deposited 14 August 2021. https://doi.org/10.6084/m9.figshare.15167175.

31. Biran J, Palevitch O, Ben-Dor S, Levavi-Sivan B. Neurokinin Bs and neurokinin B receptors in zebrafish-potential role in controlling fish reproduction. *Proc Natl Acad Sci USA*. 2012;109(26):10269–10274.

32. Hollander-Cohen L, Golan M, Aizen J, Shpilman M, Levavi-Sivan B. Characterization of carp gonadotropins: Structure, annual profile, and carp and zebrafish pituitary topographic organization. *Gen Comp Endocrinol*. 2018;264:28–38.

33. Fontaine R, Affaticati P, Yamamoto K, Jolly C, Bureau C, Baloche S, Gonnet F, Vernier P, Dufour S, Pasqualini C. Dopamine Inhibits Reproduction in female zebrafish (*Danio rerio*) via three pituitary D2 receptor subtypes. *Endocrinology*. 2013;154(2):807–818.

34. Matsuda K, Maruyama K, Nakamachi T, Miura T, Uchiyama M, Shioda S. Inhibitory effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) on food intake in the goldfish, *Carassius auratus*. *Peptides*. 2005;26(9):1611–1616.

35. Zang L, Shimada Y, Nishimura Y, Tanaka T, Nishimura N. Repeated blood collection for blood tests in adult zebrafish. *J Vis Exp*. 2015;(102):e53272.

36. Holland MCH, Gothilf Y, Meiri I, King JA, Okuzawa K, Elizur A, Zohar Y. Levels of the native forms of gnrh in the pituitary of the gilthead seabream, *Sparus aurata*, at several characteristic stages of the gonadal cycle. *Gen Comp Endocrinol*. 1998;112(3):394–405.

37. King JA, Millar RP. Multiple molecular forms of gonadotropin-releasing hormone in teleost fish brain. *Peptides*. 1985;6(4):689–694.

38. RRID:AB_11125143. https://scicrunch.org/resolver/RRID:AB_11125143.

39. Marvel M, Levavi-Sivan B, Wong T-T, Zmora N, Zohar Y. Gnrh2 maintains reproduction in fasting zebrafish through dynamic neuronal projection changes and regulation of gonadotropin synthesis, oogenesis, and reproductive behaviors. *Sci Rep*. 2021;11(1):6657.

40. RRID:AB_2878233. https://scicrunch.org/resolver/RRID:AB_2878233.

41. Stemmer M, Thumberger T, Keyer M del S, Wittbrodt J, Mateo JL. CCTop: An intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. *PloS One*. 2015;10(4):e0124633.

42. Cai M, Si Y, Zhang J, Tian Z, Du S. Zebrafish embryonic slow muscle is a rapid system for genetic analysis of sarcomere organization by CRISPR/Cas9, but not NgAgo. *Mar Biotechnol*. 2018;20(2):168–181.

43. RRID:AB_2892090. https://scicrunch.org/resolver/RRID:AB_2892090.

44. R core team. *R: A Language and Environment for Statistical Computing*. Version 4.0.3. Vienna, Austria: R foundation for Statistical Computing; 2021. https://www.R-project.org/.

45. Van Der Beek EM, Wiegant VM, Donk HA, Van Den Hurk R, Buijs RM. Lesions of the suprachiasmatic nucleus indicate the presence of a direct vasoactive intestinal polypeptide-containing projection to gonadotrophin-releasing hormone neurons in the female rat. *J Neuroendocrinol*. 1993;5(2):137–144.

46. Kriegsfeld LJ, Silver R, Gore AC, Crews D. Vasoactive intestinal polypeptide contacts on gonadotropin-releasing hormone neurones increase following puberty in female rats. *J Neuroendocrinol*. 2002;14(9):685–690.

47. Van Der Beek EM, Horvath TL, Wiegant VM, Van Den Hurk R, Buijs RM. Evidence for a direct neuronal pathway from the suprachiasmatic nucleus to the gonadotropin-releasing hormone system: Combined tracing and light and electron microscopic immunocytochemical studies. *J Comp Neurol*. 1997;384(4):569–579.

48. Mitchell K, Mikwar M, Fonte DD, Lu C, Tao B, Peng D, Erandani WKCU, Hu W, Trudeau VL. Secretoneurin is a secretogranin-2 derived hormonal peptide in vertebrate neuroendocrine systems. *Gen Comp Endocrinol*. 2020:113588.

49. Mitchell K, Zhang WS, Lu C, Tao B, Chen L, Hu W, Trudeau VL. Targeted mutation of secretogranin-2 disrupts sexual behavior and reproduction in zebrafish. *Proc Natl Acad Sci USA*. 2020:202002004.

50. Zhao E, Basak A, Wong A, Ko W, Chen A, López G, Grey C, Canosa L, Somoza G, Chang J, Trudeau V. The secretogranin II-derived peptide secretoneurin stimulates luteinizing hormone secretion from gonadotrophs. *Endocrinology*. 2009;150(5):2273–2282.

51. Samson WK, Freeman ME. Vasoactive intestinal peptide: A neural modulator of endocrine function. In: Craig AJ, Charles DB, eds. Brain-gut peptides and reproductive function. Boca Raton, FL: CRC Press; 1991:83–103.

52. Abraham E, Palevitch O, Gothilf Y, Zohar Y. The zebrafish as a model system for forebrain GnRH neuronal development. *Gen Comp Endocrinol*. 2009;164(2–3):151–160.

53. Hanna RN, Daly SCJ, Pang Y, Anglade I, Kah O, Thomas P, Zhu Y. Characterization and expression of the nuclear progestin receptor in zebrafish gonads and brain. *Biol Reprod*. 2010;82(1):112–122.

54. Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O, Pakdel F. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. *Biol Reprod*. 2002;66(6):1881–1892.

55. Fontaine R, Affaticati P, Bureau C, Colin I, Demarque M, Dufour S, Vernier P, Yamamoto K, Pasqualini C. Dopaminergic neurons controlling anterior pituitary functions: Anatomy and ontogenesis in zebrafish. *Endocrinology*. 2015;156(8):2934–2948.

56. Herzog W, Zeng X, Lele Z, Sonntag C, Ting J-W, Chang C-Y, Hammerschmidt M. Adenohypophysis formation in the zebrafish and its dependence on sonic hedgehog. *Dev Biol*. 2003;254(1):36–49.

57. Mathieu M, Tagliafierro G, Angelini C, Vallarino M. Organization of vasoactive intestinal peptide-like immunoreactive system in the brain, olfactory organ and retina of the zebrafish, *Danio rerio*, during development. *Brain Res*. 2001;888(2):235–247.

58. Chen S, Reichert S, Singh C, Oikonomou G, Rihel J, Prober DA. Light-dependent regulation of sleep and wake states by prokineticin 2 in zebrafish. *Neuron*. 2017;95(1):153-168.e6.

59. Yamashita J, Nishiike Y, Fleming T, Kayo D, Okubo K. Estrogen mediates sex differences in preoptic neuropeptide and pituitary hormone production in medaka. *Commun Biol*. 2021;4(1):948.

60. Sherwood N, Krueckl S, McRory J. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev*. 2000;21(6):619–670.

61. Chang JP, Wirachowsky NR, Kwong P, Johnson JD. PACAP stimulation of gonadotropin-ii secretion in goldfish pituitary cells: Mechanisms of action and interaction with gonadotropin releasing hormone signalling. *J Neuroendocrinol*. 2001;13(6):540–550.

62. Wong AOL, Li WS, Lee EKY, Leung MY, Tse LY, Chow BKC, Lin HR, Chang JP. Pituitary adenylate cyclase activating polypeptide as a novel hypophysiotropic factor in fish. *Biochem Cell Biol*. 2000;78(3):329–343.

63. Enjalbert A, Arancibia S, Ruberg M, Priam M, Bluet-Pajot MT, Rotsztejn WH, Kordon C. Stimulation of in vitro prolactin release by vasoactive intestinal peptide. *Neuroendocrinology*. 1980;31(3):200–204.

64. Lin C, Jiang X, He M, Zhao L, Huang T, Bian Z, Wong AOL. Mechanisms for PACAP-induced prolactin gene expression in grass carp pituitary cells. *J Endocrinol*. 2017;233(1):37–51.

65. Weick RF, Stobie KM. Role of VIP in the regulation of LH secretion in the female rat. *Neurosci Biobehav Rev*. 1995;19(2):251–259.

66. Sun Y, Shu J, Kyei K, Neal-Perry GS. Intracerebroventricular infusion of vasoactive intestinal peptide rescues the luteinizing hormone surge in middle-aged female rats. *Front Endocrinol*. 2012;3:24.

67. Paredes J, Cowan M, López-Olmeda J, Muñoz-Cueto J, Sánchez-Vázquez F. Daily rhythms of expression in reproductive genes along the brain-pituitary-gonad axis and liver of zebrafish. *Comp Biochem Physiol Part A: Mole Integr Physiol.* 2019; doi:231: 158–169.

68. Dufour S, Sebert M-E, Weltzien F-A, Rousseau K, Pasqualini C. Neuroendocrine control by dopamine of teleost reproduction. *J Fish Biol*. 2010;76(1):129–160.

69. Chang JP, Peter RE, Crim LW. Effects of dopamine and apomorphine on gonadotropin release from the transplanted pars distalis in goldfish. *Gen Comp Endocrinol*. 1984;55(3):347–350.

70. Gissis A, Levavisivan B, Rubinkedem H, Ofir M, Yaron Z. The effect of gonadotropin-releasing-hormone superactive analog and dopamine antagonists on gonadotropin level and ovulation in tilapia hybrids. *Isr Journal Aquac.* 1991;43(4):123–136.

71. Drori S, Ofir M, Levavi-Sivan B, Yaron Z. Spawning induction in common carp (*Cyprinus carpio*) using pituitary extract or GnRH superactive analogue combined with

metoclopramide: analysis of hormone profile, progress of oocyte maturation and dependence on temperature. *Aquaculture*. 1994;119(4):393–407.

72. Abraham E, Palevitch O, Gothilf Y, Zohar Y. Targeted gonadotropin-releasing hormone-3 neuron ablation in zebrafish: Effects on neurogenesis, neuronal migration, and reproduction. *Endocrinology*. 2009;151(1):332–40.

73. Lin S-W, Ge W. Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish—An in vitro approach. *Gen Comp Endocrinol*. 2009;160(2):183–193.

74. Dolatshad H, Campbell EA, O'Hara L, Maywood ES, Hastings MH, Johnson MH. Developmental and reproductive performance in circadian mutant mice. *Hum Reprod*. 2006;21(1):68–79.

75. Saha S, Singh KM, Gupta BBP. Melatonin synthesis and clock gene regulation in the pineal organ of teleost fish compared to mammals: Similarities and differences. *Gen Comp Endocrinol*. 2019;279:27–34.

76. Whitmore D, Foulkes NS, Sassone-Corsi P. Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature*. 2000;404(6773):87–91.

77. Tamai TK, Carr AJ, Whitmore D. Zebrafish circadian clocks: cells that see light. *Biochem Soc Trans*. 2005;33(5):962–966.

78. Whitmore D, Foulkes NS, Strähle U, Sassone-Corsi P. Zebrafish clock rhythmic expression reveals independent peripheral circadian oscillators. *Nat Neurosci*. 1998;1(8):701–707.

Figure 1. VipA neurons are localized in the preoptic area and hypothalamus extending their fibers to the pituitary proximal pars distalis. (A) Lateral view (anterior to left) of the adult zebrafish brain showing the position of coronal sections. (B) Top panel, images of *vipa* expressing neurons in PPa, Vp and VM in sexually mature WT female zebrafish brain, detected by in situ hybridization. Bottom panel, high magnification images showing a close up view of the boxed area on the top images. Scale bars, 80 µm (top); 10 µm (bottom). (C) Number of *vipa*-expressing neurons in PPa and Vp from wild type (WT) and *gnrh3*$^{-/-}$ female zebrafish (N=6 each). $P$>0.05 (two-sample Wilcoxon rank sum test). (D) Dorsal view (anterior to left) of the adult zebrafish brain showing the position of a sagittal section. (E) Confocal images of immunostaining with zebrafish VipA antibody (magenta) in the sagittal brain section (20 µm thickness; anterior is left) of sexually mature female zebrafish. Scale bar, 200 µm. (F) Confocal images of immunostaining with zebrafish VipA antibody (magenta; a) in the pituitary (a sagittal section; anterior to top left) of *Tg (gnrh3: eGFP; lhβ: mCherry)* showing GnRH3 neuronal fibers (green; b) and LH cells (gray; c) and a merged image showing all immunostaining (d). The dashed line in the merged image indicates the shape of whole pituitary. Zoomed image (e) showing the boxed area on the merged image. Asterisks indicate the potential cells innervated by both VipA-ir and GnRH3 fibers. Scale bars, 50 µm. OB, olfactory bulb; Tel, telencephalon; TH, tuberal hypothalamus; TeO, tectum opticum; PPa and PPp, parvocellular preoptic nucleus, anterior (PPa) and posterior parts (PPp); Vp, postcommissural nucleus of ventral telencephalic area; VT, ventral thalamus; VM, ventromedial thalamic nucleus; P, posterior thalamic nucleus; Hv, Hc, and Hd, ventral (Hv), caudal (Hc), and dorsal (Hd) zones of periventricular hypothalamus; LH, lateral hypothalamic nucleus; RPD, rostral pars distalis; PPD, proximal pars distalis; PI, pars intermedia.

Figure 2. VipA does not induce an increase in LH secretion and *lhβ* expression at the pituitary level. (A) Pituitary LH release in the culture media from in vitro whole pituitary incubation with vehicle (control), VipA alone, a combination of Vipa with $E_2$ (VipA+$E_2$), GnRH3, a combination of GnRH3 with $E_2$ (GnRH3+$E_2$), and $E_2$ alone (N=5 each). Different letters indicate statistically significant differences, $P$<0.05 (Kruskal-Wallis test followed by Dunn's *post hoc* test). (B) Levels of *lhβ* relative

expression in the pituitary from sexually mature females after in vivo intraperitoneal administration with vehicle (control), VipA alone, a combination of VipA with DOM, a dopamine D2 receptor antagonist (VipA+DOM), GnRH3 alone, a combination of GnRH3 with DOM (GnRH3+DOM) or DOM alone (N=5 each except for the control for which N=4 and GnRH3 alone and DOM alone for which N=3). *, $P<0.05$ [versus control; one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test].

Figure 3. VipA intracerebroventricular (ICV) administration induces an increase of plasma LH levels, but not brain *gnrh3* expression and GnRH pituitary content, at 1 hour post-injection. (A) LH level in plasma of sexually mature females (left, WT; right, *gnrh3*$^{-/-}$) after in vivo ICV administration with vehicle (control) or VipA. (B) LH level in plasma of WT sexually mature females after ICV administration with vehicle (control) or GnRH3. After ICV administration with vehicle or VipA in WT females, (C) *gnrh3* relative mRNA levels in the forebrain and midbrain, (D) GnRH3 protein content in the pituitary, and (E) GnRH2 protein content in the pituitary. *, $P<0.05$; **, $P<0.01$ (two-sample Student's t-test except for the results of pituitary GnRH3 and GnRH2 protein content tested by two-sample Wilcoxon rank sum test).

Figure 4. Attenuation of VipA signaling induces an increase of plasma LH levels. LH level in plasma of sexually mature females after 2 and 18 hours post-in vivo ICV administration with rabbit serum (control) or anti-mouse Vip antibody (mVip ab). **, $P<0.01$; ***, $P<0.001$ [two-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference test].

Figure 5. *vipa*$^{-/-}$ and *vipa*$^{-/-}$; *gnrh3*$^{-/-}$ females display normal fertility. (A) Schematic diagram of *vipa* gene. Arrow indicates targeted region of CRISPR/Cas9 gene editing. White boxes indicate an exon, and black boxes indicate an open reading frame. The shaded box represents the position of VipA

peptide sequence. Lines indicate an intron. The sequence shows the sgRNA target sequence with a PAM sequence, and the 8 bp deletion in *vipa*$^{-/-}$ zebrafish used in this study. (B) Confocal images of immunostaining with zebrafish VipA antibody (magenta) with nuclear counterstaining (blue) in the parvocellular preoptic nucleus PPa and Vp, and pituitary PPD of *vipa*$^{+/+}$ (WT siblings) and *vipa*$^{-/-}$ zebrafish females. Inset, a zoomed image showing the boxed area of the PPa. Arrowheads indicate VipA-ir somas. Noted that the color shown in the images of *vipa*$^{-/-}$ samples is potentially non-specific binding within the blood vessels. Scale bars, 50 µm. (C) The number of fertilized eggs per spawn (fecundity) and percentage of fertilized eggs (fertility) from *vipa*$^{+/+}$ (N=6) and *vipa*$^{-/-}$ (N=5) females paired with WT males. (D) The number of fertilized eggs per spawn (fecundity) and percentage of fertilized eggs (fertility) from *vipa*$^{+/+}$; *gnrh3*$^{+/+}$ (N=4) and *vipa*$^{-/-}$; *gnrh3*$^{-/-}$ (N=5) females paired with WT males. $P>0.05$ (two-sample Wilcoxon rank sum test). PPa, parvocellular preoptic nucleus, anterior part; Vp, postcommissural nucleus of ventral telencephalic area; PPD, proximal pars distalis.

Figure 6. Proposed diagram of the different pathways regulating LH secretion in sexually mature female zebrafish. (a) VipA acts on unknown neurons or (b) in tandem with a permissive factor(s) to regulate immediate LH secretion from the pituitary (c) Dopaminergic neurons may directly innervate pituitary LH cells (33) and possibly downregulate *lhβ* expression by preventing GnRH3 from inducing *lhβ* expression. Dopamine actions need to be neutralized in order for GnRH3 to exert its effect. (d) Estradiol-17β ($E_2$) from the ovaries directly induces LH secretion through an unknown mechanism.

Table 1. Sequences of primers used for real-time quantitative PCR

Figure 1.

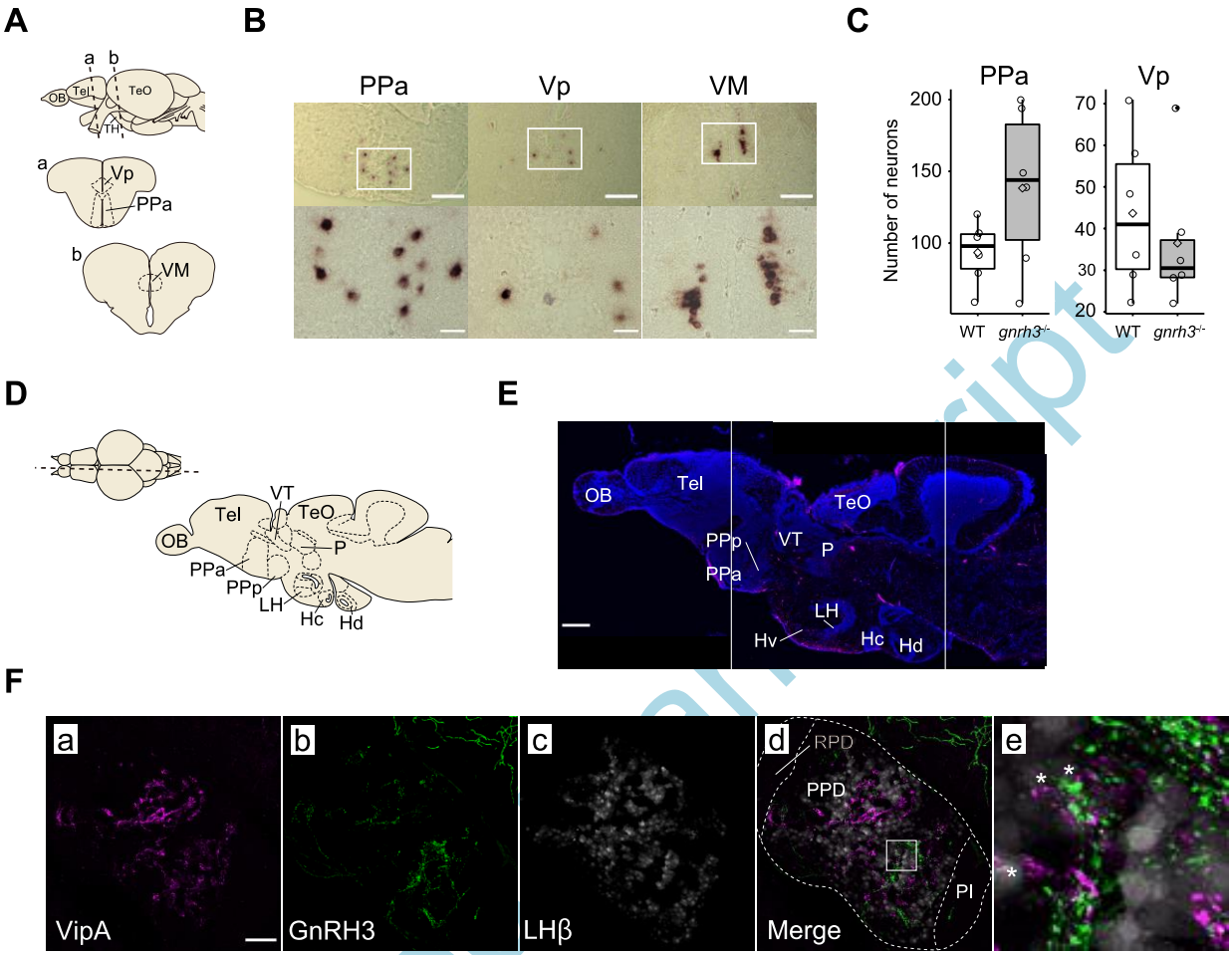

Figure 2.

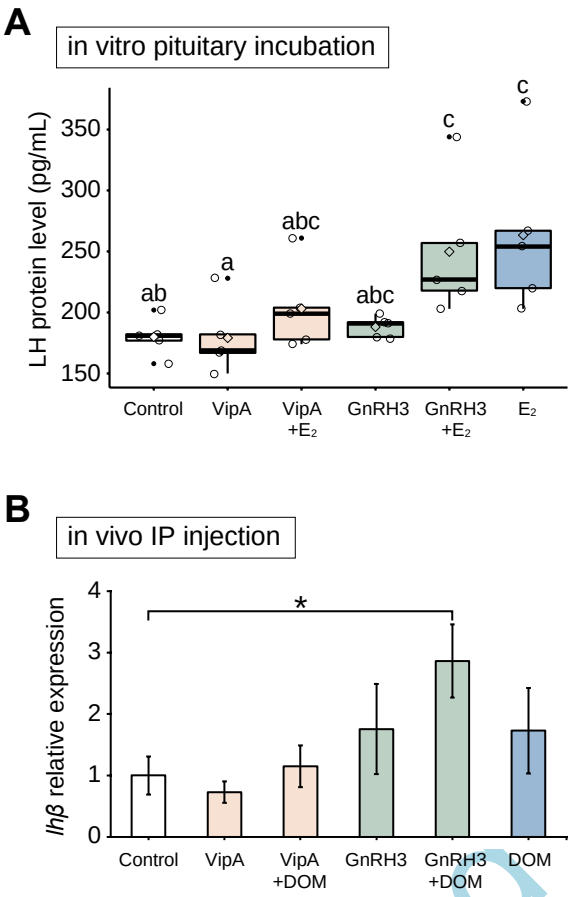

Figure 3.

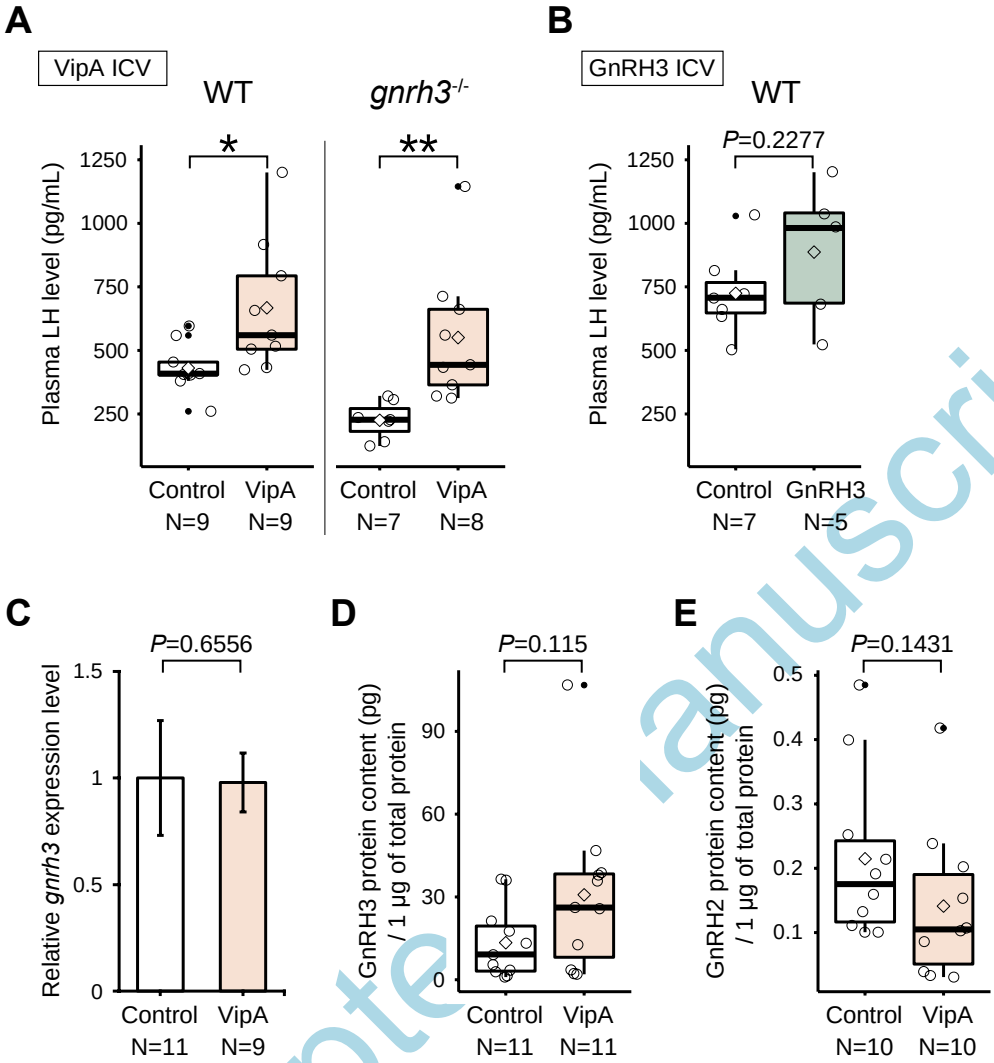

Figure 4.

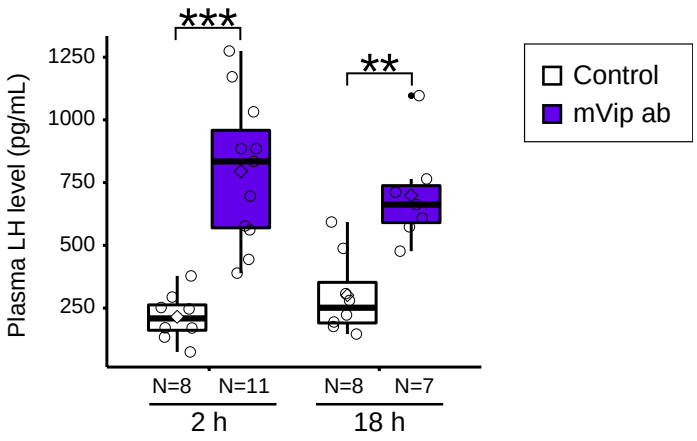

Figure 5.

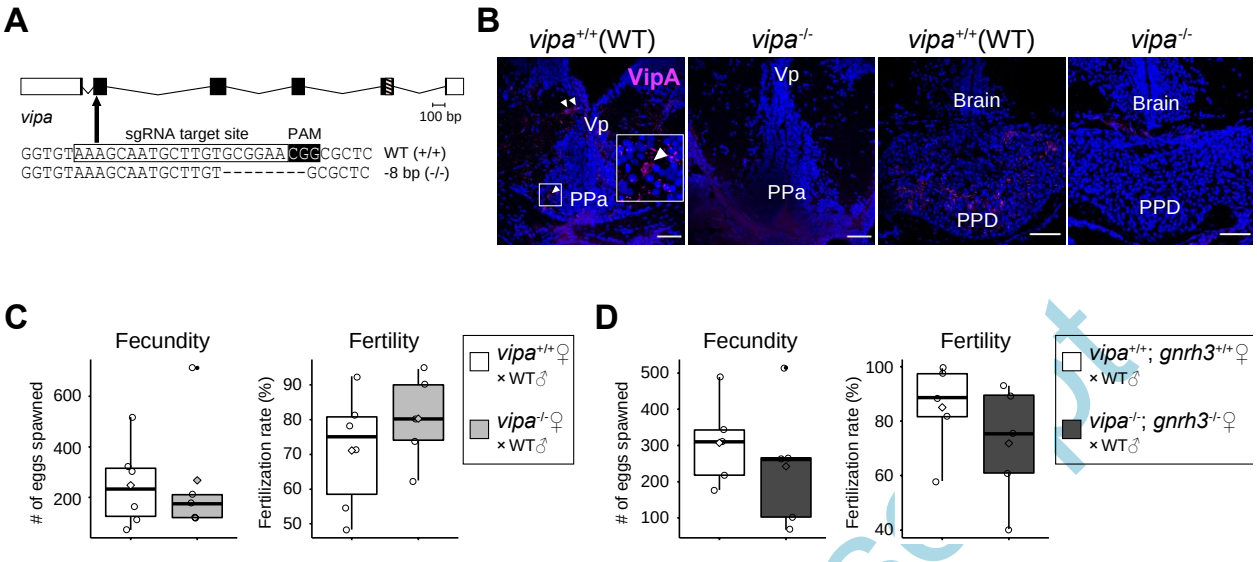

Figure 6.

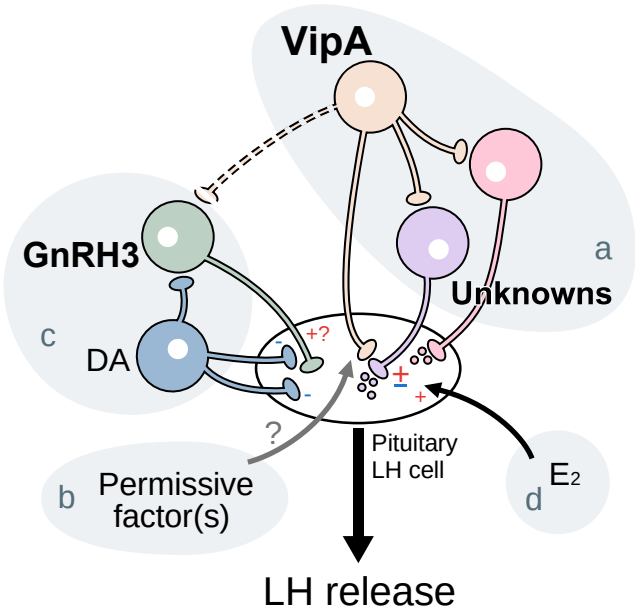

Table 1.

| Gene | GenBank accession # | Forward (5' to 3') | Reverse (5' to 3') |
|---|---|---|---|
| *eef1α1* | NM_131263.1 | ACTTCAACGCTCAGGTCATC | CTCCTTGAGCTCAGCAAACT |
| *lhβ* | NM_205622.2 | GGCTGGAAATGGTGTCTTCT | CCACCGATACCGTCTCATTTAC |
| *gnrh3* | NM_182887.2 | TGGAGGCAACATTCAGGATGT | CCACCTCATTCACTATGTGTATTG |