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Research Article
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Research Article

The gonadotropin-inhibitory hormone (Lpxrfa) system's regulation of reproduction in the brain–pituitary axis of the zebrafish (*Danio rerio*)†

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Abstract

Gonadotropin-inhibitory hormone (GNIH) was discovered in quail with the ability to reduce gonadotropin expression/secretion in the pituitary. There have been few studies on GNIH orthologs in teleosts (LPXRFamide (Lpxrfa) peptides), which have provided inconsistent results. Therefore, the goal of this study was to determine the roles and modes of action by which Lpxrfa exerts its functions in the brain–pituitary axis of zebrafish (*Danio rerio*). We localized Lpxrfa soma to the ventral hypothalamus, with fibers extending throughout the brain and to the pituitary. In the preoptic area, Lpxrfa fibers interact with gonadotropin-releasing hormone 3 (Gnrh3) soma. In pituitary explants, zebrafish peptide Lpxrfa-3 downregulated luteinizing hormone beta subunit and common alpha subunit expression. In addition, Lpxrfa-3 reduced *gnrh3* expression in brain slices, offering another pathway for Lpxrfa to exert its effects on reproduction. Receptor activation studies, in a heterologous cell-based system, revealed that all three zebrafish Lpxrfa peptides activate Lpxrf-R2 and Lpxrf-R3 via the PKA/cAMP pathway. Receptor activation studies demonstrated that, in addition to activating Lpxrf receptors, zebrafish Lpxrfa-2 and Lpxrfa-3 antagonize Kisspeptin-2 (Kiss2) activation of Kisspeptin receptor-1a (Kiss1ra). The fact that *kiss1ra*-expressing neurons in the preoptic area are innervated by Lpxrfa-ir fibers suggests an additional pathway for Lpxrfa action. Therefore, our results suggest that Lpxrfa may act as a reproductive inhibitory neuropeptide in the zebrafish that interacts with Gnrh3 neurons in the brain and with gonadotropes in the pituitary, while also potentially utilizing the Kiss2/Kiss1ra pathway.

Summary Sentence

Lpxrfa regulates reproduction in the zebrafish brain–pituitary axis through inhibitory effects on gonadotropins and Gnrh3, and Lpxrfa elicits these effects by utilizing Lpxrf receptors, as well as receptors of other reproductive neuropeptides.

Key words: Lpxrfa, Gnrh3, zebrafish, GNIH, reproduction.

Introduction

Vertebrate reproduction is regulated by the hypothalamus–pituitary–gonad axis, which translates internal and external cues into endocrine signals and reproductive output. The axis's control mechanisms include a complex network of neuropeptides that work at the level of the brain and/or the pituitary, including gonadotropin-releasing hormone (GNRH). All of the previously discovered neuropeptides that regulate gonadotropin release have had stimulatory effects, many of which include RFamide peptides. RFamide peptides are classified by the presence of an Arg-Phe-NH_2 motif at the C-terminus. While the majority of reproductive RFamide peptides induce a stimulatory effect, the discovery of gonadotropin-inhibitory hormone (GNIH) in Japanese quail (*Coturnix japonica*) [1] introduced one of the first inhibitory neuropeptides. This dodecapeptide was found in the paraventricular nucleus with projections to the median eminence and inhibits the release of luteinizing hormone (LH) in vitro [1]. In birds, GNIH has an inhibitory effect of follicle-stimulating hormone (FSH) and LH synthesis (mRNA expression) and release from the pituitary [2–6].

GNIH orthologs were later identified in other vertebrates, including amphibians [7, 8], mammals [9, 10], and fish [11–13]. In mammals, GNIH orthologs are referred to as "RFamide-related peptides" (RFRPs), and RFRP-3 is a functional ortholog of avian GNIH [14]. In addition to innervation of the median eminence by GNIH/RFRP [1, 15], it has been demonstrated that GNIH/RFRP also interacts with GNRH neurons in avian and mammalian brains [16], supported by the expression of the GNIH/RFRP receptor (G protein-coupled receptor (GPCR) 147; GPR147) on GNRH neurons in birds and mammals [6, 17]. This interaction is also evident by the ability of RFRP-3 to decrease the firing rate of GNRH1 neurons in mouse brain slices [18]. Therefore, GNIH/RFRP may affect the reproductive axis either by directly affecting pituitary gonadotropes via the median eminence or indirectly by attenuating the activity of GNRH neurons.

In teleosts, GNIH orthologs are referred to as "LPXRFamide (Lpxrfa) peptides," and the cDNA-encoding Lpxrfa peptides have been cloned in goldfish (*Carassius auratus*) [11], zebrafish (*Danio rerio*) [13], puffer (*Takifugu* spp.) [12], Nile tilapia (*Oreochromis niloticus*) [19], orange-spotted grouper (*Epinephelus coioides*) [20], and the European seabass (*Dicentrarchus labrax*) [21]. While some studies show an inhibitory effect of Lpxrfa peptides on gonadotropins [13, 20, 22], some reveal a stimulatory effect [19, 23], which can depend on gonadal stage or peptide administration methods [23]. Therefore, the functional roles of GNIH orthologs in teleosts are not as consistent as that found in birds and mammals. In addition, teleost Lpxrfa has been shown to functionally interact with Gnrh2 and Gnrh3 in the goldfish [24, 25] and to reduce *gnrh1* mRNA levels in European seabass (specifically Lpxrfa-2) [22] and orange-spotted grouper [20].

Another teleost Lpxrfa system, that of the model organism, the zebrafish, is composed of a 198-amino-acid precursor peptide that is cleaved into Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3 peptides and of three GPCRs (Lpxrf-R1, Lpxrf-R2, and Lpxrf-R3) [13]. The goal of this study was to elucidate the mode of action of each component of the Lpxrfa/Lpxrf-R system in the zebrafish and explore their relationships with major components of the zebrafish reproductive axis to determine Lpxrfa's roles in the regulation of reproduction. Specifically, we sought to understand the neuroanatomical and functional relationships between Lpxrfa and the pituitary gonadotropes and between Lpxrfa and Gnrh3 (the hypophysiotropic Gnrh in zebrafish). The relationships of the Lpxrfa peptides with the three cognate Lpxrf receptors were examined through activation potencies. Finally, we reveal an additional reproductive neuropeptide pathway that may be utilized by Lpxrfa peptides.

Materials and methods

Animals

All zebrafish originated from the Institute of Marine and Environmental Technology in Baltimore, MD. Zebrafish were maintained in a recirculating system at 28°C with a photoperiod of 14 h light and 10 h dark and were fed twice daily with a commercial flake food or pellets ad libitum. Adult males weighed on average 400 mg, while adult females weighed on average 650 mg. For pituitary immunohistochemistry, *fsh:eGFP* (ZIRC number hjr1Tg) and *lh:mCherry* (ZIRC number hjr2Tg) transgenic lines [26] from The Hebrew University in Jerusalem (Rehovot, Israel) were used. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland Baltimore School Of Medicine.

Peptides

All zebrafish Lpxrfa peptides (Lpxrfa-1: SLEIQDFTLNVAPTSGGA SSPTILRLHPIIPKPAHLHAN-LPLRF-NH_2, Lpxrfa-2: APKSTIN LPQRF-NH_2, and Lpxrfa-3: SGTGPSATLPQRF-NH_2) were synthesized at a 95% purity by Genscript.

Lpxrfa immunohistochemistry in pituitaries

For pituitary visualization, heads of male and female adult *fsh:eGFP* and *lh:mCherry* fish [26] were fixed with 4% paraformaldehyde in PBS at 4°C overnight after removal of the eyes, jaw, gills, and other soft tissues. Afterward, heads were decalcified for 5–7 days with 0.5M EDTA (pH 8.0) at 4°C. Heads were transferred to 30% sucrose in PB overnight at 4°C for cryoprotection. Tissues were frozen in OCT, sectioned coronally at 15 μm thickness, transferred to charged slides, and stored at –80°C until processed. Lpxrfa immunohistochemistry on heads was conducted as described for brains [27]. The zebrafish Lpxrfa antibody validation data is in Supplementary Figure S1.

Lpxrfa and Gnrh3 immunohistochemistry in brains

For determining the structural interactions between zebrafish Lpxrfa and Gnrh3 in the brain, we conducted double immunohistochemistry on WT male and female adult brains. Fixation, cryopreservation, and sectioning of brains were conducted as described for heads without decalcification. Briefly, dried sections were fixed in prechilled acetone for 2 min. Sections were washed with 0.5% H_2O_2 in PBS for 30 min at room temperature to quench endogenous HRPs. Sections were blocked with 5% goat serum for 1 h and incubated overnight at 4°C with rabbit anti-zebrafish Lpxrfa (1:5000). Sections were incubated for 1 h with goat anti-rabbit HRP (1:1000). The signal was detected using the TSA Plus kit (Perkin Elmer), and fluorescence was obtained via the Cy3 dye from the kit. After quenching HRPs with 0.02N HCl for 10 min, blocking and immunostaining for Gnrh3 were conducted in the same manner with the rabbit anti-zebrafish Gnrh3 Gap [27],

Table 1. QPCR primers used in quantifying gene transcripts in zebrafish brains and pituitaries.

Gene	Type	Sequence (5′ to 3′)	Tm[a] (°C)	GC %[b]	Amplicon size (bp)
gnrh2	For	CAGAGGTTTCAGAGGAAGTGAAGC	57.5	50.0	102
gnrh2	Rev	TGAGGGCATCCAGCAGTATTG	57.2	52.4	
gnrh3	For	TGGAGGCAACATTCAGGATGT	56.6	47.6	105
gnrh3	Rev	CCACCTCATTCACTATGTGTATTGG	55.4	44.0	
fshb	For	GCTGGACAATGGATCGAGTTTA	54.9	45.5	92
fshb	Rev	CTCGTAGCTCTTGTACATCAAGTT	54.5	41.7	
lhb	For	GGCTGGAAATGGTGTCTTCT	55.1	50.0	107
lhb	Rev	CCACCGATACCGTCTCATTTAC	55.1	50.0	
cga	For	TCCGGTCTATCAGTGCGT	55.6	55.6	
cga	Rev	GGATATTCGTGGCAACCATTT	53.5	42.9	148
ef1a	For	AAGACAACCCCAAGGCTCTCA	58.6	52.4	255
ef1a	Rev	CCTTTGGAACGGTGTGATTGA	55.5	47.6	

[a]Tm = melting temperature.
[b]GC% = guanine-cytosine percentage.

except that fluorescence was obtained via the FITC dye from the TSA Plus kit.

Effects of Lpxrfa-3 on pituitary in vitro

In order to determine if the effects of zebrafish Lpxrfa on pituitary gonadotropin expression are similar to that of GNIH/RFRP in birds/mammals, we used a modified protocol from a pituitary culture experiment for striped bass (*Morone saxatillis*) [28, 29]. Whole pituitaries were dissected from adult male wild-type zebrafish and placed in precooled phenol red-free L-15 media (Himedia) supplemented as described previously [28, 29] in a 24-well sterile cell culture plate. The pituitaries were placed in sterile Millicell cell culture inserts (0.4 μm and 12 mm diameter; Millipore). After washing with media for 6 h at 28°C and 30 revolutions per minute (rpm) on an orbital shaker, pituitary explants were incubated with different concentrations (0, 1, 10, 100, and 1000 pM) of zebrafish Lpxrfa-3 in media at 28°C and 30 rpm (n = 6 per concentration). Of the three Lpxrfa peptides encoded in the zebrafish Lpxrfa precursor, Lpxrfa-3 was chosen for further investigation because of its high homology to goldfish Lpxrfa-3, which was demonstrated to be a mature peptide by mass spectrometry [11], and because of its ability to reduce serum Lh levels in the goldfish in vivo [13]. After 18 h, pituitaries were frozen on dry ice and stored at –80°C for RNA extraction. QPCR for follicle-stimulating hormone beta subunit (*fshb*; standard curve coefficient correlation (R^2) = 0.999), luteinizing hormone beta subunit (*lhb*; R^2 = 0.995), and common alpha subunit (*cga*; R^2 = 0.998) was then conducted. This study was conducted twice in two independent experiments.

Effects of Lpxrfa-3 on brain in vitro

In order to determine the effects of zebrafish Lpxrfa-3 on adult brains in vitro, we used a modified protocol for a striped bass brain slice culture [28, 29]. Whole brains of adult male zebrafish were dissected and immediately placed into precooled media (Dulbecco Modified Eagle Medium with 25 mM D-glucose and HEPES supplemented as described previously [28, 29]). Whole brains were sliced at 200–300 μm thickness with a mechanical tissue chopper, and all of the brain tissue was returned to media. Tissues were held in sterile inserts in 12-well sterile cell culture plates. Brains were washed with media 3 × 1 h at 45 rpm and 28°C. Brain slices were incubated for 6 h with different concentrations (0, 0.1, 0.5, 1, and 5 nM) of zebrafish Lpxrfa-3 in media with slight agitation at 28°C (n = 6 per concentration). After 6 h, brain tissues were frozen on dry ice and stored at –80°C for RNA extraction. QPCR for *gnrh2* (R^2 = 0.952) and *gnrh3* (R^2 = 0.960) was then conducted. This study was conducted twice in two independent experiments.

Quantification of gene transcripts

Total RNA extraction, cDNA synthesis, and QPCR were conducted as previously described [27]. One microgram total RNA of brains and 100 ng total RNA of pituitaries were used, and the final reaction volume for all cDNA synthesis reactions was 20 (brains) or 10 (pituitaries) μL. Gene-specific QPCR primers for each of the target genes are listed in Table 1. Absolute copy number was calculated for the tested reactions and normalized against the housekeeping gene *ef1a*.

Lpxrf-R activation assay

The goal of this study was to generate activation profiles for each zebrafish Lpxrf receptor with the three zebrafish Lpxrfa peptides in order to determine their affinities and potencies. To accomplish this, we compared two signal transduction pathways in a COS7 cell line: cyclic AMP (cAMP)-response element (CRE) through PKA/cAMP and serum-response element (SRE) through PKC/Ca^{2+}. The full cDNA coding regions of each Lpxrfa receptor, *lpxrfr1*, *lpxrfr2*, and *lpxrfr3* (GenBank accession numbers: GU290219.1, GU290220.1, and GU290221.1, respectively), were individually cloned into the pcDNA3.1 expression vector (Invitrogen). Transfection, incubation, peptide administration, and assessment of bioluminescence were conducted as previously described [29]. All Lpxrfa peptides were individually applied in a 10x serial dilution (10 pM to 10 μM), and all experiments were performed in triplicate with at least three independent experiments.

Lpxrfa activation/inhibition of Kiss receptors

After multiple failed attempts to histologically localize the zebrafish Lpxrf receptors with in situ hybridization (see Discussion), we hypothesized that the Lpxrf receptors might be expressed at such low levels and/or be too widespread to allow detection with histological methods. Consequently, we considered that, because Lpxrfa and Kisspeptin are both reproductive RFamide peptides, zebrafish Lpxrfa might elicit its functions through Kisspeptin receptors, in addition to Lpxrf receptors. Therefore, we attempted to determine if zebrafish Lpxrfa peptides are capable of activating Kisspeptin receptor-1a

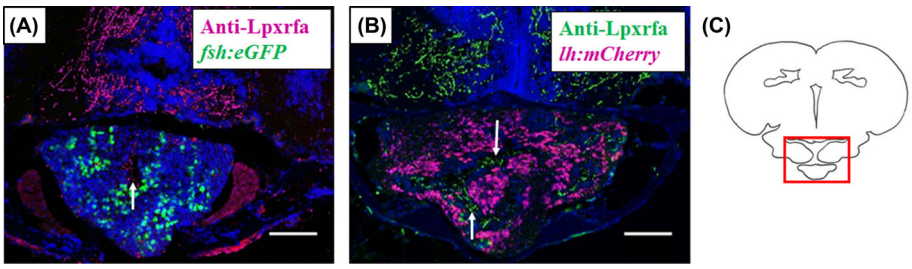

Figure 1. Zebrafish Lpxrfa-ir fibers innervate the pituitary. Coronal sections (C) of *fsh:eGFP* adult brain/pituitary (Fsh-labeled cells in green (A) and *lh:mCherry* adult brain/pituitary (Lh-labeled cells in magenta, (B) immunostained with anti-zebrafish Lpxrfa in magenta (A) and green (B) (see white arrows). Scale bars = 100 μm.

(Kiss1ra; Kiss2r) and Kiss1rb (Kiss1r) through the SRE pathway, the dominant pathway for Kiss/Kissr activation in teleosts [30, 31]. Receptor activation studies were conducted using previously described methodology [29]. As a first step, Kiss1ra and Kiss1rb were incubated with serial dilutions of Lpxrfa-2 or -3 to test receptor activation via the SRE pathway, using the same methods for the Lpxrf receptors. Additionally, to test whether Lprxfa-2 or -3 interferes with the activation of Kiss receptors by Kisspeptin graded concentrations (10 pM to 1 μm) of Lpxrfa-2 or -3 were added concomitantly to 100 nM Kisspeptin-1 (Kiss1; for Kiss1rb) or 10 nM Kisspeptin-2 (Kiss2; for Kiss1ra) to determine the levels of inhibition.

Neuroanatomical localization of Lpxrfa and *kiss1ra*

To determine if there is any neuroanatomical relationship between Lpxrfa neurons and Kiss1ra receptors, we conducted simultaneous in situ hybridization for *kiss1ra* and immunohistochemistry for Lpxrfa on male and female adult brains. An antisense DIG-labeled riboprobe was synthesized from the cDNA clone of the full *kiss1ra* coding region, using RNA polymerase (Roche Diagnostics), and the in situ hybridization protocol was followed as described previously [32]. The signal was detected using the TSA Plus kit, according to the manufacturer's protocol, using anti-DIG HRP (1:200; Roche Diagnostics) and the Cy3 dye. After quenching HRPs, Lpxrfa immunohistochemistry was conducted as described in the "Lpxrfa and Gnrh3 Immunohistochemistry in Brains" section with anti-zebrafish Lpxrfa and the FITC dye from the TSA Plus kit.

Microscopy

All sections were mounted with antifading solution with DAPI and imaged with one of three microscopes. COS7 cells were imaged with a Zeiss Axioplan 2 microscope with an Attoarc HBO100 W power source, equipped with a CCD Olympus DP70 camera. Pituitary sections were imaged with a Leica Microsystems SP6 confocal microscope. Brain sections were imaged with a Leica Microsystems DMi8 confocal microscope or with the Zeiss Axioplan 2 microscope. All images were analyzed with Image J and/or Adobe Photoshop.

Statistics

For QPCR values in the in vitro experiments, the mean of each concentration was compared to the control (0 nM or pM Lpxrfa-3) by a one-tailed Student *t*-test. GraphPad Prism (Version 3) was used to plot dose–response activation curves and calculate EC_{50}s and IC_{50}s for the receptor activation/inhibition assays. The IC50 was calculated using log(inhibitor) vs. response-variable slope (four parameters), and the EC50 used the formula log(agonist) vs. response-variable slope (four parameters). In addition, relative luciferase activity values for the inhibition of Kiss-activated Kiss receptors by Lpxrfa peptides were compared to the control (activation of Kiss receptor by Kiss) by a one-tailed Student *t*-test. Statistical significance was established if $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, and $^{***}P \leq 0.0005$.

Results

Neuroanatomical localization of Lpxrfa

Lpxrfa immunohistochemistry in pituitary

The anti-zebrafish Lpxrfa antibody was used to localize Lpxrfa within the pituitary in brain-pituitary sections of adult *fsh:eGFP* and *lh:mCherry* fish. Fsh-labeled (Figure 1A) and Lh-labeled (Figure 1B) cells were located within the proximal pars distalis with a higher prevalence of Lh-labeled cells than Fsh-labeled cells, as seen in previous zebrafish studies [26]. Overall, Lpxrfa-ir innervation (Figure 1A and B) in the pituitary was detectable, though not as intense as in the hypothalamus. In addition, Lpxrfa-ir fibers in the pituitary did not interact with and were not in direct contact with either Fsh-labeled or Lh-labeled cells. The Lpxrfa-ir fibers in the pituitary appeared to be localized to the medial region of the neurohypophysis (Figure 1A and B).

Lpxrfa and Gnrh3 immunohistochemistry in brain

To determine whether Lpxrfa neurons interact with Gnrh3 neurons, double Lpxrfa and Gnrh3 immunohistochemistry in adult brain sections was conducted. We found that Lpxrfa-ir soma are located in a distinct cluster in the ventral zone of the periventricular hypothalamus with Lpxrfa-ir fibers extending throughout the forebrain, midbrain, and hindbrain (Figure 2A and C). Gnrh3 soma were found within the terminal nerve/ventral telencephalon and preoptic area/hypothalamus, and Gnrh3-ir fibers were found to project posteriorly through the hypothalamus and then ventrally to the pituitary, as reported previously [33, 34]. Overall, Lpxrfa-ir and Gnrh3-ir fibers were found in similar regions of the brain, while their soma were in very distinct regions. In the regions of the forebrain that contained Gnrh3-ir soma, particularly the preoptic area, we found that Lpxrfa-ir fibers interact with and strongly project to Gnrh3-ir soma (Figure 2E–G).

Functional effects of Lpxrfa

Lpxrfa-3 effects on pituitary in vitro

We incubated adult male pituitary explants with Lpxrfa-3 to determine whether Lpxrfa directly influences the expression of pituitary gonadotropins. After 18 h, samples were assessed for mRNA levels of *fshb*, *lhb*, and *cga*. While there were no changes in *fshb* levels (Figure 3A), both *lhb* (Figure 3B) and *cga* (Figure 3C) levels were

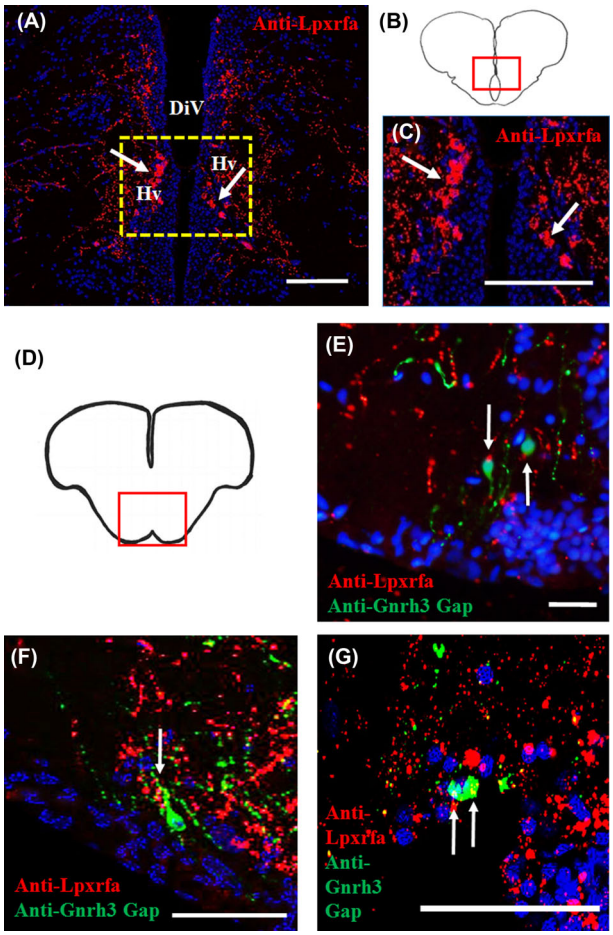

Figure 2. Zebrafish Lpxrfa-ir soma are located in the ventral hypothalamus and interact with and project to Gnrh3-ir soma in the forebrain. (A–C) Coronal sections of the hypothalamus (B) of adult WT brains immunostained with anti-zebrafish Lpxrfa (red, A and C). The Lpxrfa-ir soma (white arrows) are located in a cluster in the ventral zone of the periventricular hypothalamus that surrounds the diencephalic ventricle (A and C). C is the magnified version of the yellow square in A. (D–G) Coronal sections of the preoptic area (D) of adult wild-type brains immunostained with anti-zebrafish Lpxrfa (red) and anti-zebrafish Gnrh3 Gap (green). Three examples of Lpxrfa-ir neuron fibers (red) interacting with and making direct contact with Gnrh3-ir soma (green), as indicated by the white arrows (E–G). Hv = ventral zone of the periventricular hypothalamus. DiV = diencephalic ventricle. Scale bars = 100 μm.

reduced, in response to Lpxrfa-3. Two concentrations of Lpxrfa-3 (10 and 100 pM) significantly reduced *lhb* mRNA levels compared to the control (Figure 3B), while 1, 10, and 100 pM concentrations of Lpxrfa-3 significantly reduced *cga* levels compared to the control (Figure 3C). Interestingly, for both *lhb* and *cga*, 1000 pM Lpxrfa-3 did not affect mRNA levels (Figure 3B and C).

Lpxrfa-3 effects on brain in vitro

To determine a possible direct effect of Lpxrfa on Gnrh3, we incubated adult male brain slices with zebrafish Lpxrfa-3 to determine if Lpxrfa directly influences brain mRNA levels of *gnrh3* and *gnrh2*. After 6 h, samples were taken to assess mRNA levels of *gnrh3* and *gnrh2* via QPCR. While only the lowest zebrafish Lpxrfa-3 concentration tested (0.1 nM) slightly increased *gnrh2* levels (Figure 4B), incubation with all of the zebrafish Lpxrfa-3 concentrations resulted in significant reductions (approximately 50%) in *gnrh3* levels (Figure 4A). Therefore, the inhibitory effect of Lpxrfa-3 on the brain is more specific to *gnrh3* than to *gnrh2*.

Receptor activation/inhibition assays

Lpxrf receptors

To generate activation profiles for each of the Lpxrf receptors, we measured luciferase bioactivity in the COS7 cell line, using the Dual Luciferase Reporter Assay System (Promega). No dose response of the SRE pathway (PKC/Ca^{2+}) was detected by any of the three Lpxrfa peptides with any of the three Lpxrf receptors (Figure 5). However, Lpxrf-R2 and Lpxrf-R3 exhibited dose–response activation curves within the CRE pathway (PKA/cAMP) by all three Lpxrfa peptides (Figure 6B and C). For both receptors, Lpxrfa-2 and Lpxrfa-3 were the stronger elicitors of a dose response, while Lpxrfa-1 (the longest peptide) was the weakest (Figure 6B and C). The EC_{50}s of Lpxrf-R2 via CRE were 2.43×10^{-8} M ($\pm$ 2.12×10^{-8} M), 4.64×10^{-9} M ($\pm$ 5.96×10^{-10} M), and 1.88×10^{-9} M ($\pm$ 2.39×10^{-9} M) for peptides Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3, respectively. The EC_{50}s of Lpxrf-R3 via CRE were 5.72×10^{-8} M ($\pm$ 3.78×10^{-8} M), 6.91×10^{-10} M ($\pm$ 3.20×10^{-10} M), and 4.75×10^{-9} M ($\pm$ 4.28×10^{-9} M) for peptides Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3, respectively. Lpxrf-R1, on the other hand, did not demonstrate any response of the CRE pathway by any of the Lpxrfa peptides (Figure 6A).

An alignment of the amino acid sequences of all three zebrafish Lpxrf receptors was conducted previously [13]. When analyzing the homologies of the three major regions (N-terminus, transmembrane domains, and C-terminus) of the three zebrafish Lpxrf GPCRs, we demonstrated the following: the N-terminus is 27%–30% conserved among the three receptors, while the transmembrane domains are highly conserved (62%–72%), typical of GPCRs [35, 36]. For the C-terminus, however, there is 11%–40% homology among the three Lpxrf receptors. In the C-terminus, the GPCR region that tends to exhibit the most variation [35, 36], there is 40% homology between Lpxrf-R2 and -R3. However, Lpxrf-R1 has 11% and 24% homology with Lpxrf-R2 and Lpxrf-R3, respectively.

Kiss receptors

Next, we sought to determine if zebrafish Lpxrfa-2 and Lpxrfa-3 could activate either Kiss1ra or Kiss1rb, which are normally activated by the RFamide peptides Kiss2 and Kiss1, respectively. Using the PKC/Ca^{2+} pathway, there was no activation of Kiss1ra or Kiss1rb by neither Lpxrfa-2 nor Lpxfa-3 (Figure 7). However, Kiss2 and Kiss1 activated Kiss1ra and Kiss1rb, respectively, in a dose-dependent manner (Figure 7), as seen previously [29]. Therefore, Lpxrfa-2 and Lpxrfa-3 were subsequently used to determine if they could inhibit Kiss1 and Kiss2 activation of Kiss1rb and Kiss1ra, respectively. Regarding Kiss1ra, most of the lower concentrations of Lpxrfa-3 inhibited Kiss2 activation, while almost all of the Lpxrfa-2 concentrations inhibited Kiss2 activation ($IC_{50} = 5.915 \times 10^{-13}$ M; Figure 8A). For Kiss1rb, none of the concentrations of Lpxrfa-3 inhibited Kiss1 activation, while only the highest concentrations (10^{-7} and 10^{-6} M) of Lpxrfa-2 slightly inhibited Kiss1 activation (Figure 8B).

Neuroanatomical localization of *kiss1ra* and Lpxrfa

Because zebrafish Lpxrfa-2 and Lpxrfa-3 antagonized Kiss2 activation of Kiss1ra, we conducted simultaneous in situ hybridization for *kiss1ra* and immunohistochemistry for Lpxrfa in the brain to determine if the relationship between Lpxrfa and Kiss1ra can be determined neuroanatomically. The *kiss1ra*-expressing cells

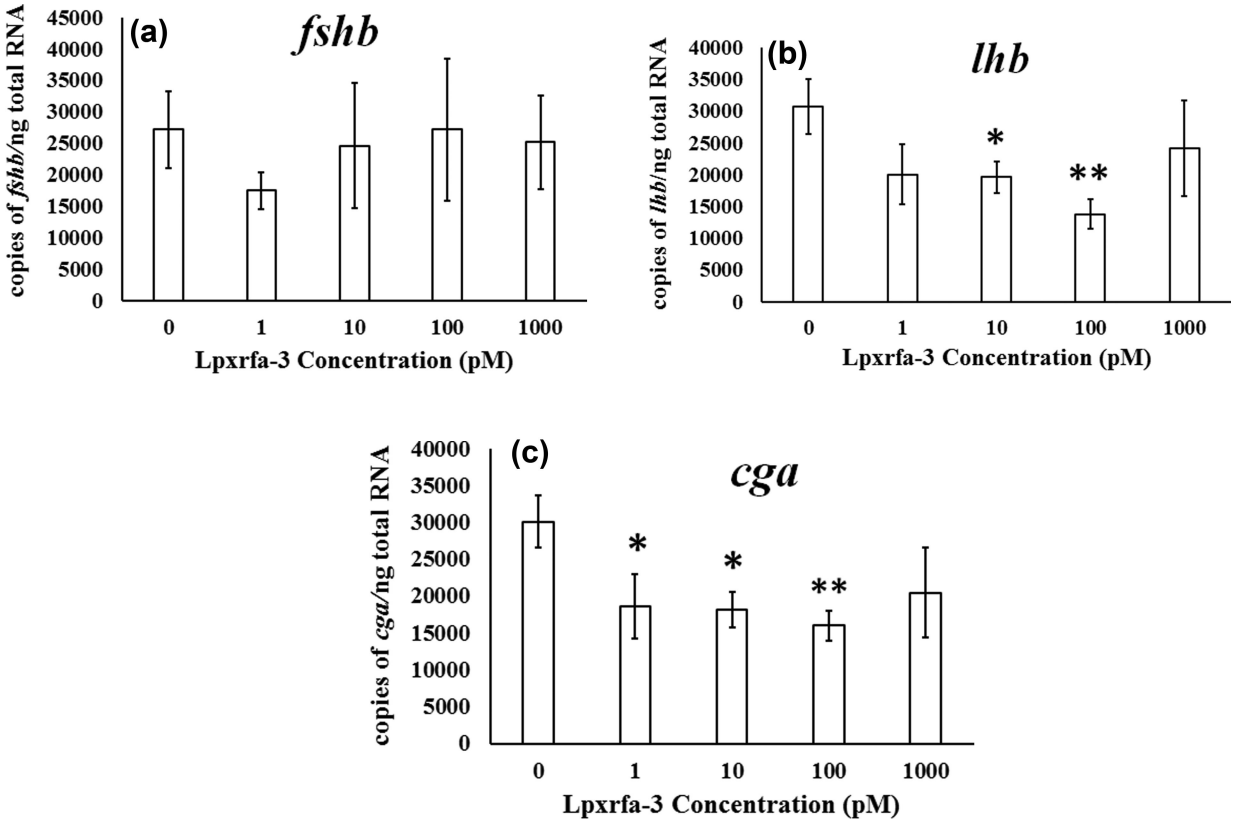

Figure 3. Zebrafish Lpxrfa-3 administration reduces *lhb and cga* mRNA levels in adult male zebrafish pituitaries in vitro. After 18 h of incubation with zebrafish Lpxrfa-3, whole pituitary explants from adult male zebrafish did not have altered *fshb* mRNA levels (A) but did exhibit reduced *lhb* levels (B) at 10 and 100 pM and *cga* levels (C) at 1, 10, and 100 pM in vitro. With two independent experiments, n = 6 per concentration with duplicates for QPCR. Absolute mRNA levels were normalized to *ef1a* levels and are presented as mean ± SEM. Differences between each Lpxrfa-3 concentration and the control (0 pM) were determined by a one-tailed Student *t*-test and are considered statistically significant when $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, and $^{***}P \leq 0.0005$.

were widespread throughout the zebrafish brain, as seen in the European sea bass [37] and striped bass [32]. In the preoptic area/hypothalamus, we found several *kiss1ra*-expressing cells that were innervated by Lpxrfa-ir fibers in slides treated with the antisense riboprobe and anti-zebrafish Lpxrfa (Figure 9A and D). All slides treated with the sense riboprobe revealed no *kiss1ra*-expressing cells (Figure 9C and F).

Discussion

We have characterized the Lpxrfa/Lpxrf-R system in terms of neuroanatomical distribution, functional effects, receptor activation, and utilization of additional receptor pathways in the brain–pituitary axis of the zebrafish. Our study demonstrates that Lpxrfa exerts inhibitory effects on the reproductive axis by acting on gonadotropes in the pituitary and by interacting with the hypophysiotropic Gnrh3 neurons in the brain. We also provide another route by which Lpxrfa may exert its effects via inhibiting the activation of Kiss1ra by Kiss2.

Neuroanatomical Distribution of Lpxrfa

Brain localization of Lpxrfa soma in this study revealed a single cluster in the ventral zone of the periventricular hypothalamus, similar to the case in goldfish [11]. Some fish species possess Lpxrfa soma in other brain regions, including the nucleus reticularis and octaval nucleus in the hindbrain for Indian major carp (*Labeo rohita*) [38] and the posterior ventricular nucleus of the caudal preoptic area, the nucleus posterioris periventricularis, and the nucleus olfacto-retinalis for cichlids [39, 40]. On the other hand, European sea bass Lpxrfa-2-ir soma are found in multiple brain regions: olfactory bulbs-terminal nerve, ventral telencephalon, caudal preoptic area, dorsal mesencephalic tegmentum, and rostral rhombencephalon [22]; however, there are no Lpxrfa-ir soma in the sea bass hypothalamus [22]. Therefore, the anatomical localization of Lpxrfa soma in the brain can be species specific, with perhaps more evolutionary advanced fish (perciforms) exhibiting more extensive soma distribution than less evolved fish species (cyprinids). There is also the possibility that sex and reproductive status affect the expression and anatomical localization of these neuropeptides in the brain [32, 41]. However, no major sex-specific differences in Lpxrfa-ir soma and fiber distribution were noted in this study.

In the brains of adult zebrafish, axons of Lpxrfa and Gnrh3 neurons mutually project and terminate in close contact of each other, particularly in the preoptic area, the major brain region in which hypophysiotropic Gnrh3 soma reside [33]. This finding was also reported in rats/mice [42], sheep [15], and starlings [6], and between Lpxrfa and Gnrh in the Indian major carp [38]. However, in another teleost, the tilapia, Lpxrfa does not associate with neither Gnrh3 nor hypophysiotropic Gnrh1 neurons in the brain [40]. Because tilapia exhibit a stimulatory effect of Lpxrfa on gonadotropins [19], it is possible that this species does not utilize an Lpxrfa-Gnrh

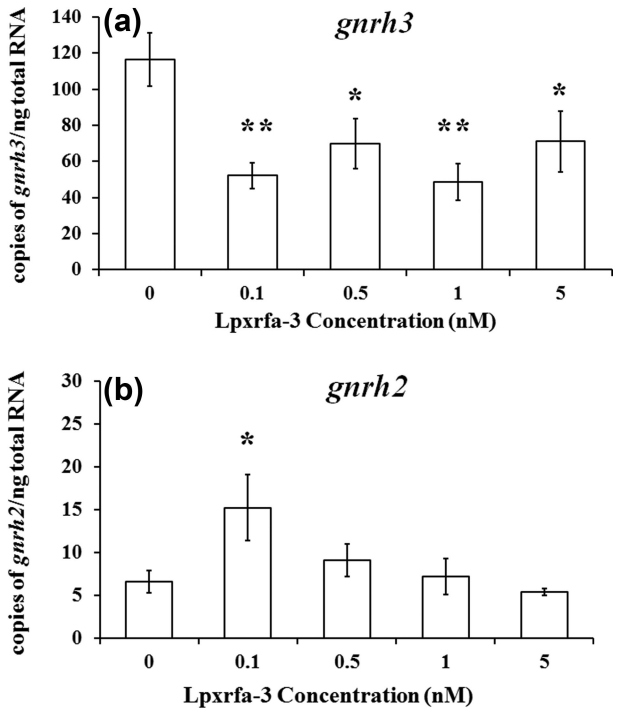

Figure 4. Zebrafish Lpxrfa-3 administration reduces *gnrh3* gene expression in the male adult zebrafish brain in vitro. After incubation with zebrafish Lpxrfa-3, brain slices from adult males had reduced *gnrh3* mRNA levels (A) at all of the concentrations tested in vitro. Zebrafish Lpxrfa-3 had a slight stimulatory effect on *gnrh2* at the lowest concentration tested (B). With two independent experiments, n = 6 per concentration with duplicates for QPCR. Absolute mRNA levels were normalized to *ef1a* levels and are presented as mean ± SEM. Differences between each treatment and the control (0 nM) were determined by a one-tailed Student *t*-test and are considered statistically significant when $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, and $^{***}P \leq 0.0005$.

relationship and that tilapia Lpxrfa may influence gonadotropin secretion solely by direct pituitary contact.

In addition to distribution in the brain, this study demonstrated that zebrafish Lpxrfa can be considered a "hypophysiotropic" neuropeptide due to the axons innervating the pituitary, as seen in almost all fish examined to date [11, 19, 21, 38]. Thus, it is not surprising that Lpxrfa directly regulates gonadotropin synthesis/release in many of these species. Because Lpxrfa-ir fibers did not reveal direct contact with gonadotropes but appeared in the neurohypophysis, it is possible that Lpxrfa peptides reach the adenohypophysis via blood vasculature, as was recently shown for Gnrh3 in zebrafish, instead of direct innervation of the gonadotrope cells [26]. In the European sea bass, on the other hand, Lpxrfa-ir fiber innervations in the pituitary are closely associated with Fsh- and Lh-producing cells [21]. Most importantly, the innervation of the zebrafish pituitary by Lpxrfa is apparently sufficient to induce an effect on gonadotropin expression, which probably requires low doses (pM) of Lpxrfa, as demonstrated by the inhibitory effects seen in the pituitary explant study.

Functional effects of Lpxrfa

In this study, zebrafish Lpxrfa-3 exhibited a direct inhibitory effect on *lhb* and *cga* mRNA levels at low physiological levels, as low as 10 pM, in the pituitary explant study. These results are supported by previous results [13], which demonstrated that goldfish serum Lh levels can be reduced by zebrafish Lpxrfa-3. An inhibitory effect of Lpxrfa on gonadotropin synthesis/release has been demonstrated in other fish species, including the male European sea bass [22], common carp (*Cyprinus carpio*) [43], and the cichlid *Cichlasoma dimerus* [39]. However, some species exhibit stimulatory effects, such as the tilapia [19], puffer [12], and goldfish [23], which is occasionally influenced by gonadal state [23]. Interestingly, there was consistently no effect of the highest concentration of Lpxrfa-3 tested on both *lhb* and *cga* expression. As is seen in other teleost in vitro culture systems [29], high concentrations of neuropeptides that are above typical physiological levels can have effects on gene expression that are different or even opposite from those effects seen at lower physiological levels.

Because the zebrafish used in the current study are held under laboratory conditions and are not exposed to seasonal changes of temperature and photoperiod, reproduction occurs on a continuously daily basis, in which the ovaries exhibit asynchronous oocyte development [44]. This monotonous state in the zebrafish probably manifests as only one effect of Lpxrfa on gonadotropin production, which was determined to be inhibitory in the current study. The observation that Lpxrfa exhibits stimulatory and/or inhibitory effects on gonadotropins in teleosts may be reflected by the suggestion that this class is evolutionary intermediate [45, 46], in which the role of Lpxrfa is a transitional stage from jawless fish (stimulatory) to birds and mammals (inhibitory). Alternatively, the dual roles of Lpxrfa peptides (and the potential multiple roles of their cognate receptors) arose separately during evolution in response to the vast array of reproductive strategies (e.g., daily vs seasonal spawning) found among teleost species.

In addition to affecting the expression of gonadotropins, zebrafish Lpxrfa-3 also consistently reduced *gnrh3* expression in the brain in vitro. Thus, it appears that zebrafish Lpxrfa has an inhibitory impact on the hypophysiotropic Gnrh neurons, which has been found in some teleosts, including the goldfish Lpxrfa-2 and Lpxrfa-3 peptides on *gnrh3* [24] and the sea bass Lpxrfa-1 peptide on *gnrh1* [22]. This result supports the reports in sheep that RFRP-3 reduces GNRH-stimulated gonadotropin release [15, 47]; thus, RFRP-3 moderates the stimulatory effect of GNRH on gonadotropin release. This finding is also in agreement with that of a study on mice that demonstrated that RFRP-3 application to brain slices reduces GNRH1 neuron firing rates [18]. Therefore, this negative impact of RFRP-3/Lpxrfa on hypophysiotropic Gnrh neurons seems to be conserved across vertebrate classes.

Lpxrf receptors

In the zebrafish, all three Lpxrfa peptides are capable of activating both Lpxrf-R2 and Lpxrf-R3 through the PKA/cAMP pathway. The lack of activation of Lpxrf-R1 could be partially explained by its high variation in the C-terminus sequence, compared with Lpxrf-R2 and Lpxrf-R3, leading to its potential inability to initiate the proper signaling cascade within the cell. Thus, Lpxrf-R1 is probably a nonfunctional receptor or is potentially utilizing a completely different signaling pathway. The utilization of the PKA/cAMP pathway has been seen in other teleost Lpxrfa systems, including tilapia [19] and orange-spotted grouper [20]. Both these species possess only one Lpxrf receptor, compared to the three of the cyprinids, the zebrafish [13] and goldfish [24]. Thus, it is possible that teleosts could have lost the other two Lpxrf receptors before the emergence of perciforms (e.g., tilapia and grouper) and birds/mammals, which possess only one receptor and also utilize both the PKA and PKC pathways [19, 20, 48, 49].

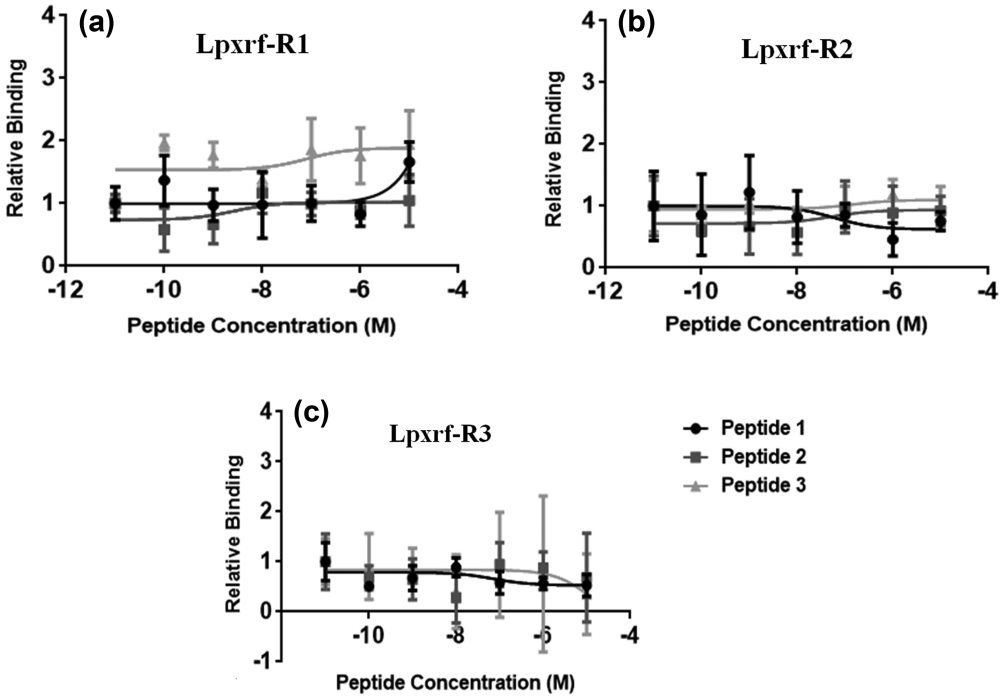

Figure 5. The activation of the PKC/Ca^{2+} pathway (SRE) in all three zebrafish Lpxrf receptors by all three zebrafish Lpxrfa peptides. The zebrafish Lpxrf-R1, Lpxrf-R2, and Lpxrf-R3 were exposed to either Lpxrfa-1 (circles; A), Lpxrfa-2 (squares; B), or Lpxrfa-3 (triangles; C), using a dual luciferase reporter assay. None of the peptides (at any concentration tested) activated any of the receptors through the PKC/Ca^{2+} pathway. With three independent experiments for the PKC/Ca^{2+} pathway, n = 3 in duplicate/concentration/receptor. Values are presented as mean ± SEM.

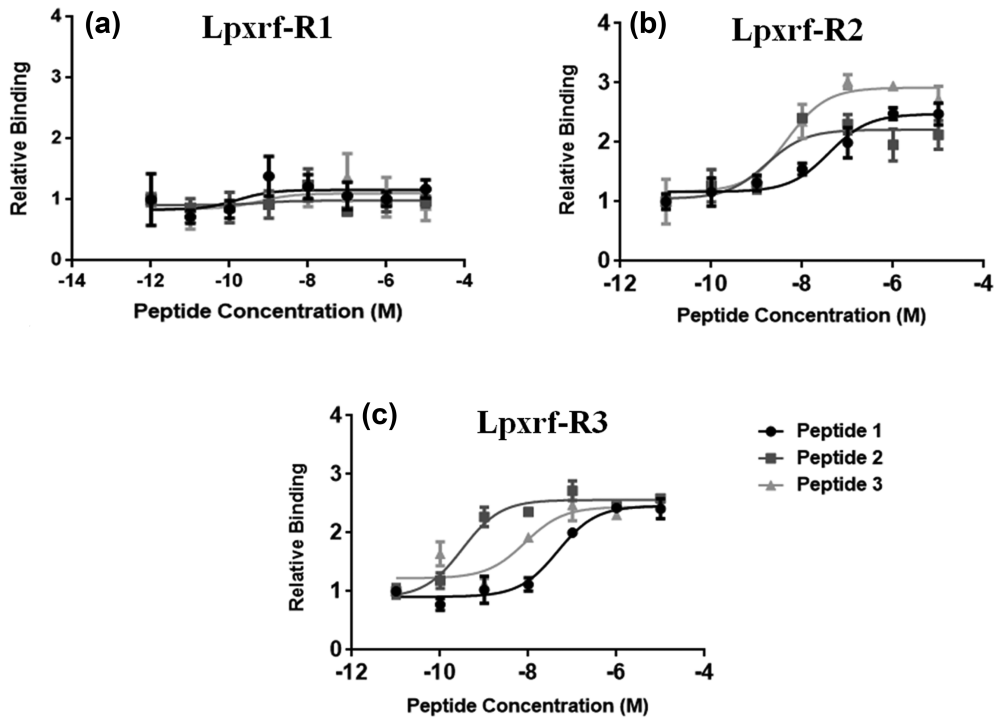

Figure 6. The activation of the PKA/cAMP pathway (CRE) in all three zebrafish Lpxrf receptors by all three zebrafish Lpxrfa peptides. The zebrafish Lpxrf-R1, Lpxrf-R2, and Lpxrf-R3 were exposed to either Lpxrfa-1 (circles; A), Lpxrfa-2 (squares; B), or Lpxrfa-3 (triangles; C), using a dual luciferase reporter assay. All of the peptides activated Lpxrf-R2 and Lpxrf-R3 through the PKA/cAMP pathway. The EC_{50}s of Lpxrf-R2 via CRE were 2.43×10^{-8} M ($\pm 2.12 \times 10^{-8}$ M), 4.64×10^{-9} M ($\pm 5.96 \times 10^{-10}$ M), and 1.88×10^{-9} M ($\pm 2.39 \times 10^{-9}$ M) for peptides Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3, respectively. The EC_{50}s of Lpxrf-R3 via CRE were 5.72×10^{-8} M ($\pm 3.78 \times 10^{-8}$ M), 6.91×10^{-10} M ($\pm 3.20 \times 10^{-10}$ M), and 4.75×10^{-9} M ($\pm 4.28 \times 10^{-9}$ M) for peptides Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3, respectively. However, Lpxrf-R1 was not activated by any of the peptides through this pathway. With three independent experiments for the PKA/cAMP pathway, n = 3 in duplicate/concentration/receptor. Values are presented as mean ± SEM.

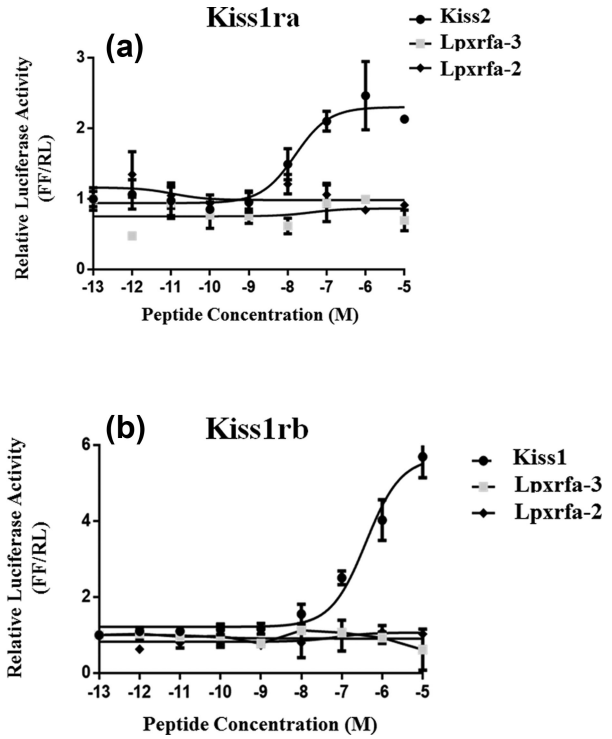

Figure 7. The activation of the PKC/Ca^{2+} pathway (SRE) in zebrafish Kiss1ra and Kiss1rb by Kiss1/Kiss2, Lpxrfa-2, and Lpxrfa-3. Lpxrfa-2 and Lpxrfa-3 did not activate either Kiss1ra (A) or Kiss1rb (B); however, Kiss2 and Kiss1 activated Kiss1ra and Kiss1rb, respectively, through the PKC/Ca^{2+} pathway. For this pathway, n = 3 in duplicate/concentration/receptor. Values are presented as mean ± standard deviation. Circles, Kiss1/2; squares, Lpxrfa-3; diamonds, Lpxrfa-2.

Kiss receptors

Our attempts to histologically detect zebrafish *lpxrfr2* and *lpxrfr3* in the brain/pituitary using in situ hybridization were unsuccessful. This was surprising because the cDNA of all three zebrafish Lpxrf receptors was detectable using end-point PCR and/or QPCR in this study and in a previous study [13]. Our trials included different stages of fish and different methods as follows. (1) We employed riboprobes that recognized the entire cDNA coding region of each receptor and riboprobes that recognized only the cDNA region of the C-terminus. (2) Using the TSA Plus Kit (that usually detects very low levels of mRNA), a signal amplification of the in situ hybridization signal was tested, alongside the nonamplified procedure. (3) We selected samples in which the mRNA levels determined via QPCR were highest (adult vs juvenile, male vs female, and morning vs evening). (4) A positive control using an antisense riboprobe against *lpxrfa* was employed at each run. However, none of these methods allowed the Lpxrf-R mRNA signals to be visualized histologically. Therefore, we assume that the brain mRNA expression of zebrafish Lpxrf receptors is extremely low and that the receptors might be very widespread, allowing histological detection of mRNA to be nearly impossible. It is noteworthy that being a daily spawner, there might be circadian changes in zebrafish Lpxrf-R expression that were not captured by our sampling methods. Other changes that are related to translational rates and affinities of the receptors may also occur. Therefore, further studies examining circadian receptor levels and affinities are needed. In addition, it is possible that the difficulty in histologically detecting these transcripts is due to their expression being dependent on the physiology of the organism. This phenomenon may be widespread in teleosts as Lpxrf-R mRNA localization in the brain has proven to be challenging [40].

Due to the inability to localize Lpxrf receptors in the zebrafish brain–pituitary axis, we consequently examined other possible pathways for Lpxrfa action. We focused on Kisspeptins that, like the Lpxrfa peptides, belong to the RFamide peptide family. Kisspeptin, known for its positive effect on reproduction, is a direct stimulator of GNRH expression/secretion [50], and Kiss receptors are widely distributed throughout the zebrafish brain [51]. While zebrafish Lpxrfa-2 and Lpxrfa-3 peptides do not directly activate zebrafish Kiss1ra or Kiss1rb, they do antagonize Kiss2 activation of Kiss1ra, the reproductively related Kiss receptor [51], at very low physiological levels (10 pM). This suggests that low levels of Lpxrfa peptides are needed to exert this antagonistic effect and that the inhibition of Kiss1ra is more sensitive (and perhaps more readily used) than Lpxrfa-2's and -3's activation of Lpxrf receptors. (The EC50s for Lpxrfa-2 activation of Lpxrf-R2 and Lpxrf-R3 are 4640 pM and 691 pM, respectively, and the EC50s for Lpxrfa-3 activation of Lpxrf-R2 and Lpxrf-R3 are 1880 pM and 4750 pM, respectively.) Because Lpxrfa-ir fibers also innervate *kiss1ra*-expressing cells, which are highly expressed and distributed in multiple, yet distinct, regions of the zebrafish brain [51], it is reasonable to assume that zebrafish Lpxrfa-2 and Lpxrfa-3 elicit their functions through Kiss2/Kiss1ra, at least partially. Thus, in addition to possibly acting through one of the three Lpxrf receptors, Lpxrfa may also utilize the Kisspeptin pathway by antagonizing Kiss2's effects on Kiss1ra to exert its inhibitory effects.

In conclusion, the zebrafish Lpxrfa/Lpxrf-R system has been characterized in terms of descriptive and functional assays. We have localized zebrafish Lpxrfa soma to the ventral zone of the periventricular hypothalamus of the brain with fibers extending throughout the brain and pituitary. Lpxrfa-3 is capable of reducing *lhb* and *cga* mRNA levels at low physiological concentrations in the pituitary in vitro. In addition, Lpxrfa projections to Gnrh3 soma in the preoptic area are in accordance with the findings that zebrafish Lpxrfa-3 downregulates *gnrh3* expression in the brain in vitro. Therefore, zebrafish Lpxrfa most likely exhibits an inhibitory effect on gonadotropins, using pathways to the pituitary and through Gnrh3 neurons. Additionally, receptor activation studies revealed that all three zebrafish Lpxrfa peptides activate Lpxrf-R2 and Lpxrf-R3 via the PKA/cAMP pathway. Finally, by demonstrating antagonistic activities of Lpxrfa-2 and Lpxrfa-3 on Kiss1ra and neuroanatomical relationships between Lpxrfa and *kiss1ra*, we offer an additional pathway, the Kiss2/Kiss1ra system, by which Lpxrfa may elicit its effects. In general, zebrafish Lpxrfa appears to be a functional reproductive inhibitory neuropeptide in the brain/pituitary, potentially utilizing pathways of other reproductive neuropeptides to exert its functions.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Figure S1. Anti-zebrafish Lpxrfa polyclonal antibody is specific to zebrafish Lpxrfa. The cDNA coding region of zebrafish *lpxrfa* (from 157 bp to 423 bp of GenBank #GU290218.1 encompassing the nucleotide sequence encoding for Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3 peptides) was cloned into the pET-15b vector and expressed in Rosetta-gami B(DE3)pLysS *E. coli* cells (Novagen) as N-terminal His-tagged recombinant proteins. The proteins were collected, purified, and used to develop a polyclonal antibody in rabbits. In order to verify the specificity of the zebrafish Lpxrfa antibody, we used two approaches: (1) tested recognition of zebrafish Lpxrfa

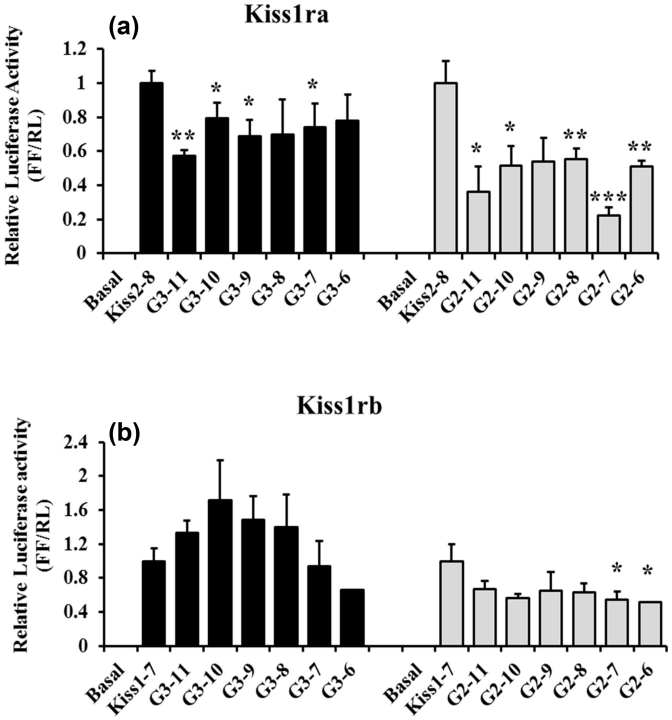

Figure 8. Inhibition of Kiss-stimulated activation of Kiss receptors by zebrafish Lpxrfa peptides in the PKC/Ca^{2+} (SRE) pathway. High concentrations of Lpxrfa-2 slightly inhibited Kiss1 activation of Kiss1rb (B), while most of the concentrations of Lpxrfa-2 inhibited Kiss2 activation of Kiss1ra ($IC_{50} = 5.915 \times 10^{-13}$ M; A). Lpxrfa-3 also inhibited Kiss2 activation of Kiss1ra (A) at many of the tested concentrations but had no effect on Kiss1 activation of Kiss1rb (B). For this pathway, n = 3 in duplicate/concentration/receptor. Values are presented as mean ± standard deviation. Differences between the effects of the control (activation by Kiss) and each treatment were determined by a one-tailed Student *t*-test and are considered statistically significant when $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, and $^{***}P \leq 0.0005$. G2 = Lpxrfa-2 (gray columns). G3 = Lpxrfa-3 (black columns). Numbers after the ligand name represent the molarity concentrations of each ligand. For instance, "G3-11" is Lpxrfa-3 at 10^{-11} M.

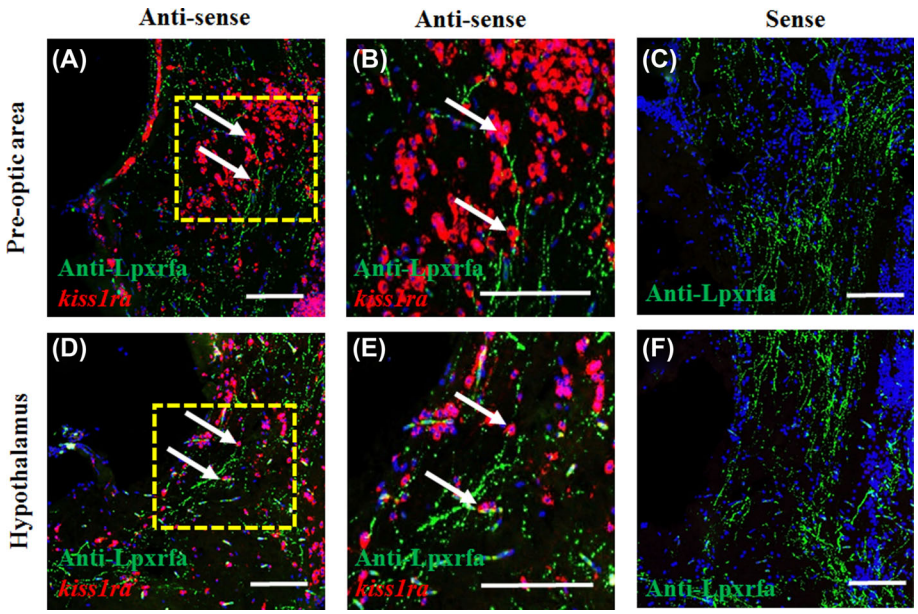

Figure 9. Zebrafish *kiss1ra*-expressing cells are innervated by Lpxrfa-ir fibers in the forebrain. The antisense *kiss1ra* riboprobe stained cells (red) in many regions of the brain of the adult zebrafish, especially the preoptic area (A) and the hypothalamus (D), and these cells were innervated (white arrows) by Lpxrfa-ir fibers (green). B and E are the magnified versions of the yellow squares in A and D, respectively. The sense riboprobe did not stain any cells (red) in any regions of the brain (C, F). Scale bars = 100 μm.

expressed in a COS7 cell line by Lpxrfa immunohistochemistry and (2) examined co-localization of *lpxrfa* using in situ hybridization and Lpxrfa using immunohistochemistry. (A) COS7 cells were transfected with either a control pcDNA3.1 plasmid (Aa, Ac) or an *lpxrfa*-pcDNA3.1 plasmid (Ab, Ad) and immunostained with either anti-zebrafish Lpxrfa (Aa, Ab) or pre-immune serum (Ac, Ad) from the rabbit used to make the polyclonal antibody. The cells that express zebrafish Lpxrfa and are immunostained with anti-zebrafish Lpxrfa (green) are indicated by white arrows, verifying the specificity of the anti-zebrafish Lpxrfa polyclonal antibody (Ab). (B) Simultaneous *lpxrfa* in situ hybridization and Lpxrfa immunohistochemistry on sections of adult brain slices. Co-localization (see white arrows) of *lpxrfa* mRNA (red; Ba) and Lpxrfa-ir soma (green; Bb) in the ventral zone of the periventricular hypothalamus (yellow; Bc) demonstrates the specificity of the anti-zebrafish Lpxrfa antibody. Scale bars = 100 μm.

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