APPROVAL SHEET

Title of Dissertation: MOLECULAR MECHANISMS INVOLVED IN LPXRFA AND GNRH3 REGULATION OF THE BRAIN-PITUITARY-GONAD AXIS OF THE ZEBRAFISH (*Danio rerio*)

Name of Candidate: Olivia Smith Spicer
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Dissertation and Abstract Approved: _____
Yonathan Zohar, Ph.D.
Professor and Chair
Department of Marine Biotechnology

Date Approved: _____

ABSTRACT

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Olivia Smith Spicer, Doctor of Philosophy, 2016

Directed By: Professor and Chair, Yonathan Zohar, Ph.D., Department of Marine Biotechnology

Vertebrate reproduction is regulated by the brain-pituitary-gonad axis, which translates internal/external cues into reproductive output. Gonadotropin-releasing hormone (GNRH) stimulates the production of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary that induce gonadal development. Recently, gonadotropin-inhibitory hormone (GNIH) was implicated in the inhibition of gonadotropin release via direct pituitary contact or GNRH neurons in birds and mammals. While teleost GNIH (Lpxrfa) exhibits stimulatory and/or inhibitory effects on gonadotropins, there is little information on the modes of action by which Lpxrfa exerts its functions. The goal of this study was to elucidate the mechanisms by which Lpxrfa and hypophysiotropic Gnrh3 coordinately regulate reproduction in the zebrafish, by neuroanatomical distribution, functional effects, and ligand-receptor relationships. In adults, Lpxrfa soma are localized to the ventral zone of the periventricular hypothalamus and project throughout the brain/pituitary. Pituitary explants incubated with zebrafish Lpxrfa-3 demonstrate that Lpxrfa acts directly on gonadotropes to downregulate *lhβ* and *cgα*. Receptor activation studies *in*

vitro demonstrated that Lpxrfa peptides activate only Lpxrf-R2 and -R3 via the PKA/cAMP pathway. Concurrently, zebrafish Lpxrfa-2 and -3 are capable of antagonizing Kiss2's activation of Kiss1ra at lower concentrations than those needed to activate Lpxrf-Rs, offering another, more sensitive pathway for Lpxrfa to elicit its functions. This study demonstrated that Lpxrfa also acts on Gnrh3 to exert its negative effect on reproduction. Lpxrfa-3 downregulates *gnrh3* expression in the brain *in vitro*, and Lpxrfa fibers interact with forebrain Gnrh3 soma. Although we could not establish an *lpxrfa*$^{-/-}$ line, we generated a *gnrh3*$^{-/-}$ line and determined how *lpxrfa* is affected by the loss of Gnrh3. While developmental expression profiles of *lpxrfa* indicate that Gnrh3 may upregulate *lpxrfa*, *lpxrfa* expression in *gnrh3*$^{-/-}$ adult female brains differs from that in wild-type only in the evening, suggesting that Lpxrfa and Gnrh3 participate in the female's reproductive cyclicity. The *gnrh3*$^{-/-}$ fish also exhibited no major reproductive phenotypes, having normal gametogenesis and reproduction, suggesting that a compensatory mechanism is being activated. In conclusion, Lpxrfa and Gnrh3 are central neuropeptides in the zebrafish that interact to coordinate and exert functions upon the gonadotropins to regulate the neuroendocrine control of reproduction.

MOLECULAR MECHANISMS INVOLVED IN LPXRFA AND GNRH3 REGULATION OF THE BRAIN-PITUITARY-GONAD AXIS OF THE ZEBRAFISH (*Danio rerio*)

By

Olivia Smith Spicer

Dissertation submitted to the Faculty of the Graduate School of the
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Advisory Committee:

Professor Yonathan Zohar, Ph.D., Chair
Associate Professor Rachel Brewster, Ph.D.
Associate Professor Shaojun "Jim" Du, Ph.D.
Associate Professor L. Curry Woods, Ph.D.
Assistant Professor Edward Orlando, Ph.D.

Dedication

This work is dedicated to the memory of my grandmother, Wilma Alpha, who passed during my graduate studies and who taught me that unseen acts are the most important.

Acknowledgements

First and foremost, I want to thank my mentor, Dr. Yonathan Zohar, for taking me into his lab and introducing me to a world of opportunity. My participation in his lab has been some of the best years of my life and has allowed me to participate in amazing biotechnology research in Baltimore and around the globe. Working under him has allowed me to widely expand my scientific skill set and work on subjects that I never dreamed I could work on, which has made my life all the richer. I also must thank the Institute of Marine and Environmental Technology and the Department of Marine Biotechnology at UMBC for supporting me as a graduate student. In addition, I want to express much gratitude to the Gudelsky Family Foundation for supporting my dissertation research.

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To my dear parents, Denise and Dan Smith, I carry you in my heart no matter where life takes me. Thank you for your boundless love and care throughout all of these years of school. None of this would have been possible without your confidence and love from day one, and I hope I can prove to you how forever grateful I will always be.

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Chapter 1: General Introduction

VERTEBRATE REPRODUCTION

In vertebrates, reproduction is regulated by the brain-pituitary-gonad (BPG) axis, which translates internal and external cues into endocrine signals and, ultimately, 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. Gonadotropin-releasing hormone (GNRH), the major regulator of neuroendocrine control of reproduction, stimulates the synthesis and release of two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the gonadotropes in the anterior pituitary. During the past decade, several more neuropeptides that function upstream of and alongside GNRH, such as kisspeptin (KISS), neurokinin B (NKB), and gonadotropin-inhibitory hormone (GNIH), have been discovered (for reviews, see Navarro et al. 2009, Tsutsui et al. 2010, Zohar et al. 2010). After their release from the pituitary, FSH and LH travel to the gonads via the bloodstream, where they initiate steroidogenesis, gametogenesis, final gamete maturation, and spawning. Gonadal hormones (e.g., steroids, activin, inhibin, etc.) are also secreted and feed back to the brain and pituitary, where they positively and/or negatively regulate the production of GNRH, additional neuropeptides, and gonadotropins.

BRAIN-PITUITARY AXIS

While the neuropeptides, neurohormones, and neurotransmitters that control reproduction and other endocrine processes are relatively conserved amongst

vertebrates, the structure of the brain-pituitary axis and the method in which neuropeptides access the pituitary vary across species. In many vertebrates (birds and mammals), neurons in the brain interact with their corresponding cell types (i.e., gonadotropes, somatotropes, thyrotropes, etc.) in the anterior pituitary via a hypophyseal portal blood system that carries the secreted neuropeptide from the ventral hypothalamus to the appropriate cells in the anterior pituitary gland (Zohar et al. 2010; Figure 1.1A). In the medio-basal hypothalamus, neuropeptides are released into the median eminence, and the adjacent primary plexus of capillaries transports the peptides through the portal system and directly into the anterior pituitary, while not entering the posterior pituitary gland (Zohar et al. 2010). In teleosts, on the other hand, the median eminence and hypophyseal portal blood system are absent, and instead, neurosecretory fibers in the hypothalamus directly innervate the appropriate cells in the anterior pituitary (Zohar et al. 2010; Figure 1.1B). These fibers transverse through the posterior pituitary gland before branching out into the different regions of the anterior pituitary gland. Recently, however, neurosecretory fibers in teleosts have been shown to also secrete neuropeptides into the vasculature in the pituitary and not only directly innervate the specialized pituitary cells (Golan et al. 2015). Additionally, differences in the brain-pituitary axis of teleosts and other vertebrates exist in that, in teleosts, pituitary cells of a specific type are often grouped together in similar regions of the pituitary, whereas the pituitary cells of other vertebrates are not arranged in specialized groups (Zohar et al. 2010). Therefore, the anatomy of the pituitary gland will affect the physical infrastructure and distribution of neuropeptides/neurohormones/neurotransmitters from the brain into the pituitary.

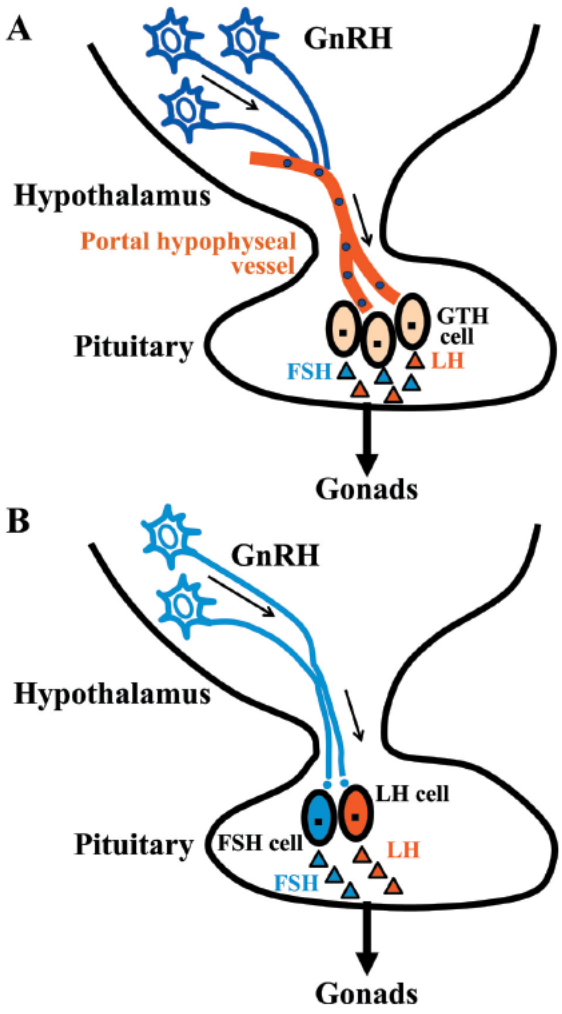

Figure 1.1. Anatomy of brain-pituitary axis in birds and mammals (A) and in teleosts (B; Amano 2010).

GNRH

Discovery of GNRH

The first major hypothalamic neuropeptide conclusively shown to control pituitary gonadotropin release was identified in the 1970s, based on its ability to induce the release of LH from porcine and ovine gonadotropes (Amoss et al. 1971,

Matsuo et al. 1971). Initially, this decapeptide (pEHWSYGLRPG-NH_2) was termed LH-releasing hormone (LHRH); however, after it was demonstrated to also induce the release of FSH from the pituitary, the name was changed to gonadotropin-releasing hormone. The GNRH decapeptide travels to the anterior pituitary via a hypophyseal portal blood system in birds and mammals and via a direct innervation of neurosecretory fibers or a neurosecretory fiber-vasculature network in teleosts (Zohar et al. 2010, Golan et al. 2015). In the anterior pituitary, GNRH activates its cognate receptors on gonadotropes and, in turn, the synthesis and release of gonadotropins FSH and LH, which has been repeatedly shown in multiple model systems during the past four decades (for review, see Zohar et al. 2010). Thus, downstream activity in the reproductive BPG axis is dependent on activation by the GNRH neuropeptide.

GNRH Multiplicity

Multiple isoforms of GNRH can exist within a single species; however, the functions of each specific isoform are not necessarily known. In mammals, there are two Gnrh isoforms: the hypophysiotropic mammalian GNRH (GNRH1) and the ubiquitous chicken GNRH-II (GNRH2), except for rodents, which lack GNRH2. Of the approximately 14 identified GNRH isoforms in vertebrates, nine are currently found only in fish, while two (mammalian GNRH and chicken GNRH-II) are present in fish and other vertebrates. Therefore, fish represent the most diverse group of Gnrh isoforms, and fish are also the only vertebrates to possess three Gnrh isoforms in some species. In many teleosts (e.g., perciforms), there are three types of Gnrhs: the hypophysiotropic seabream/pejerrey Gnrh (Gnrh1), the chicken Gnrh-II (Gnrh2),

and salmon Gnrh (Gnrh3; Powell et al. 1994), while in other fish (e.g., cyprinids and salmonids), only two types exist: Gnrh2 and Gnrh3 (Steven et al. 2003). Originally, GnRH isoforms were originally named for the organism in which they were discovered (salmon, seabream, chicken, guinea pig, etc.), even though they can be found in multiple species of different vertebrate classes. However, the current nomenclature is determined by phylogenetic analysis of known sequences and location of expression (Fernald and White 1999). The GNRH1 branch consists of the hypophysiotropic variants located in the pre-optic area/hypothalamus of fish, amphibians, and mammals. The GNRH2 branch consists of the ubiquitous variant that is expressed in the midbrain of almost all vertebrates from fish to mammals (except rodents), and the Gnrh3 branch represents the fish (salmon Gnrh) variants that are located in the terminal nerve/ventral telencephalon. In fish with only two Gnrh isoforms (e.g., cyprinids), Gnrh3 is located in the terminal nerve/ventral telencephalon and in the pre-optic area/hypothalamus and is believed to assume the non-redundant roles of Gnrh1 (Okubo and Aida 2001).

GNRH Structures

All GNRH precursors are translated into one peptide, which includes the following components: a signal peptide, the GNRH decapeptide, a processing tripeptide (Gly-Lys-Arg), and a GNRH-associated peptide (GAP), and the GNRH decapeptide of each isoform is conserved across multiple vertebrate classes (Zohar et al. 2010). The GNRH decapeptide is most variable at the 5, 7, and 8 amino acid positions (Lethimonier et al. 2004), determining the specific isoform of the decapeptide. After the signal peptide is cleaved, the remaining GNRH precursor

peptide is transported to the Golgi apparatus, where prohormone convertases and exopeptidases expose the GNRH decapeptide. Further post-translational modifications to the decapeptide include amidation of the glycine at the C-terminus and conversion of the glutamate at the N-terminus into pyroglutamate. The decapeptide and the GAP are packed into neurosecretory granules and transported to the axon terminals for release (Andersen et al. 1988, Rangaraju et al. 1991). Finally, the mature GNRH decapeptide travels to the anterior pituitary to activate its cognate receptors on the gonadotropes. Of the different regions of the GNRH precursor peptide, the most divergent region within and among species is the GAP. For instance, in the European sea bass (*Dicentrarchus labrax*), Gap nucleotide sequences from Gnrh1, Gnrh2, and Gnrh3 precursor peptides do not exhibit more than 42% homology (Zmora et al. 2002). However, the functions of the GAP, unlike the GNRH decapeptide, are largely unknown.

GNRH Functions

As mentioned previously, the neuroanatomical localization and, therefore, functions of GNRH neurons determine their classification as GNRH1-, GNRH2-, or GNRH3-type neurons. While the functions of the ubiquitous GNRH2 and of GNRH3 (in fishes with three Gnrhs) are mostly unknown, the functions of hypophysiotropic GNRH1 (or Gnrh3 in some teleosts) are widely researched in a variety of species. Since its discovery over four decades ago, GNRH has consistently and reliably demonstrated to be capable of stimulating gonadotropin (FSH and LH) production from the anterior pituitary in multiple vertebrate species, allowing it to be considered the master regulator of reproduction. However, relatively recent research has

demonstrated that additional neuropeptides upstream of GNRH can influence its production and that these neuropeptides can sometimes relay environmental cues (e.g., photoperiod) to GNRH and the reproductive axis. Due to the activation from one of these upstream neuropeptides, the kisspeptin system (described below), GNRH is also responsible for initiating the activation of the reproductive axis during puberty (Clarkson et al. 2010). Additionally, in mammals, GNRH is released in coordinated and simultaneous pulsatile secretions from multiple axon terminals in the median eminence, which is essential for the regulation of the estrous cycle (Hotchkiss and Knobil 1994).

The importance of GNRH functions in reproduction is evident in that GNRH loss-of-function studies demonstrate major reproductive phenotypes that often include hypogonadotropic hypogonadism and sterility (Cattanach et al. 1977, Chan et al. 2009). In 1977, Cattanach et al. (1977) reported the discovery of a natural *Gnrh1* mutant mouse (*hpg*), in which individuals possess a 33.5 kb deletion that includes the latter half of the *Gnrh1* gene (Mason et al. 1986). These *hpg* mice exhibit reduced pituitary content and circulating levels of FSH and LH and display hypogonadotropic hypogonadism, in which all individuals are sterile (Cattanach et al. 1977). In addition, some humans with hypogonadotropic hypogonadism are characterized by a failure to undergo puberty and have been described to possess one of approximately six different forms of mutations in *GNRH1* (Chan et al. 2009), in which one of the mutational "hot spots" tends to be in the region encoding the decapeptide (Mengen et al. 2015). Other humans with hypogonadotropic hypogonadism are often characterized as having Kallmann syndrome, in which a cessation of GNRH1 soma

migration (due to a mutation in the *KAL1* gene) during development and subsequent hypogonadism are observed (Soussi-Yanicostas et al. 1998), thereby demonstrating the importance of the migration and early development of GNRH neurons (Abraham et al. 2008).

In addition to mammals, Gnrh has been demonstrated to be essential to the ontogeny of its neurons and to reproduction in teleosts, particularly the zebrafish (*Danio rerio*). The knockdown of hypophysiotropic *gnrh3* by anti-sense morpholino (MO) oligonucleotides resulted in misguided migration of Gnrh3 soma during early zebrafish development, suggesting that Gnrh3 itself is needed for proper migration of its neurons (Abraham et al. 2008). In addition, successful laser ablation of Gnrh3-expressing cells in the olfactory region during early zebrafish development (4-6 days post-fertilization; dpf) has led to the production of all females, in which oocyte development is arrested at stage II (cortical alveolus/pre-vitellogenic follicles), and sterility (Abraham et al. 2010). Therefore, Gnrh3 neurons originating in the olfactory region during early development are most likely essential to full gamete maturation and spawning in zebrafish, at least in females.

GNRH Receptors

In order for GNRHs to elicit their effects on the pituitary, multiple isoforms of GNRH receptors are utilized. GNRH receptors are G protein-coupled receptors (GPCRs) that utilize the PKA/cAMP and PKC/Ca^{2+} pathways to exert their functions (Shimizu and Bédécarrats 2010). In the pituitary, GNRH receptors have been found on gonadotropes, somatotropes, and lactotropes, and in the striped bass (*Morone saxatillis*), Gnrh2 is the most potent activator of the pituitary Gnrh receptor, more so

than Gnrh3 and the hypophysiotropic Gnrh1 (Alok et al. 2000). The expression of the GNRH receptor is highly dependent on steroid feedback, especially in mammals, as estradiol can increase the release of GNRH and, in turn, GNRH receptor expression and/or as estradiol can directly affect GNRH receptor expression in the pituitary (Klenke 2006). In addition, teleost research has indicated that Gnrh itself is a positive regulator of its own receptors, as observed *in vitro* and *in vivo* in the tilapia (*Oreochromis niloticus*), while dopamine downregulates the Gnrh receptors (Okuzawa et al. 2002, Levavi-Sivan et al. 2004).

NON-GNRH FACTORS THAT REGULATE REPRODUCTION IN THE BRAIN

In addition to GNRH, other neuropeptides and neurotransmitters (e.g., KISS, NKB, dopamine, etc.) have been identified that influence GNRH and/or gonadotropin production. The recent discovery of the kisspeptins and their cognate receptors (GPCR 54; GPR54) has revolutionized our understanding of the neuroendocrine regulation of reproduction (de Roux et al. 2003, Seminara et al. 2003). Dubbed the "gatekeeper of puberty and reproduction," kisspeptin controls the onset of puberty through GNRH regulation (Han et al. 2005) and relays environmental and metabolic signals to the BPG axis in mammals (Tena-Sempere 2006, Greives et al. 2007). Neurokinin B is another important neuropeptide that stimulates GNRH and gonadotropin release and can also be co-localized with kisspeptin neurons (Ramaswamy et al. 2010, Biran et al. 2012). The neurokinin B system's regulation of GNRH and gonadotropins is evident in that mice and humans with mutated neurokinin B receptors are infertile (Young et al. 2010, Yang et al. 2011). In addition

to neuropeptides, catecholamine neurotransmitters, such as dopamine, are capable of affecting GNRH in the brain and gonadotropins in the pituitary. In teleosts, in which the research on dopamine's effects on reproduction is quite extensive, dopamine inhibits the production of both Gnrh and gonadotropins (Chang and Peter 1983, Yu et al. 1991). However, this effect is not observed in some marine teleosts (Copeland and Thomas 1989).

RFAMIDE PEPTIDES

Some of the non-GNRH neuropeptides that regulate reproduction in the brain belong to the RFamide peptide family, whose members are classified by the presence of an Arg-Phe-NH_2 motif at the C-terminus and participate in the endocrine control of reproduction, as well as many other physiological processes. The first RFamide peptide to be discovered (Phe-Met-Arg-Phe-NH_2, FMRFamide peptide) was isolated from the ganglia of the venus clam (*Macrocallista nimbosa*) and acts as a cardioexcitatory neuropeptide (Price and Greenberg 1977). Six years later, the first vertebrate RFamide peptide (Leu-Pro-Leu-Arg-Phe-NH_2, LPLRFamide) was identified in the chicken (Dockray et al. 1983). However, it was later determined that this peptide was most likely a degraded fragment of the GNIH dodecapeptide that was later identified in Japanese quail (*Coturnix japonica*; Tsutsui et al. 2000). Besides reproductive function, RFamide peptides play functional roles within a variety of physiological processes, such as feeding and muscle excitatory actions (McFarlane et al. 1987, Tachibana et al. 2005, Pineda et al. 2010b).

The RFamide peptide family can be divided into five groups: PQRFamide peptide group (e.g., neuropeptide FF), LPXRFamide (Lpxrfa) peptide (X is L or Q)

group (e.g., GNIH), prolactin-releasing peptide (PrRP) group (e.g., PrRP31), kisspeptin group (e.g., KISS1), and 26RFamide/pyroglutamylated RFamide peptide group (e.g., QRFP; Ukena and Tsutsui 2005, Osugi et al. 2011). Many of these peptides are found throughout the central nervous system and have important roles in influencing the release of a wide range of hormones from a wide range of tissues, especially the anterior pituitary (Matsumoto et al. 1999). Before 2000, all of the previously discovered neuropeptides that regulate gonadotropin release from the anterior pituitary have had a stimulatory effect on the synthesis/release of gonadotropins. However, a particular RFamide peptide discovery, that of GNIH, has significantly changed our understanding of hypophysiotropic neurohormones and their cascade effects on gonadotropin production and the entire BPG axis.

GNIH

Discovery of GNIH

In 2000, Tsutsui et al. (2000) discovered a novel hypothalamic neuropeptide in the brain of Japanese quail. Using a combination of high-performance liquid chromatography and mass spectrophotometry on acetic extracts of quail brains, the dodecapeptide (SIKPSAYLPLRF-NH_2) was identified as a member of the RFamide peptide family (Tsutsui et al. 2000). Cell bodies expressing the peptide were immunohistochemically localized to the paraventricular nucleus of the hypothalamus, while immunoreactive (-ir) cell fibers were found in the median eminence leading to the pituitary (Tsutsui et al. 2000). Using cultured quail anterior pituitaries, the dodecapeptide was shown to inhibit the release of LH, but not FSH or prolactin, in a dose-dependent manner (Tsutsui et al. 2000). Thus, this novel peptide was termed

gonadotropin-inhibitory hormone and became the first hypothalamic neuropeptide identified in vertebrates to inhibit LH release.

The GNIH precursor peptide encodes 173 amino acids, which consists of GNIH and two related peptides (GNIH-RP-1 and GNIH-RP-2), each containing the LPXRF-NH_2 (X is L or Q) motif at the C-terminus (Satake et al. 2001). Each peptide is followed by a glycine amidation signal at the C-terminus and is flanked by a single basic amino acid (K or R) at both termini, signaling endoproteolytic sites (Satake et al. 2001). GNIH and GNIH-RP-2 are both mature peptides expressed within the diencephalon (Satake et al. 2001). Later studies revealed the presence of GNIH and GNIH-RPs in other avian species, demonstrating that the GNIH precursor peptide conserves similar structures across many avian species (Osugi et al. 2004, Ubuka et al. 2008). The peptide's cognate receptor was also identified in Japanese quail (Yin et al. 2005). This GPCR (GPR147) binds GNIH and GNIH-RPs in a concentration-dependent manner and is expressed in the pituitary and multiple brains regions (Yin et al. 2005).

GNIH Orthologs

Mammals—After the discovery of GNIH in multiple avian species, research began to uncover GNIH orthologs in mammals, "RFamide-related peptides" (RFRPs), and in teleosts, "Lpxrfa peptides." RFRP precursors typically encode two peptides: RFRP-1 and RFRP-3 with both peptides containing the LPXRF-NH_2 motif at the C-terminus. Based on existing literature for mammalian RFRPs, it appears that RFRP-3 is a functional ortholog of avian GNIH (Bentley et al. 2010). After the identification of the human GNIH orthologs, nucleotide sequence analysis of *RFRP2*

revealed an RSamide peptide instead of an RFamide peptide (Ubuka et al. 2009a), and failed attempts to isolate the RFRP-2 peptide uncovered that the alleged C-terminus of RFRP-2 was actually a part of the N-terminus of RFRP-3 (Bentley et al. 2010). Therefore, many mammals do not have an RFRP-2 peptide. In addition, RFRP receptors (GPR147) have been identified in many mammals, including humans (Ubuka et al. 2009a) and sheep (Dardente et al. 2008).

Teleosts—The first teleost Lpxrfa peptide identified was that of the goldfish (*Carassius auratus*), which encodes for three peptides, and goldfish Lpxrfa-3 peptide was determined to be a mature peptide (Sawada et al. 2002). Qi et al. (2013) identified the three receptors (GPCRs) of goldfish Lpxrfa. Similar to the goldfish, the zebrafish's Lpxrfa peptide precursor encodes three Lpxrfa peptides, and three receptors have also been identified in this species (Zhang et al. 2010). In the tilapia, the Lpxrfa peptide precursor encodes three Lpxrfa peptides, which utilize a single Lpxrfa receptor (Biran et al. 2014), while the European sea bass's Lpxrfa precursor uniquely encodes for two Lpxrfa peptides (Paullada-Salmerón et al. 2016a). Additionally, Osugi et al. (2011, 2012) identified GNIH orthologs in the agnathans, the brown hagfish (*Paramyxine atami*) and lamprey, the most ancestral vertebrate class to date identified to possess GNIH orthologs.

GNIH/RFRP/Lpxrfa Neuroanatomical Distribution

Birds—Since GNIH's discovery, several studies have demonstrated the distribution of GNIH neurons in regions of the brain associated with reproduction. As shown in quail (Tsutsui et al. 2000), GNIH-ir cells are located within the paraventricular nucleus in many avian species (Bentley et al. 2003, Osugi et al. 2004,

Tobari et al. 2010). GNIH-ir fibers project to several diencephalic and mesencephalic regions of the avian brain and to the median eminence of the hypophyseal portal system (Bentley et al. 2003, Ukena et al. 2003, Ubuka et al. 2008, Tobari et al. 2010). *GNIH* precursor mRNA, on the other hand, is expressed solely within the paraventricular nucleus of the quail brain (Satake et al. 2001, Ukena et al. 2003), confirming the synthesis of *GNIH* within this region, and also in the dorsomedial nucleus of the hypothalamus in the zebra finch (*Taeniopygia guttata*; Tobari et al. 2010).

Mammals—RFRP distribution in the mammalian brain has also been undertaken to determine if the areas that contain RFRP cell bodies and fibers are similar to that of GNIH in birds. RFRP cell bodies are found within the dorsal medial hypothalamus of hamsters (Kriegsfeld et al. 2006) and rats (Gibson et al. 2008), while they are found within this region, the lateral superior olive, and the nucleus of the solitary tract in mice (Ukena and Tsutsui 2001). In the rodent brain, RFRP-ir fibers project to multiple brain regions but, in particular, to the median eminence (Ukena and Tsutsui 2001, Gibson et al. 2008). In sheep, RFRP-3 cell bodies are localized to the dorsal medial hypothalamus and the paraventricular hypothalamus with RFRP-3-ir fibers projecting to several regions, including the median eminence (Clark et al. 2008, Dardente et al. 2008), while *GPR147* expression is detected in multiple brains regions of sheep (Dardente et al. 2008), indicating potential multiple functions for RFRP-3 in the ovine brain. In non-human primates, *RFRP* mRNA and RFRP-ir cell bodies are located in the intermediate periventricular nucleus of the hypothalamus with RFRP-ir fibers exhibiting a wide distribution, including the median eminence

(Ubuka et al. 2009a, 2009b), while *GPR147* mRNA in humans has also been localized to the hypothalamus and gonadotropes (Ubuka et al. 2009a). Therefore, RFRP's neuroanatomical distribution of the brain seems to be conserved across several mammalian species.

Teleosts—Unlike mammals, the neuroanatomical localization of Lpxrfa peptides has been determined in only a few piscine species. Nonetheless, in teleosts, Lpxrfa gene and protein expression follow a similar pattern to that of birds and mammals: *lpxrfa* mRNA expression and Lpxrfa-ir cell bodies are found specifically within the periventricular nucleus of the hypothalamus in the goldfish, and Lpxrfa-ir fibers project throughout the brain and pituitary (Sawada et al. 2002). Similarly, in the tilapia, Lpxrfa-ir soma are localized to the posterior ventricular nucleus of the caudal preoptic area (Ogawa et al. 2016). Interestingly, both the grass puffer (*Takifugu niphobles*) and tilapia exhibit *lpxrfa* expression in the pituitary, unlike the zebrafish (Zhang et al. 2010), indicating a potential local role for Lpxrfa in the puffer and tilapia pituitary. In the European sea bass, the available neuroanatomical localization is much more descriptive and extensive as Lpxrfa-ir soma are found in the olfactory bulbs/terminal nerve, ventral telencephalon, caudal preoptic area, dorsal mesencephalic tegmentum, and rostral rhombencephalon with Lpxrfa-ir fiber innervation including the majority of the brain but especially in the preoptic area, hypothalamus, and optic tectum (Paullada-Salmerón et al. 2016a). The sea bass pituitary is also innervated by Lpxrfa-ir fibers that most likely originate from the telencephalon and preoptic area Lpxrfa-ir fibers, and these pituitary innervations are

closely associated with gonadotropes and somatotropes (Paullada-Salmerón et al. 2016a).

GNIH/RFRP/Lpxrfa Functions

Birds—After Tsutsui et al. (2000) demonstrated the role of GNIH in reducing LH release from the anterior pituitary, research began focusing on the specific functions of GNIH within the BPG axis and how it affects gonadotropin release (Figure 1.2). Ubuka et al. (2006) demonstrated that GNIH reduces LH release in quail pituitaries *in vivo* and also its synthesis by analysis of *LHβ* mRNA expression. The effect of GNIH is not limited to LH as GNIH can also reduce expression of the common gonadotropin α subunit (*CGA*) and the *FSHβ* subunit, as seen in the chicken (Ciconne et al. 2004). GNIH has also been shown to reduce GNRH-induced increases in gonadotropin levels in birds *in vivo* (Osugi et al. 2004), indicating that GNIH may exert its functions through GNRH.

Mammals—In mammals, the functions of RFRPs were found to be similar to that of birds, with RFRP having an inhibitory effect on gonadotropin synthesis and release (Figure 1.2). In the hamster, intracerebroventricular and peripheral administration of RFRP rapidly inhibited LH secretion (Kriegsfeld et al. 2006). Pineda et al. (2010b) found that intracerebroventricular administration of RFRP-3 to gonadectomized female rats resulted in decreased levels of LH secretion, and intravenous administration of RFRP-3 to gonadectomized male rats resulted in decreased circulating levels of both FSH and LH. Additionally, RFRP-3 inhibited basal and GNRH-stimulated LH levels from the pituitaries of gonadectomized male rats *in vitro*, and this effect was blocked by the addition of RF9, an RFRP receptor

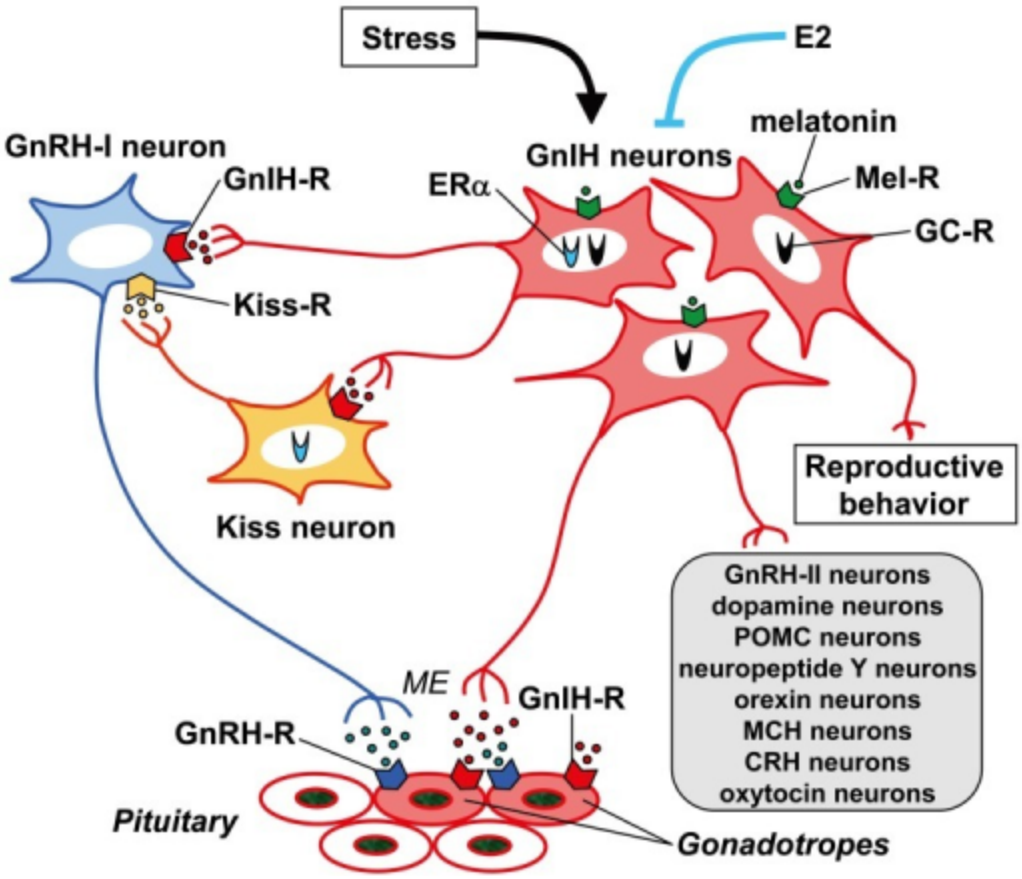

Figure 1.2. Neuroanatomical and functional interactions between GNIH (or its orthologs), GNRH, the pituitary, and other neuropeptides within the vertebrate brain (Ubuka et al. 2012b).

antagonist (Pineda et al. 2010b). In sheep, RFRP-3 has also been demonstrated to inhibit GNRH-stimulated mRNA expression of *FSHβ* and *LHβ in vitro* (Clarke et al. 2008)

Teleosts—Although GNIH and RFRP appear to act as strong inhibitors of gonadotropin release in birds and mammals, respectively, the functions of Lpxrfa peptides in fish are more variable with some species exhibiting stimulatory effects, which can depend on factors such as reproductive stage. The first study on Lpxrfa

function in fish determined that the administration of all three goldfish Lpxrfa peptides to cultured sockeye salmon (*Oncorhynchus nerka*) pituitaries resulted in increased levels of Fsh and Lh release (Amano et al. 2006). The grass puffer demonstrated similar results with the administration of puffer Lpxrfa causing increased mRNA expression levels of *fshβ* and *lhβ* but not *cgα in vitro* (Shahjahan et al. 2011). However, the first *in vivo* study on fish Lpxrfa function demonstrated that, in goldfish, the administration of zebrafish Lpxrfa-3 resulted in reduced serum levels of Lh (Zhang et al. 2010). However, the effects of goldfish Lpxrfa on mRNA levels of pituitary gonadotropins in the goldfish seem to be both stimulatory and inhibitory, depending on the seasonal reproductive state of the organism (Moussavi et al. 2012).

Recently, the results in teleosts have expanded to include more species and more variability in Lpxrfa's effects on the pituitaries. In the tilapia, increased levels of Fsh and Lh, but not growth hormone (Gh), were observed *in vivo* after administration of tilapia Lpxrfa-2 (Biran et al. 2014). However, in the European sea bass, decreased mRNA levels of *fshβ* and *lhβ* were reported with *in vivo* administration of sea bass Lpxrfa-2 (Paullada-Salmerón et al. 2016b), while grouper Lpxrfa-2 inhibited mRNA levels of *lhβ in vivo* in the orange-spotted grouper (*Epinephelus coioides*; Wang et al. 2015). In addition, the common carp (*Cyprinus carpio*) also revealed an inhibitory effect of carp Lpxrfa-3 on the expression of pituitary gonadotropins (Peng et al. 2016).

While the results in teleosts have indicated that Lpxrfa can have stimulatory and/or inhibitory roles on influencing gonadotropin synthesis and release, research in agnathans has pointed more towards an exclusively stimulatory role. In the brown

hagfish, Lpxrfa increased the mRNA expression levels of the gonadotropin β subunit in cultured pituitary cells (Osugi et al. 2011), while in the lamprey, a high concentration of Lpxrfa-2 stimulated the gene expression of the gonadotropin β subunit in the pituitary and increased Gnrh3 protein concentration in the hypothalamus *in vivo* (Osugi et al. 2012). Therefore, it has been proposed that Lpxrfa stimulates gonadotropin release in ancient fishes (Agnatha), while GNIH/RFRP inhibits gonadotropin release in later vertebrates (Aves and Mammalia). Within teleosts, however, Lpxrfa appears to have an intermediate role by displaying stimulatory and/or inhibitory effects on gonadotropins, sometimes depending on factors, such as the sex or reproductive state of the organism (Osugi et al. 2012, Tsutsui et al. 2012).

Relationships between GNIH/RFRP/Lpxrfa and GNRH

Structural—Because of its location within the hypothalamus and because of its impact on gonadotropin regulation, researchers were interested to determine if GNIH interacted anatomically and functionally with other hypophysiotropic factors within the brain, including the major regulator of vertebrate reproduction: GNRH. A possible GNIH/GNRH interaction could offer another pathway by which GNIH could elicit its effects on the pituitary (Figure 1.2). In an initial study, GNIH-ir fibers were demonstrated to be in close proximity to GNRH1 neurons and fibers in the pre-optic area and to GNRH2 neurons in the midbrain of songbirds (Bentley et al. 2003). Expanding on this research, Ubuka et al. (2008) discovered that GNIH-ir axon terminals in the European starling (*Sturnus vulgaris*) interact directly with and project to GNRH1 and GNRH2 neurons in the pre-optic area and midbrain, which also

express *GPR147* mRNA. This information indicates that GNIH potentially mediates its effects via direct interaction with the pituitary and/or with GNRH neurons in the avian brain. Additionally, the interaction of GNIH-ir fibers and GNRH1-ir fibers at the median eminence in birds implies the potential of GNIH affecting the release of GNRH1 from axons within the avian median eminence (Bentley et al. 2003).

Since these discoveries, other research projects have demonstrated similar results in mammals and fish (Figure 1.2). In mice, RFRP-ir fibers make contact with GNRH1 neurons in the preoptic area, and in the hamster, more than 40% of GNRH1 neurons receive projections from RFRP-ir fibers (Kriegsfeld et al. 2006). In the rhesus macaque (*Macaca mulatta*), RFRP-3-ir fibers make putative contacts with GNRH1 neurons in the pre-optic area (Ubuka et al. 2009b). In sheep, RFRP-3 neurons make contact with GNRH neurons, and these contacts are significantly reduced during the breeding season (Smith et al. 2008). Importantly, RFRP-3 has been extracted from the hypophyseal portal system of sheep, indicating that it is, indeed, a hypophysiotropic hormone in mammals (Smith et al. 2012). In teleosts, few studies have determined the physical relationships between Lpxrfa and Gnrh neurons. Structural information between these two neuropeptides in teleosts is mostly limited to neuroanatomical studies that demonstrate the signals of such proteins in similar regions of the brain, such as in the Indian major carp (*Labeo rohita*; Lpxrfa and Gnrh; Biswas et al. 2015) and in the lamprey (Lpxrfa and Gnrh3; Osugi et al. 2012). However, in the tilapia, Lpxrfa-ir soma are surprisingly not closely associated nor in direct contact with hypophysiotropic Gnrh1 or Gnrh3 neurons (Ogawa et al. 2016).

Functional—Because GNIH/RFRP/Lpxrfa has been demonstrated to interact with GNRH neurons and to influence GNRH-stimulated gonadotropin release (Clarke et al. 2008, Sari et al. 2009), research has uncovered the direct impacts of GNIH/RFRP/Lpxrfa administration on GNRH neurons (Figure 1.2). Anderson et al. (2009) found that chronic infusion of RFRP-3 resulted in 50 to 60 percent inhibition of GNRH neuron activation during the pre-ovulatory surge in LH in rats; however, a bolus injection of RFRP-3 did not influence the pulsatile frequency, pulsatile amplitude, or mean concentration of LH (Anderson et al. 2009). Similarly, Ducret et al. (2009) found that RFRP-3 administration to cultured mouse brain slices resulted in a decreased firing rate of a subset of GNRH neurons. In teleosts, intraperitoneal injections of goldfish Lpxrfa-2 and Lpxrfa-3 reduce hypophysiotropic *gnrh3* mRNA levels *in vivo*, while goldfish Lpxrfa-3 inhibits Gnrh-stimulated *fshβ* and *lhβ* expression in pituitary cells *in vitro* (Qi et al. 2013). In addition, European sea bass and the orange-spotted grouper both exhibit reduced *gnrh1* expression levels in response to sea bass Lpxrfa-1 and to all three of the grouper Lpxrfa peptides, respectively (Wang et al. 2015, Paullada-Salmerón et al. 2016b). Therefore, in most teleosts studied so far, Lpxrfa peptides negatively regulate gene expression of Gnrhs.

GNIH/RFRP/Lpxrfa Cell Signaling

Because GNIH and its orthologs are relatively newly discovered neuropeptides, there are still many gaps in our knowledge of how these peptides mediate their effects on cellular and molecular levels. However, research has begun to uncover some of the cell signaling pathways that facilitate GNIH/RFRP/Lpxrfa's effects. In the chicken, Shimizu and Bédécarrats (2010) demonstrated that the GNIH

receptor inhibits cAMP production by coupling to the $G_{\alpha i}$ protein and that this inhibition can reduce the GNRH-induced cAMP activation via the $G_{\alpha s}$ protein (Figure 1.3). Similarly in the sheep, RFRP-3 is capable of abolishing the GNRH-induced phosphorylation of ERK, thereby inhibiting the cAMP-dependent pathway for gene activation (Sari et al. 2009). In the mouse, Son et al. (2012) used a gonadotrope cell line (LβT2) to demonstrate that mouse RFRP-3 inhibits GNRH-induced cAMP activation (via inhibition of AC) and ERK phosphorylation. RFRP-3 also inhibited GNRH-induced, but not basal, gonadotropin subunit gene expression (Son et al. 2012). In addition, an AC inhibitor (MDL) and a PKA inhibitor (H89) reduced GNRH-induced gonadotropin subunit expression, while a PKC inhibitor (GF) did not (Son et al. 2012). Therefore, RFRP-3 inhibits GNRH-induced gonadotropin subunit expression by inhibiting PKA- (and not PKC-) dependent ERK phosphorylation in the mouse. In addition to the cAMP pathway, RFRPs have also been shown to inhibit GNRH-induced gonadotropin expression via the mobilization of intracellular calcium (Clarke et al. 2008).

ZEBRAFISH AS A MODEL ORGANISM FOR LPXRFA/GNRH3 RESEARCH

In order to best understand the functional roles of Lpxrfa and Gnrh3 in teleosts, we use the zebrafish as our model species, which will also be useful in clarifying some of the variability surrounding how Lpxrfa regulates gonadotropin release (stimulator vs inhibitor) in teleosts. The zebrafish is a commonly used vertebrate model for genetic and developmental studies because of many convenient characteristics: ease in obtaining large numbers of embryos from a single spawn, fast embryonic and larval development, transparent embryos and larvae that allow for

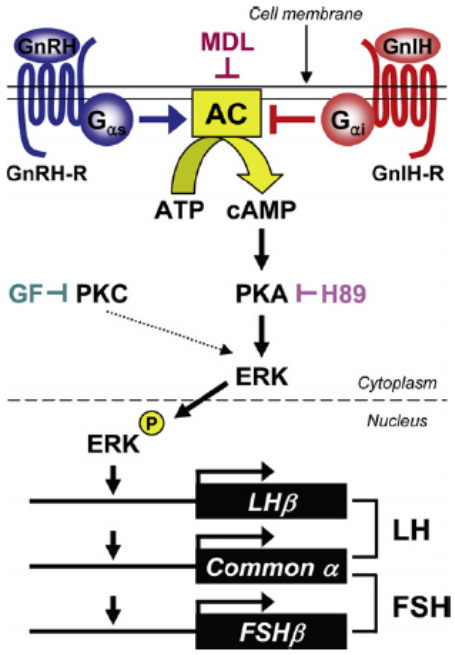

Figure 1.3. **Illustration of GNIH and GNRH's effects on the PKA/cAMP-dependent pathway (Ubuka et al. 2013).** Caption from Ubuka et al. (2013): Model of the inhibitory mechanism of GnIH on GnRH-induced gonadotropin subunit, LHb, FSHb, and common a, gene transcriptions. The inhibitors of adenylate cyclase (AC)/cAMP/protein kinase A (PKA) pathway, MDL (inhibitor of AC) and H89 (inhibitor of PKA), effectively inhibited GnRH-stimulated gonadotropin expressions. On the contrary, the inhibitor of protein kinase C (PKC), GF, did not inhibit GnRHstimulated gonadotropin expressions. Accordingly, mouse GnIH may inhibit GnRHinduced gonadotropin subunit gene transcriptions by inhibiting AC/cAMP/PKAdependent ERK activation in LβT2 cells.

easy visualization, rapid reproductive maturation, and ease of raising fish within a laboratory setting (Abraham 2008). In addition to being a convenient laboratory model, there are several molecular tools available for zebrafish research, which include (but are not limited to) sequenced genome, transgenesis, knockdown techniques, over-expression techniques, and knockout techniques. Many of these techniques can also be implemented in conditional scenarios, in which tissue-specific expression regulates the desired effect (e.g., activation of a promoter that drives the over-expression of a particular gene).

In the zebrafish, a single Lpxrfa precursor peptide is cleaved into three individual Lpxrfa peptides: Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3 (Figure 1.4), which allegedly utilize the three zebrafish Lpxrf receptors, all of which are GPCRs (Zhang et al. 2010). In goldfish, Lpxrfa-3 peptide was found to be a mature peptide by mass spectrometry (Sawada et al. 2002), and this dodecapeptide differs from the zebrafish Lpxrfa-3 dodecapeptide by one amino acid. However, the exact functions of zebrafish Lpxrfa peptides are unknown. Although the zebrafish is such a useful model, the research on Lpxrfa in this species is limited to one study (Zhang et al. 2010), where zebrafish Lpxrfa and its receptors were cloned and characterized in terms of tissue distribution, developmental expression, and function on gonadotropins, in which intraperitoneal injections of zebrafish Lpxrfa-3 reduced serum Lh levels in mature goldfish. However, there is no existing information on the molecular mechanisms that Lpxrfa uses in the brain or pituitary to regulate gonadotropin release, in particular, and reproduction, in general, in the zebrafish.

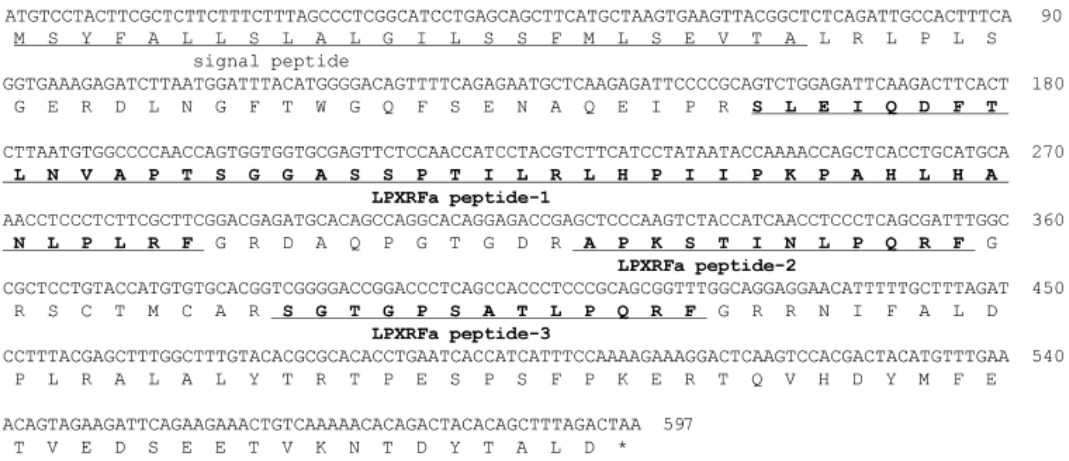

Figure 1.4. The nucleotide and amino acid sequences for the zebrafish Lpxrfa precursor peptide (Zhang et al. 2010). After translation, the precursor is cleaved into three individual peptides, all of which contain the LPXRF-NH_2 motif (X is L or Q) at the C-terminus. Each peptide is followed by a glycine amidation signal at the C-terminus and is flanked by a single basic amino acid (K or R) at both termini, signaling endoproteolytic sites as determined by Satake et al. 2001 in Japanese quail. Caption from Zhang et al. (2010): Sequence analysis of the zebrafish gnih precursor. Panel (A): the nucleotide sequence and the deduced aa sequence of zebrafish gnih. The putative signal peptide is underlined. The putative zebrafish LPXRFamide peptides are underlined and shown in bold.

In the zebrafish, two Gnrh isoforms exist: Gnrh2 and Gnrh3. Gnrh3 assumes the neuroanatomical distribution and non-redundant roles of Gnrh1 in cyprinids (Okubo and Aida 2001) and is the hypophysiotropic form in the zebrafish (Steven et al. 2003, Abraham et al. 2008). The Gnrh3 system in the zebrafish, unlike the Lpxrfa system, is very well characterized in terms of its neuroanatomical distribution,

regulation of forebrain neurogenesis, hypophysiotropic effects on the pituitary, and regulation of fertility. The distribution of zebrafish Gnrh3 has been described in both the developing (Abraham et al. 2008) and adult (Steven et al. 2003) brains, with soma migrating during development and eventually residing in the terminal nerve/ventral telencephalon and pre-optic area/hypothalamus and projecting to the pituitary of the adult. In the developing zebrafish embryo/larvae, Gnrh3 is responsible for controlling the migration of Gnrh3 soma, which also includes the involvement of netrin proteins (Abraham 2008). On the other hand, in the adult zebrafish, Gnrh3 is responsible for controlling the fertility of females, as Gnrh3-cell ablated larvae result in an all-female, sterile population (Abraham et al. 2010). Therefore, Gnrh3 regulates multiple reproductive processes in the developing and adult zebrafish brains. However, the molecular mechanisms by which this Gnrh3 regulation occurs are mostly unknown and require further investigation to determine their modes of action.

PROJECT HYPOTHESES, OBJECTIVES, AND ORGANIZATION

The overall goal of this research was to elucidate the mechanisms by which Lpxrfa and the hypophysiotropic Gnrh3 coordinately regulate reproduction in the zebrafish, by neuroanatomical distribution, neuronal interactions, functional effects, ligand-receptor relationships, and loss-of-function assays. In order to achieve this goal, we postulated the following three hypotheses, representing the next three chapters of this thesis. Each hypothesis was tested via the following specific objectives and methodology.

Hypothesis 1: Zebrafish Lpxrfa is a functional neuropeptide in the zebrafish brain-pituitary that inhibits the production of gonadotropins from the pituitary via direct innervation of the pituitary and through the use of Lpxrf receptors.

To test hypothesis 1, our objectives were to 1) localize zebrafish Lpxrfa in the brain and pituitary of adults by immunohistochemistry, 2) determine the effects of zebrafish Lpxrfa administration on adult gonadotropin expression *in vitro* and *in vivo*, and 3) understand the roles and relationships between the three Lpxrfa peptides and the three Lpxrf receptors in the zebrafish through receptor activation studies, receptor sequence comparisons, and neuroanatomical localization.

To achieve these objectives, we 1) generated and validated a polyclonal antibody in rabbits against the zebrafish Lpxrfa precursor, 2) exposed adult zebrafish pituitaries *in vitro* and adult zebrafish *in vivo* to zebrafish Lpxrfa-3 and measured mRNA levels of pituitary gonadotropin genes, 3) utilized a Dual Reporter Luciferase Assay to assess the activation potencies of each zebrafish Lpxrf receptor with each zebrafish Lpxrfa peptide through two major signal transduction pathways, and 4) attempted to histologically detect the mRNA of Lpxrf receptors in the adult zebrafish brain and pituitary.

Hypothesis 2: Based on the above studies, we further hypothesized that zebrafish Lpxrfa also exerts its functions on the pituitary via Gnrh3 neurons in the brain of zebrafish, and this relationship is present during both development and adulthood.

To test hypothesis 2 during development, our objectives were to 1) characterize the wild-type (WT) developmental expression profiles of *lpxrfa* and

gnrh3, 2) achieve transient *lpxrfa* knockdown in early development and determine its effects on *gnrh3* expression, and 3) determine the effects of the inherited loss of Gnrh3 on *lpxrfa* expression during early development. To test hypothesis 2 during adulthood, our objectives were to 1) localize the neuroanatomical relationships between Lpxrfa and Gnrh3 in the adult zebrafish brain, 2) determine the *in vitro* and *in vivo* effects of Lpxrfa on *gnrh* expression in the adult brain, and 3) determine the effects of the inherited loss of Gnrh3 on *lpxrfa* expression in adult male and female brains.

Originally, we planned to generate and validate an *lpxrfa*$^{-/-}$ knockout line, in addition to the *gnrh3*$^{-/-}$ knockout line, to determine how Gnrh3 is affected by the loss of Lpxrfa. However, due to technical difficulties explained later in this document, we could not generate a true *lpxrfa*$^{-/-}$ knockout line. Therefore, in this hypothesis (chapter), we describe how *lpxrfa* is affected during development and adulthood by the loss of Gnrh3 in the *gnrh3*$^{-/-}$ knockout line, while the next hypothesis describes the generation, validation, and characterization of the *gnrh3*$^{-/-}$ line.

To achieve the objectives of hypothesis 2 during development, we 1) quantified mRNA levels of *lpxrfa* and *gnrh3* during development, 2) achieved *lpxrfa* knockdown with *lpxrfa*-targeting anti-sense MO oligonucleotides and quantified resulting mRNA levels of *gnrh3*, and 3) established a *gnrh3*$^{-/-}$ knockout line using the transcription activator-like effector nuclease (TALEN) technology to determine how mRNA levels of *lpxrfa* are affected in the developing mutant embryo/larva. To achieve the objectives of hypothesis 2 during adulthood, we 1) developed and validated a polyclonal antibody against the zebrafish Gnrh3 Gap to localize Gnrh3,

alongside Lpxrfa, 2) exposed adult zebrafish brains *in vitro* and adult zebrafish *in vivo* to zebrafish Lpxrfa-3 and measured resulting mRNA levels of *gnrh3* and *gnrh2*, and 3) used the *gnrh3*$^{-/-}$ knockout line to determine how *lpxrfa* mRNA levels are affected in male and female brains, separately, at different times of the day.

Hypothesis 3: The generation of a valid *gnrh3*$^{-/-}$ knockout line will result in the production of embryonic/larval zebrafish with misguided Gnrh3 neuronal migration and adult zebrafish that exhibit disrupted gametogenesis and sterility.

To test hypothesis 3, our objectives were to 1) generate and validate a *gnrh3*$^{-/-}$ line in the zebrafish with the TALEN technology, 2) quantify important genes/peptides in the reproductive axis during development and adulthood in the *gnrh3*$^{-/-}$ line, 3) determine if the ontogeny and migration of Gnrh3 neurons during development are affected by the inherited loss of Gnrh3, and 4) determine how gametogenesis and reproductive performance in adults are affected by the inherited loss of Gnrh3.

To achieve these objectives, we 1) generated the *gnrh3*$^{-/-}$ knockout line and validated it at the gene, transcript, and peptide levels, 2) quantified mRNA and peptide levels of reproductive genes and peptides during development and adulthood, 3) examined Gnrh3 neuronal migration in *gnrh3*$^{-/-}$ *gnrh3:tdTomato* juveniles, 4) observed gross gonadal morphology and gonadal histology with hematoxylin and eosin staining in adults, and 5) measured fecundity, fertilization rate, and offspring survival of different spawning combinations of adults.

Chapter 2: Neuroanatomical Distribution and Functional Roles of the Lpxrfa/Lpxrf-R System in the Zebrafish Brain-Pituitary Axis

ABSTRACT

GNIH and its orthologs have been widely described in terms of their neuroanatomical distribution and functional roles in gonadotropin synthesis/release in birds and mammals. However, this information is highly lacking in teleosts. Consequently, the purpose of this chapter is to characterize the neuroanatomical distribution and functional roles of the Lpxrfa/Lpxrf-R system in the brain-pituitary axis of the zebrafish. The Lpxrfa system in zebrafish is composed of a single *lpxrfa* gene that encodes for three Lpxrfa peptides (Lpxrfa-1-3) and of three different receptors (Lpxrf-R1-3). The neurons expressing Lpxrfa were neuroanatomically localized, using a polyclonal antibody (developed for this purpose) against the zebrafish Lpxrfa precursor. Zebrafish Lpxrfa soma were confined to a single cluster in the ventral zone of the periventricular hypothalamus adjacent to the diencephalic ventricle with Lpxrfa fibers extending throughout the fore-, mid-, and hind-brains and even into the pituitary. However, our attempts to histologically detect the zebrafish Lpxrf receptors, in order to determine the target sites of Lpxrfa neurons, were unsuccessful, despite extensive efforts. In order to determine the effects of Lpxrfa on gonadotropins, we employed *in vitro* and *in vivo* assays with zebrafish Lpxrfa-3. Through an *in vitro* assay, zebrafish Lpxrfa-3 reduced the expression of *lhβ* and *cgα* mRNA levels in the pituitary, suggesting that Lpxrfa plays an inhibitory role on gonadotropins in the zebrafish; however, these effects were not observed *in vivo*. We also characterized the relationships between the three zebrafish Lpxrfa peptides and

the three zebrafish Lpxrf receptors in terms of activation capacity. Using receptor activation assays, we demonstrated that zebrafish Lpxrf-R2 and Lpxrf-R3 are activated by all three zebrafish Lpxrfa peptides via only the PKA/cAMP pathway, while Lpxrf-R1 is not activated by any of the three peptides. Lastly, due to the inability to histologically detect Lpxrf receptor mRNA, we explored additional pathways and found that zebrafish Lpxrfa-2 and Lpxrf-3 inhibit Kiss2's activation of the Kiss1ra receptor. In zebrafish, *kiss1ra* is expressed in high levels and is abundant throughout the brain, including in the preoptic area and the hypothalamus, and indeed, we found that Lpxrfa-ir fibers innervate *kiss1ra*-expressing cells, indicating an additional pathway for zebrafish Lpxrfa to exert its functions. Overall, zebrafish Lpxrfa appears to be a functionally active reproductive neuropeptide in the brain that directly reduces *lhβ* and *cgα* expression in the pituitary, while potentially utilizing pathways of other reproductive neuropeptides, including Kiss2, to elicit its functions.

INTRODUCTION

Since the discovery of GNIH in 2000 (Tsutsui et al. 2000), GNIH orthologs have been discovered and characterized (neuroanatomically and functionally) in a number of different vertebrate species. Depending on the class of the particular species, the GNIH ortholog terminology can change, but the peptides almost consistently contain the LPXRF-NH_2 motif (X is L or Q) at the C-terminus. For instance, GNIH orthologs in mammals are referred to as "RFamide-related peptides" (RFRPs), and RFRP-3 has reliably demonstrated to be an active inhibitor of gonadotropin synthesis and release in many species, including cows (Kadokawa et al. 2009), rats (Pineda et al. 2010a), and pigs (Li et al. 2013). In fish, however, GNIH

orthologs are referred to as "Lpxrfa peptides," which have been identified in a number of species, including tilapia (Biran et al. 2014), goldfish (Sawada et al. 2002), European seabass (Paullada-Salmerón et al. 2016a), zebrafish (Zhang et al. 2010), and others.

While neuroanatomical localization studies of Lpxrfa peptides are, for the most part, scarce in teleosts, there is abundant information on the localization of GNIH and its orthologs in birds and mammals. In these vertebrates, neurons expressing *GNIH* or *RFRP* mRNA are primarily found within the diencephalon, particularly the paraventricular nucleus in the hypothalamus (Satake et al. 2001, Ukena et al. 2003, Kriegsfeld et al. 2006, Ubuka et al. 2009a). In accordance, GNIH-ir/RFRP-ir cells are found in the paraventricular nucleus (Tsutsui et al. 2000, Osugi et al. 2004, Kriegsfeld et al. 2006, Clarke et al. 2008), while GNIH/RFRP-ir fibers project to several diencephalic and mesencephalic regions and to the median eminence (Bentley et al. 2003, Kriegsfeld et al. 2006, Clarke et al. 2008). Nonetheless, in teleosts, Lpxrfa gene and protein expression follow a similar pattern to that of birds and mammals: *lpxrfa* mRNA expression and Lpxrfa-ir cell bodies are found specifically within the periventricular nucleus of the hypothalamus in the goldfish, and Lpxrfa-ir fibers project throughout the brain and to the pituitary (Sawada et al. 2002). In the European sea bass, the available neuroanatomical localization is much more descriptive as Lpxrfa-ir soma are found in the olfactory bulbs/terminal nerve, ventral telencephalon, caudal preoptic area, dorsal mesencephalic tegmentum, and rostral rhombencephalon but not the hypothalamus, with Lpxrfa-ir fiber innervation including the majority of the brain but especially the

preoptic area, hypothalamus, and optic tectum (Paullada-Salmerón et al. 2016a). The sea bass pituitary is also innervated by Lpxrfa-ir fibers that most likely originate from the telencephalon and preoptic area Lpxrfa-ir fibers, and these pituitary innervations are in close approximation to gonadotropes and somatotropes (Paullada-Salmerón et al. 2016a). Thus, the distribution of the Lpxrfa system in teleosts is species-specific.

While the effects of Lpxrfa peptides on fish gonadotropins are not as well studied, multiple studies have shown that GNIH and RFRP-3 display similar functions. After the discovery that GNIH reduces LH levels from quail pituitaries, Ubuka et al. (2006) demonstrated that GNIH reduces not only LH release in pituitaries but also its synthesis through mRNA expression. The effect of GNIH is not limited to LH, since GNIH can also reduce expression of the *CGα* subunit and the *FSHβ* subunit (Ciconne et al. 2004). Although GNIH/RFRP-3 appears to act as an inhibitor of gonadotropin release in mammals and birds, the functions of Lpxrfa in fish are more variable with some species exhibiting stimulatory effects. For instance, tilapia Lpxrfa-2 positively regulates both Fsh and Lh (but not Gh) *in vitro* and *in vivo* (Biran et al. 2014). However, the first *in vivo* study on fish Lpxrfa function demonstrated that, in goldfish, the administration of zebrafish Lpxrfa-3 results in reduced serum levels of Lh (Zhang et al. 2010). These results in teleosts have expanded to demonstrate that Lpxrfa can exert stimulatory (Moussavi et al. 2012, 2013) and/or inhibitory effects (Di Yorio et al. 2016, Paullada-Salmerón et al. 2016b, Peng et al. 2016) on gonadotropin release, sometimes depending on the species or gonadal phase. However, research in agnathans has pointed more towards a solely stimulatory role of Lpxrfa (Osugi et al. 2011, 2012). Therefore, it has been proposed

that GNIH/RFRP/Lpxrfa's roles in reproduction have evolved evolutionarily: from stimulating gonadotropin release in jawless fishes to inhibiting gonadotropin release in more evolutionarily advanced vertebrates (e.g., birds and mammals; Osugi et al. 2012, Tsutsui et al. 2012). Within teleosts, however, Lpxrfa appears to play stimulatory and/or inhibitory roles on gonadotropin release, often depending on the sex, reproductive state, or perhaps other unknown factors of the organism (Osugi et al. 2012, Tsutsui et al. 2012).

Much research has been aimed at determining how GNIH and its orthologs elicit their functions by studying the activation and pathways of GNIH receptors and their orthologs. The GNIH receptor (GPR147) mediates its effects by inhibiting basal (via the $G_{\alpha i}$ subunit) and GNRH-induced (via the $G_{\alpha s}$ subunit) cAMP production or by abolishing GNRH-induced phosphorylation of ERK (Sari et al. 2009, Shimizu and Bédécarrats 2010). In addition to the cAMP pathway, RFRP-3 is also capable of inhibiting GNRH-induced gonadotropin expression via the mobilization of intracellular calcium (Clarke et al. 2008). In teleosts, Lpxrfa peptides also utilize the PKA/cAMP and PKC/Ca^{2+} pathways, as demonstrated by the tilapia's Lpxrfa-2 activation of the tilapia Lpxrf-R through the CRE and SRE pathways, respectively (Biran et al. 2014). However, Lpxrf receptor activation studies are scarce in teleosts.

Because of the little information available regarding localization and functionality of Lpxrfa peptides and their receptors in teleosts, the goal of this study was to characterize these components and determine the modes of action and the mechanisms by which Lpxrfa elicits its functions in the model organism of the zebrafish. In the zebrafish, a single Lpxrfa precursor peptide is cleaved into three

individual Lpxrfa peptides: Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3, which allegedly utilize the three zebrafish Lpxrf receptors, all of which are GPCRs (Zhang et al. 2010). Specifically, we sought to describe the zebrafish Lpxrfa/Lpxrf-R system's neuroanatomical localization, functional effects on gonadotropins, and receptor activation pathways. Originally, this study included the generation of a *lpxrfa:eGFP* transgenic line of zebrafish in order to understand the development of the Lpxrfa system and its interactions with other reproductive factors. However, because this line could not be produced, we focused on developing a polyclonal antibody against the zebrafish Lpxrfa precursor in order to visually detect Lpxrfa, which demonstrated to be extremely useful in determining Lpxrfa's neuroanatomical localization in the zebrafish brain-pituitary axis. In addition, as thoroughly explained later, we were unable to histologically detect the zebrafish Lpxrf receptors, which led us to explore Lpxrfa's utilization of an additional RFamide peptide pathway: kisspeptin. In doing so, we found, through receptor activation/inhibition assays and double immunohistochemistry/*in situ* hybridization, that Lpxrfa may elicit its functions (at least partially) through a reproductively related kisspeptin receptor. The specific objectives of this chapter were to conduct the following:

1) Localize zebrafish Lpxrfa in the brain and pituitary of adults by immunohistochemistry,
2) Determine the effects of zebrafish Lpxrfa administration on gonadotropin expression *in vitro* and *in vivo*,

3) Understand the roles and relationships between the three Lpxrfa peptides and the three Lpxrf receptors in zebrafish through receptor activation studies, receptor sequence comparisons, and neuroanatomical localization,
4) Determine the activation/inhibition potencies of zebrafish Lpxrfa-2 and -3 on two kisspeptin receptors expressed in a COS7 cell line, and
5) Confirm this effect of zebrafish Lpxrfa-2 and -3 on a kisspeptin receptor through double immunohistochemistry/*in situ* hybridization.

METHODS

Animals

All zebrafish used in this study originated from the in-house colony at 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 monitored and fed twice daily with a commercial flake food or pellets *ad libitum*. Zebrafish embryos and larvae were raised in individual containers of freshwater until 30 dpf, before being transferred to the recirculating system. Starting at 5 dpf, larval zebrafish were fed *Paramecium* twice daily, until 14 dpf, when *Artemia* nauplii was introduced to their diet. Prior to tissue collections, adult fish were euthanized by immersion in an ice bath followed by quick decapitation. 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: SLEIQDFTLNVAPTSGGASSPTILRLHPIIPKPAHLHAN-LPLRF-NH_2, Lpxrfa-2: APKSTINLPQRF-NH_2, and Lpxrfa-3: SGTGPSATLPQRF-NH_2) were synthesized at a 95% purity by Genscript.

Generation of* lpxrfa:eGFP *Transgenic Line by BAC Recombination

In order to visualize Lpxrfa within the zebrafish, we originally attempted to establish a *lpxrfa:eGFP* transgenic line, in which the activation of the *lpxrfa* promoter would drive the expression of eGFP, allowing convenient visualization of *lpxrfa*. Initially, the 10 kb sequence upstream of the *lpxrfa* start codon was cloned and modified so that the activation of the promoter drove the expression of eGFP, whose sequence began immediately after the start codon of *lpxrfa*. When this construct was micro-injected into one- to two-cell stage WT embryos, no transient eGFP expression was observed at 48-72 hours post-fertilization (hpf), when *lpxrfa* expression is high in the developing embryo/larva (see Chapter 3; Zhang et al. 2010). It is most likely that this sequence did not contain enough of the *lpxrfa* gene's regulatory elements, so we, therefore, resorted to the use of DNA from a BAC clone (approximately 200 kb) to drive the expression of eGFP.

The BAC clone DKEY-22M8 contains the complete *lpxrfa* gene and large upstream and downstream sequences and was obtained from Source BioScience imaGenes. Modifications to the BAC DNA were conducted according to the galK-mediated recombination protocols in bacteria from Warming et al. (2005) and Sharan et al. (2009). The transgene was generated by the additions of eGFP immediately

after the start codon of *lpxrfa*, a polyA tail at the end of the eGFP, and Tol2 cassettes at either end of the transgene. The transgene was micro-injected into one- to two-cell stage WT embryos at 50 ng/mL with 25 ng/mL *tol2* transposase mRNA. At approximately 48 hpf, embryos were assessed for transient eGFP expression with a Zeiss Axioplan 2 microscope with an Attoarc HBO100 W power source, equipped with a CCD Olympus DP70 camera. Transient eGFP expression in live embryos was validated by *lpxrfa in situ* hybridization and Lpxrfa immunohistochemistry (using the anti-zebrafish Lpxrfa antibody that is described in the next section) on WT embryos to determine if the signals were localized to the same region. Once the injected embryos reached adulthood, males were stripped of sperm (~0.5 μL), which was used as a template for PCR with eGFP-specific primers to detect the presence of the transgene.

Generation of Polyclonal Antibody against Zebrafish Lpxrfa

Because the generation of the *lpxrfa:eGFP* transgenic line was not successful in providing a tool to visualize Lpxrfa in the zebrafish, we, instead, generated a polyclonal antibody against the zebrafish Lpxrfa precursor to allow histological detection of this peptide in fixed tissues. The cDNA 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 from the insoluble fraction were collected according to Brent (1997). These proteins were dissolved in 8M urea, purified with nickel-nitrolotriacetic acid columns (Promega), and de-salted on

sephadex G-15 columns (Pharmacia). The purified protein was used as an antigen for production of antiserum in rabbits (ProteinTech). The final bleed antiserum was used as the primary antibody in all zebrafish Lxprfa immunohistochemistry.

Verification of Anti-Zebrafish Lpxrfa

In order to verify the specificity of our zebrafish Lpxrfa antibody, we used two approaches: 1) Tested recognition of zebrafish Lpxrfa expressed in a COS7 cell line by Lpxrfa immunohistochemistry, and 2) Examined co-localization of *lpxrfa in situ* hybridization and Lpxrfa immunohistochemistry. Briefly, the full zebrafish *lpxrfa* cDNA coding region was cloned into the pcDNA3.1 (Life Technologies) mammalian expression vector under the control of the CMV promoter. The zebrafish *lpxrfa*-pcDNA3.1 plasmid and the control pcDNA3.1 plasmid were transfected into COS7 cells with FuGENE 6.0 (Promega). The cells were grown in 25 cm^2 sterile cell culture flasks in DMEM supplemented with 10% FBS, 1% glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Biological Industries) at 37 °C and 5% CO_2. After 48 hours of incubation, cells were transferred to Lab-Tek®II two-chamber slides (Thermo Scientific). After 24 hours, the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 1 hour at room temperature and were washed with PBS. Blocking and immunostaining for zebrafish Lpxrfa was then conducted as described in the "Neuroanatomical Localization of Lpxrfa in Brain and Pituitary" section below.

In order to conduct double *in situ* hybridization and immunohistochemistry, WT brains were dissected from adult fish and fixed overnight with 4% PFA (in PBS) at 4 °C. Before cryopreservation, brains were transferred to 30% sucrose in PB

overnight at 4 °C. Tissues were frozen in OCT, sectioned coronally at 15 µm thickness, transferred to charged slides, and stored at -80 °C until processed. An anti-sense DIG-labeled riboprobe was synthesized from the cDNA clone of the full *lpxrfa* coding region, using RNA polymerase (Roche Diagnostics). The *in situ* hybridization protocol was followed as according to Zmora et al. (2012), including incubation with 0.5% H_2O_2 to block endogenous peroxidases and a riboprobe concentration of 1000 ng/mL. The signal was detected using the Tyramide Signal Amplification (TSA) Plus kit (Perkin Elmer), according to the manufacturer's protocol and using anti-DIG HRP (1:200; Roche Diagnostics). Fluorescence was obtained via the Cy3 dye from the TSA Plus kit. After quenching HRPs with 0.02 N HCl for 10 minutes, blocking and immunostaining for Lpxrfa were conducted as described: Sections were blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Sections were incubated overnight at 4 °C with rabbit anti-zebrafish Lpxrfa (1:5000) diluted in 1% BSA and 0.3% Triton X-100 in PBS. Sections were incubated for 1 hour at room temperature with goat anti-rabbit HRP (1:1000). The signal was detected using the TSA Plus kit, and fluorescence was obtained via the FITC dye from the kit.

Neuroanatomical Localization of Lpxrfa in Brain and Pituitary

For visualization of the zebrafish brain, WT adult brains were dissected, fixed, cryopreserved, and sectioned as previously described. For visualization of the zebrafish pituitary, whole zebrafish heads of adult *fsh:eGFP* and *lh:mCherry* fish (kindly provided by the lab of Dr. Berta Levavi-Sivan at The Hebrew University of Jerusalem) were dissected after removal of the eyes, jaw, gills, and other soft tissues.

Fixation, cryopreservation, and sectioning of whole heads were conducted as described for brains, with the exception that whole heads were decalcified for 5-7 days with 0.5M EDTA (pH 8.0) at 4 °C between fixation and sucrose. Lpxrfa immunohistochemistry on brains and whole heads was conducted as briefly described: dried sections were fixed in pre-chilled acetone for 2 minutes and allowed to dry. Sections were blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Sections were incubated overnight at 4 °C with rabbit anti-zebrafish Lpxrfa (1:5000) diluted in 1% BSA and 0.3% Triton X-100 in PBS. Sections were incubated for 1 hour at room temperature with goat anti-rabbit secondary antibody Alexa 488 (Life Technologies) or Cy3 (KPL) diluted 1:300.

***Effects of Lpxrfa-3 on Whole Pituitary Explants* 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 modified the protocol from a pituitary culture experiment for striped bass (Klenke 2006). Whole pituitaries were dissected from adult male WT zebrafish and placed in pre-cooled phenol red-free L-15 media (Himedia) supplemented with 0.15% BSA, 9 mM sodium bicarbonate, 20 mM HEPES, and penicillin/streptomycin (pH 7.4) at 100 units and 12.5 units per mL, respectively, in a 24-well sterile cell culture plate with 400 µL media per well. The pituitaries were placed in sterile Millicell cell culture inserts (0.4 µm and 12 mm diameter; Millipore) in the wells of the plate. After washing with media for 6 hours at 28 °C with gentle rotation at 30 rpm, pituitary explants were incubated with different concentrations (0, 1, 10, 100, and 1000 pM) of zebrafish Lpxrfa-3 in L-15 media at 28 °C and 30 rpm (n = 6 per treatment). Of the

three Lpxrfa peptides encoded in the zebrafish Lpxrfa precursor peptide, zebrafish Lpxrfa-3 was chosen because of its high homology to goldfish Lpxrfa-3, which was demonstrated to be a mature peptide detected by mass spectrometry (Sawada et al. 2002), and because of its ability to reduce serum Lh levels in the goldfish *in vivo* (Zhang et al. 2010). After 18 hours of incubation, pituitaries were frozen on dry ice and stored at -80 °C for RNA extraction. QPCR for *fshβ* (standard curve coefficient correlation (R^2) = 0.999), *lhβ* (R^2 = 0.995), and *cgα* (R^2 = 0.998) was then conducted as described below.

***Effects of Lpxrfa-3 on Pituitary Gene Expression* in vivo**

In order to determine if the effects of zebrafish Lpxrfa-3 on gonadotropin expression occurs *in vivo* in the same manner as *in vitro*, female and male adult zebrafish (n = 3-4) were injected intraperitoneally with Lpxrfa-3 and subsequently sampled for QPCR. Males and females were injected with 0.5 µg/fish and 1 µg/fish Lpxrfa-3, respectively, in PBS with 1% Ethan's blue dye, while a separate group was injected with PBS with 1% Ethan's blue dye. Injections were administered twice, approximately 4 hours apart: first injection at 9:40 – 11:15 and second injection at 14:00 – 15:20. Three hours after the second injection, fish were sampled for pituitaries, which were frozen on dry ice and stored at -80 °C until RNA extraction. This protocol was modified from Zhang et al. (2010), in which two intraperitoneal injections (3 hours apart) of zebrafish Lpxrfa-3 into mature goldfish resulted in an inhibitory effect on serum Lh levels at 1 and 3 hours after the second injection. QPCR was then conducted for *fshβ* (R^2 = 0.986), *lhβ* (R^2 = 0.999), and *cgα* (R^2 = 0.996), as described below.

Quantification of Gene Transcripts

Total RNA was extracted from pituitaries using the manufacturer's protocol for the TRIzol® reagent (Invitrogen), and total RNA was quantified with a Nanodrop (Thermo Scientific). Total RNA (100 ng) of pituitaries was treated with gDNA wipeout buffer for 9 minutes at 42 °C and synthesized into first-strand cDNA with the Qiagen QuantiTect Reverse Transcriptase (RT) Kit. The final reaction volume for all cDNA synthesis reactions was 10 µL. The mRNA levels of the target genes in pituitaries were measured via QPCR. Gene-specific QPCR primers for each of the target genes are listed in Table 2.1. Specificity of each primer set's amplification was confirmed by a dissociation curve. Each QPCR reaction was carried out in duplicate with a final volume of 10 µL: 2x DyNAmo Flash SYBR Green QPCR mix (Life Technologies), 200 nM primer mix, 0.3x ROX (Life Technologies), 20 ng cDNA, and sterile MilliQ water, in a 7500 Fast Real-Time PCR System (Applied Biosystems). The cycle conditions were 95 °C for 7 minutes, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. Each plate included a standard curve using the reverse-transcribed RNA of the specific clone as a template. Absolute copy number was calculated for the tested reactions and normalized against the housekeeping gene *ef1α*, which has consistently and reliably been used as a housekeeping gene in zebrafish QPCR (Abraham et al. 2008, Tang et al. 2014, Zhang et al. 2015). Each plate included two no RT controls and two no template controls.

Lpxrf-R Activation Assay with Dual Luciferase Reporter 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

Table 2.1. QPCR primers used to quantify gene transcripts in pituitaries for *in vitro* and *in vivo* assays.

Gene	Type	Sequence (5' → 3')	T_m (°C)	GC%	Amplicon Size (bp)
fshβ	For	GCTGGACAATGGATCGAGTTTA	54.9	45.5	92
fshβ	Rev	CTCGTAGCTCTTGTACATCAAGTT	54.5	41.7	
lhβ	For	GGCTGGAAATGGTGTCTTCT	55.1	50.0	107
lhβ	Rev	CCACCGATACCGTCTCATTTAC	55.1	50.0	
cgα	For	TCCGGTCTATCAGTGCGT	55.6	55.6	148
cgα	Rev	GGATATTCGTGGCAACCATTT	53.5	42.9	
ef1α	For	AAGACAACCCCAAGGCTCTCA	58.6	52.4	255
ef1α	Rev	CCTTTGGAACGGTGTGATTGA	55.5	47.6	

affinities and potencies. To accomplish this, we compared two common signal transduction pathways: the cAMP-response element (CRE) through PKA/cAMP and the serum-response element (SRE) through PKC/Ca^{2+}. The 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 mammalian expression vector (Invitrogen) under the control of the CMV promoter. COS7 cells were grown in 96-well plates in DMEM supplemented with 10% FBS, 1% glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Biological Industries) under 5% CO_2 until 90% confluent. Lpxrf receptor plasmids (0.03 µg/well) were co-transfected into a COS7 cell line, using FuGENE 6.0 (Promega), with pCMV-*Renilla* luciferase (Promega; 0.03 ng/well) as an internal control and either pCRE-firefly luciferase (Invitrogen) or pSRE-firefly luciferase (Invitrogen; 0.03 ng/well) for 36 hours before the media was replaced.

After 18 hours of incubation with a HEPES-modified DMEM containing 0.5% BSA as a substitution for serum, the cells were incubated with one of the three synthetic Lpxrfa peptides (Lpxrfa-1, Lpxrfa-2, or Lpxrfa-3) in a 10x serial dilution (10 pM to 10 μM). After 6 hours, the cells were rinsed with PBS and lysed with passive lysis buffer. Cells were assessed for both firefly and *Renilla* luciferase activity, using the Dual Luciferase Reporter Assay Kit and following the manufacturer's protocol (Promega). The data was calculated as the mean ratio of firefly luciferase activity to *Renilla* luciferase activity per Lpxrfa peptide concentration for each receptor. The results were used to plot individual dose responses of Lpxrf receptors to Lpxrfa peptides, under the control of either CRE or SRE, to determine which signal transduction pathways Lpxrfa peptides and Lpxrf receptors utilize. All experiments were performed in triplicate with at least three independent experiments.

Lpxrfa Activation of Kiss Receptors and Inhibition of Kiss-Activated Kiss Receptors

After multiple failed attempts to histologically localize the zebrafish Lpxrf receptors in the brain with *in situ* hybridization, we hypothesized that the zebrafish Lpxrf receptors might be expressed at such low levels and be too widespread to allow detection with histological methods (see Discussion). 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 the Lpxrf receptors. Therefore, we attempted to determine if zebrafish Lpxrfa peptides are capable of activating Kiss1ra (Kiss2r) and Kiss1rb (Kiss1r) through the SRE pathway, which is the dominant pathway for Kiss/Kissr activation in teleosts

(Biran et al. 2008, Zmora et al. 2014). Because zebrafish Lpxrfa-1 is much longer in its amino acid sequence than zebrafish Lpxrfa-2 and Lpxrfa-3, we limited our zebrafish Lpxrfa ligands to only Lpxrfa-2 and Lpxrfa-3. As a first step, Kiss1ra or Kiss1rb was incubated with serial dilutions of Lpxrfa-2 or -3 to test whether they were able to activate the receptor via the SRE pathway, using the same methods described above for the Lpxrf receptors. Additionally, to test whether Lprxfa-2 or -3 interferes with the activation of Kiss receptors by Kiss, graded concentrations (10 pM to 1 μm) of Lprxfa-2 or -3 were added to 100 nM Kiss1 (for Kiss1rb) or 10 nM Kiss2 (for Kiss1ra) to determine levels of inhibition. Cells were incubated, washed, and assessed for bioluminescence as previously described.

Neuroanatomical Localization of Lpxrfa Neurons and* kiss1ra-*expressing Cells

Because zebrafish Lpxrfa-2 and Lpxrfa-3 demonstrated a strong ability to inhibit Kiss2's activation of Kiss1ra, we wanted to determine if there is any anatomical relationship between Lpxrfa neurons and Kiss1ra receptors. Therefore, we conducted simultaneous *in situ* hybridization for *kiss1ra* and immunohistochemistry for Lpxrfa. An anti-sense DIG-labeled riboprobe was synthesized from the cDNA clone of the full *kiss1ra* coding region, using RNA polymerase (Roche Diagnostics). The double *in situ* hybridization and immunohistochemistry protocol was conducted as described above, with the exception that a riboprobe concentration of 500 ng/mL was used. In addition, the Cy3 dye from the TSA kit was used for the *kiss1ra* signal, while the FITC dye was used for the Lpxrfa signal.

Microscopy

All sections were mounted with either anti-fading solution with DAPI or Vectashield with DAPI (Vector Labs) 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, at a resolution of 1360 x 1024 and a magnification of 20x. Pituitary sections were imaged with a Leica Microsystems SP6 confocal microscope with a resolution of 1024 x 1024. Brain sections were imaged with a Leica Microsystems DMi8 confocal microscope with a resolution of 1024 x 1024, a magnification of 20x, and a z-step size of 0.10. All images were analyzed with Image J and/or Adobe Photoshop.

Statistics

All data are represented as mean values ± standard error of the mean (SEM), unless otherwise specified. For QPCR values in the pituitary *in vitro* and *in vivo* experiments, the mean of each concentration/dose was compared to the control (0 pM Lpxrfa-3 or PBS) by a one-tailed, homoscedastic Student *t*-test. GraphPad Prism was used to plot dose-response activation curves and calculate EC_{50}s and IC_{50}s for the receptor activation/inhibition assays. In addition, relative luciferase activity values for the inhibition of Kiss receptors by Lpxrfa peptides were compared to the control (activation of Kiss receptor by Kiss ligand) by a one-tailed, homoscedastic Student *t*-test. Statistical significance was established if $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$.

RESULTS

Generation of lpxrfa:eGFP *Transgenic Line by BAC Recombination*

In order to generate an *lpxrfa:eGFP* transgenic line to better visualize zebrafish Lpxrfa, we modified BAC DNA containing the zebrafish *lpxrfa* gene so that, with the use of the Tol2 transposase, the activation of the *lpxrfa* promoter would drive the expression of eGFP. Our transgene and *tol2* mRNA (Figure 2.1A) were simultaneously micro-injected into one- to two-cell stage WT embryos (F0). At 48 hpf, transient expression of eGFP was visible in the hypothalamus of the injected embryos (Figure 2.1B). In order to confirm that the eGFP expression was within *lpxrfa*-expressing neurons, we conducted *lpxrfa in situ* hybridization on 48 hpf WT embryos and demonstrated the presence of *lpxrfa* in the same region as the transient eGFP expression (Figure 2.1C). In addition, Lpxrfa immunohistochemistry (using the anti-zebrafish Lpxrfa antibody that is described and validated in the next section) on 72 hpf WT embryos also demonstrated the presence of Lpxrfa-ir soma in the same region as the transient eGFP expression (Figure 2.1D). However, even though eGFP expression was observed in transgene-injected embryos, the transgene could not be detected by PCR in the sperm of hundreds of F0 males that were screened. In addition, screening of the transgene in hundreds of F0 male and female samples of fin clip gDNA also did not demonstrate any presence of the transgene through PCR. Therefore, we could not establish a true *lpxrfa:eGFP* line as the transgene was not detectable in the germ or somatic cells of the injected fish as adults. However, with the generation and verification of the specific zebrafish Lpxrfa antibody, we were able to successfully visualize Lpxrfa in fixed tissues.

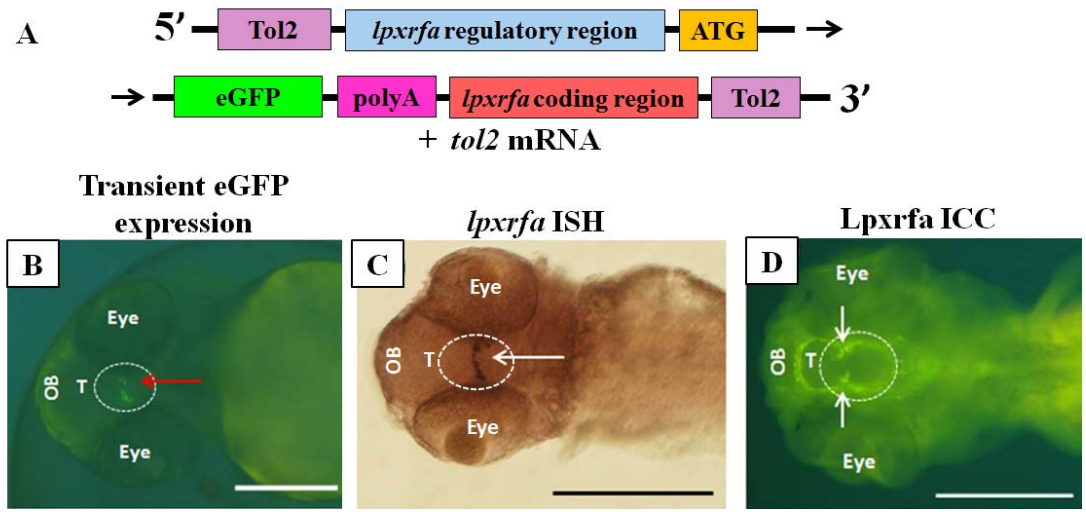

Figure 2.1. Transient expression of eGFP was observed in embryos injected with *lpxrfa:eGFP* transgene and *tol2* mRNA. (A) The transgene that was constructed from BAC DNA containing the zebrafish *lpxrfa* gene and upstream sequences. The eGFP sequence was inserted after the start codon of the *lpxrfa* gene, a polyA tail was added at the end of the eGFP sequence, and the Tol2 cassette was used (with *tol2* mRNA). (B) Transient eGFP expression (red arrow) in the hypothalamus of a 48 hpf embryo injected with construct (A), which is validated by *in situ* hybridization for *lpxrfa* (white arrow) on a 48 hpf WT embryo (C) and Lpxrfa immunohistochemistry (white arrows indicate Lpxrfa-ir soma) on a 72 hpf WT larva (D). (B-D) Ventral view with anterior towards the left. Circled region represents hypothalamus. OB = olfactory bulb region. T = telencephalon. ISH = *in situ* hybridization. ICC = immunocytochemistry. Scale bars = 250 µm.

Verification of Anti-Zebrafish Lpxrfa

In order to determine the specificity of the anti-zebrafish Lpxrfa polyclonal antibody, we transfected COS7 cells with either a control pcDNA3.1 plasmid or a

pcDNA3.1 plasmid that contained the entire coding region of zebrafish *lpxrfa*. COS7 cells transfected with either the control pcDNA3.1 plasmid or the *lpxrfa*-pcDNA3.1 plasmid and stained with the Lpxrfa pre-immune serum did not produce any signal (Figure 2.2Ac, d), while cells transfected with the control pcDNA3.1 plasmid and stained with anti-zebrafish Lpxrfa also did not produce any signal (Figure 2.2Aa). However, cells transfected with the zebrafish *lpxrfa*-pcDNA3.1 plasmid and stained with anti-zebrafish Lpxrfa did yield positive signal (Figure 2.2Ab).

We also conducted simultaneous *lpxrfa in situ* hybridization and immunostaining for Lpxrfa in adult brain sections to determine if anti-zebrafish Lpxrfa recognizes soma that express *lpxrfa*. Soma in the ventral zone of the periventricular hypothalamus were stained with an anti-sense riboprobe against *lpxrfa* (Figure 2.2Ba), and these same soma were also stained with the anti-zebrafish Lpxrfa antibody (Figure 2.2Bb). Therefore, the co-localization of these two signals indicates again that our anti-zebrafish Lpxrfa polyclonal antibody is specific to zebrafish Lpxrfa (Figure 2.2Bc).

Zebrafish Lpxrfa Localization in Brain and Pituitary

By conducting Lpxrfa immunohistochemistry on adult brains, we found that Lpxrfa soma are located solely within a distinct cluster in the ventral zone of the periventricular hypothalamus (Figure 2.3A, C) with Lpxrfa-ir fibers extending throughout the fore-, mid-, and hind-brains (data not shown). In order to determine zebrafish Lpxrfa's location within the pituitary, we used the anti-zebrafish Lpxrfa antibody on coronal sections of adult *fsh:eGFP* and *lh:mCherry* brains/pituitaries. Fsh-labeled (Figure 2.4A) and Lh-labeled (Figure 2.4B) cells were located within the

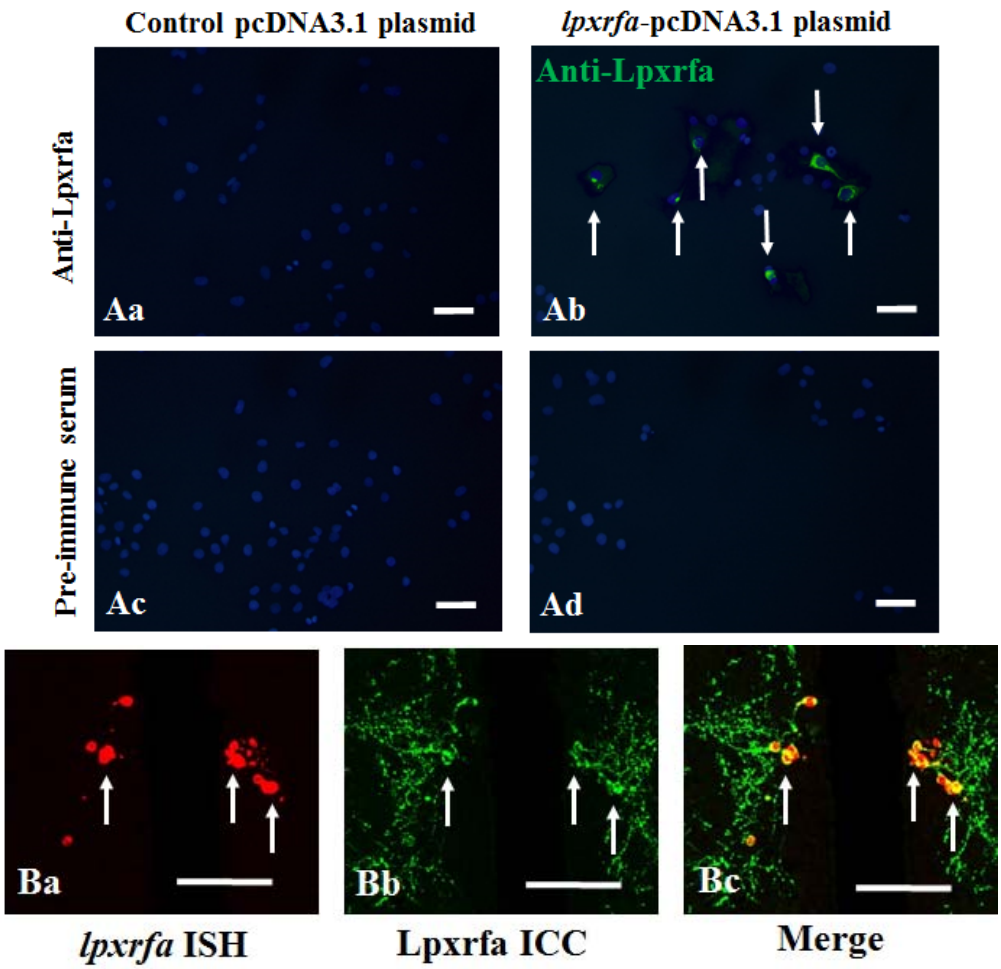

Figure 2.2. Anti-zebrafish Lpxrfa polyclonal antibody is specific to zebrafish Lpxrfa. (A) Immunostaining with anti-zebrafish Lpxrfa (Aa, Ab) or pre-immune serum (Ac, Ad) in COS7 cells transfected with control pcDNA3.1 plasmid (Aa, Ac) or with zebrafish *lpxrfa*-pcDNA3.1 plasmid (Ab, Ad). The cells that express zebrafish Lpxrfa and are immunostained with anti-zebrafish Lpxrfa (green) are indicated by white arrows (Ab). (B) 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). ISH = *in situ* hybridization. ICC = immunocytochemistry. Scale bars = 100 μm.

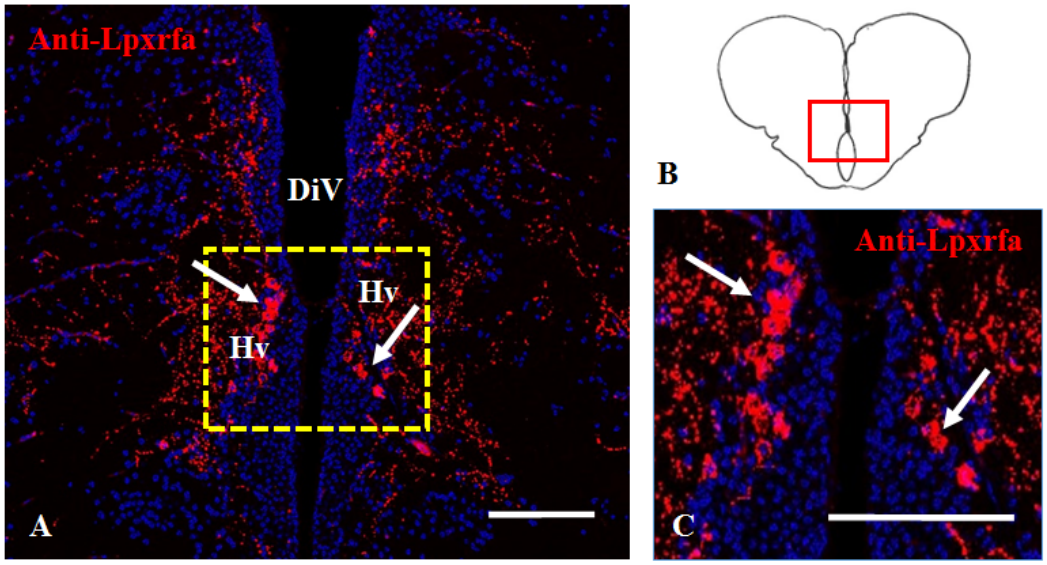

Figure 2.3. Zebrafish Lpxrfa-ir soma are located in the ventral hypothalamus. Coronal sections (B) of adult WT brains immunostained with anti-zebrafish Lpxrfa (red; A, C). The Lpxrfa-ir soma (white arrows) are located in a cluster in the ventral zone of the periventricular hypothalamus that is adjacent to the diencephalic ventricle (A). C is the magnified version of the yellow square in A. Hv = ventral zone of the periventricular hypothalamus. DiV = diencephalic ventricle. Scale bars = 100 µm.

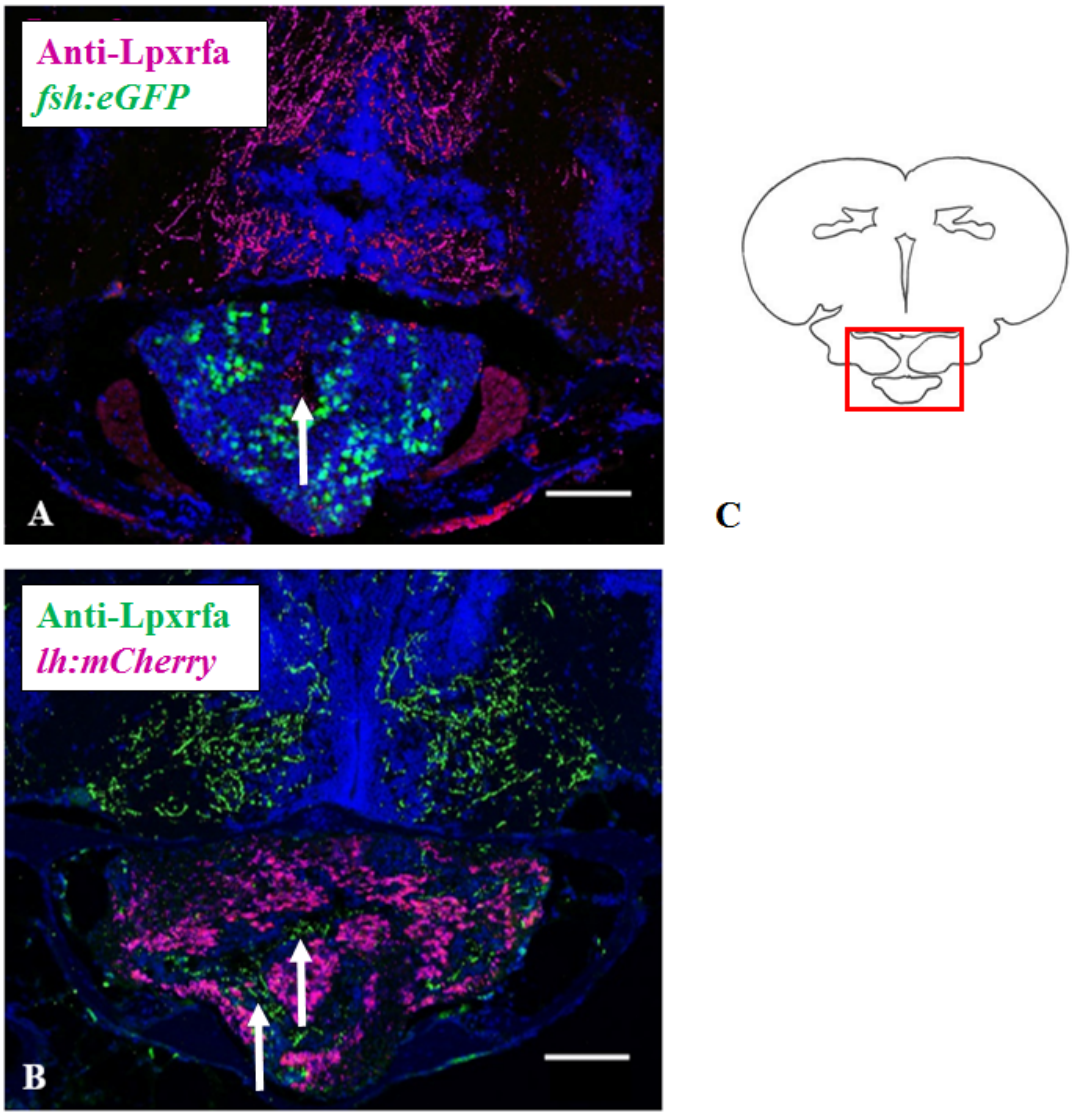

Figure 2.4. Zebrafish Lpxrfa-ir fibers weakly innervate the pituitary. Coronal sections (C) of *fsh:eGFP* adult brain/pituitary (Fsh-labeled cells in green; A) and *lh:mCherry* adult brain/pituitary (Lh-labeled cells in magenta; B) immunostained with anti-zebrafish Lpxrfa in magenta (A) and green (B) (see white arrows). Scale bars = 100 µm.

proximal pars distalis with a higher prevalence of Lh-labeled cells than Fsh-labeled cells, as seen in previous zebrafish studies (Golan et al. 2015). Overall, Lpxrfa-ir innervation (Figure 2.4A, B) in the pituitary was detectable, though very weak. In addition, Lpxrfa-ir fibers in the pituitary were not in direct contact with neither Fsh-labeled nor Lh-labeled cells and appeared to be localized to the medial region of the neurohypophysis (Figure 2.4A, B).

***Lpxrfa-3 Effects on Pituitary Gonadotropin Gene Expression* in vitro**

We incubated adult male pituitary explants with zebrafish Lpxrfa-3 to determine whether Lpxrfa directly influences the expression of pituitary gonadotropins. After 18 hours of incubation, samples were taken to assess mRNA levels of *fshβ, lhβ*, and *cgα*. While there were no changes in *fshβ* mRNA levels, both *lhβ* and *cgα* levels were reduced, in response to Lpxrfa-3 administration (Figure 2.5). Two concentrations of Lpxrfa-3 (10 and 100 pM) significantly reduced *lhβ* mRNA levels compared to the control, while 1, 10, and 100 pM concentrations of Lpxrfa-3 significantly reduced *cgα* mRNA levels compared to the control (Figure 2.5). Interestingly, for both *lhβ* and *cgα*, 1000 pM Lpxrfa-3 did not affect mRNA levels (Figure 2.5).

***Lpxrfa-3 Effects on Pituitary Gonadotropin Gene Expression* in vivo**

Because zebrafish Lpxrfa-3 was capable of inhibiting *lhβ* and *cgα* expression in the pituitary *in vitro*, we tested the administration of zebrafish Lpxrfa-3 to adult zebrafish to determine if the same effect could occur *in vivo*. Three hours after the second intraperitoneal injection of zebrafish Lpxrfa-3, zebrafish were sampled for pituitaries, which were used to conduct QPCR on *fshβ*, *lhβ*, and *cgα*. Unlike what

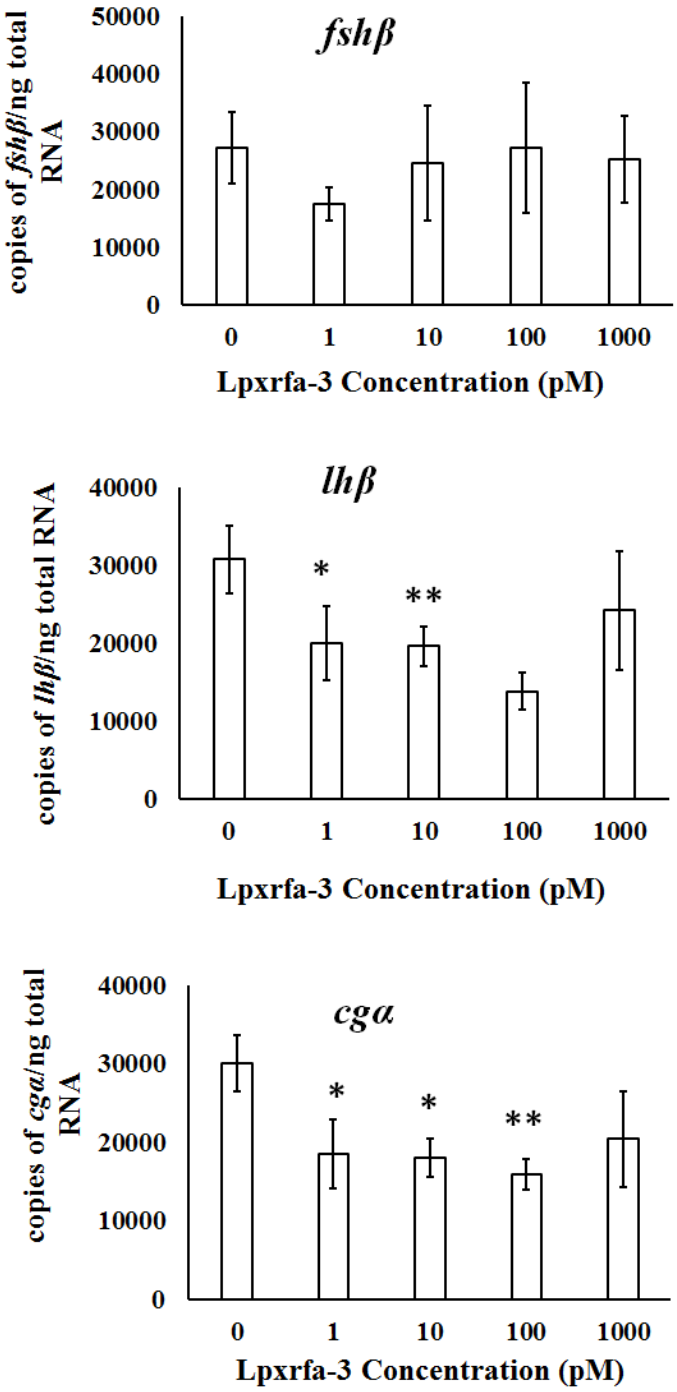

Figure 2.5. Zebrafish Lpxrfa-3 administration reduces *lhβ and cgα* mRNA levels in adult male pituitaries *in vitro*. After 18 hours of incubation with zebrafish Lpxrfa-3, whole pituitary explants from adult male zebrafish did not have altered *fshβ* mRNA levels but did have reduced *lhβ* levels at 10 and 100 pM and *cgα* levels at 1,

10, and 100 pM *in vitro*. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. Differences between each Lpxrfa-3 concentration and the control (0 pM) were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$.

was observed *in vitro*, the pituitaries of fish that were injected with Lpxrfa-3 did not have significantly different *fshβ*, *lhβ*, or *cgα* levels than those injected with the vehicle (PBS; Figure 2.6). Therefore, the effects of zebrafish Lpxrfa-3 on pituitary gonadotropin expression differs, depending on whether the experiment is conducted *in vitro* or *in vivo*.

Zebrafish Lpxrf Receptor Activation by Lpxrfa Peptides

In order to generate activation profiles for each of the Lpxrf receptors, we measured luciferase bioluminescence 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 2.7). However, Lpxrf-R2 and Lpxrf-R3 both exhibited dose-response curves within the CRE pathway (PKA/cAMP) by all three Lpxrfa peptides (Figure 2.8). For both receptors, Lpxrfa-2 and Lpxrfa-3 peptides were the stronger elicitors of a dose response, while Lpxrfa-1 peptide (the longest peptide) was the weakest. The EC_{50}s of Lpxrf-R2 via CRE were 2.43 x 10^{-8} M (± 2.12 x 10^{-8} M), 4.64 x 10^{-9} M (5.96 x 10^{-10} M), and 1.88 x 10^{-9} M (± 2.39 x 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 x

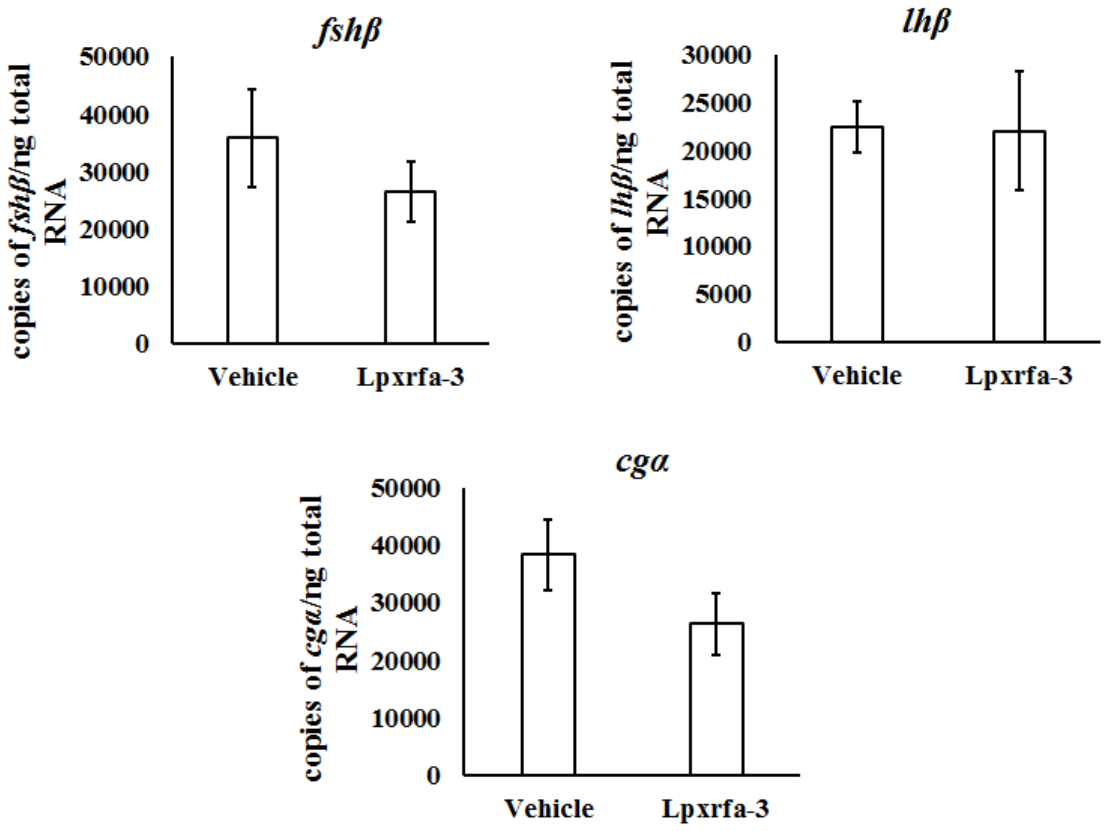

Figure 2.6. Zebrafish Lpxrfa-3 *in vivo* administration does not affect the expression of the pituitary gonadotropin genes in adults. After two consecutive injections of 0.5 µg/fish and 1 µg/fish of zebrafish Lpxrfa-3 for males and females, respectively, *fshβ*, *lhβ*, and *cgα* mRNA levels were determined via QPCR, which demonstrated no changes. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. Differences between the effects of the vehicle and the treatment were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$.

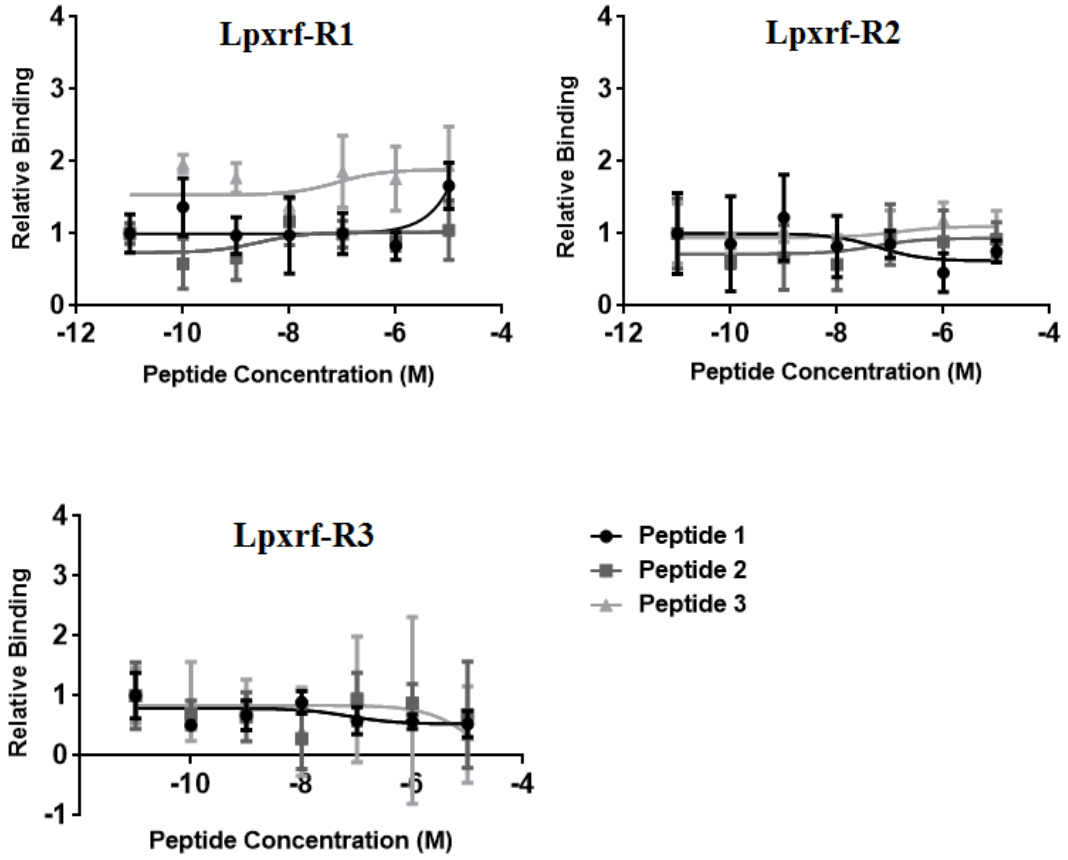

Figure 2.7. The activation of the PKC/Ca^{2+} pathway (SRE) in all three zebrafish Lpxrf receptors by all three zebrafish Lpxrfa peptides. The zebrafish Lpxrf-R1, Lpxrf-R2, and Lpxrf-R3 were exposed to either Lpxrfa-1 (black circles), Lpxrfa-2 (dark grey squares), or Lpxrfa-3 (light grey triangles), using a dual luciferase reporter assay. None of the peptides (at any concentration tested) activate any of the receptors through the PKC/Ca^{2+} pathway.

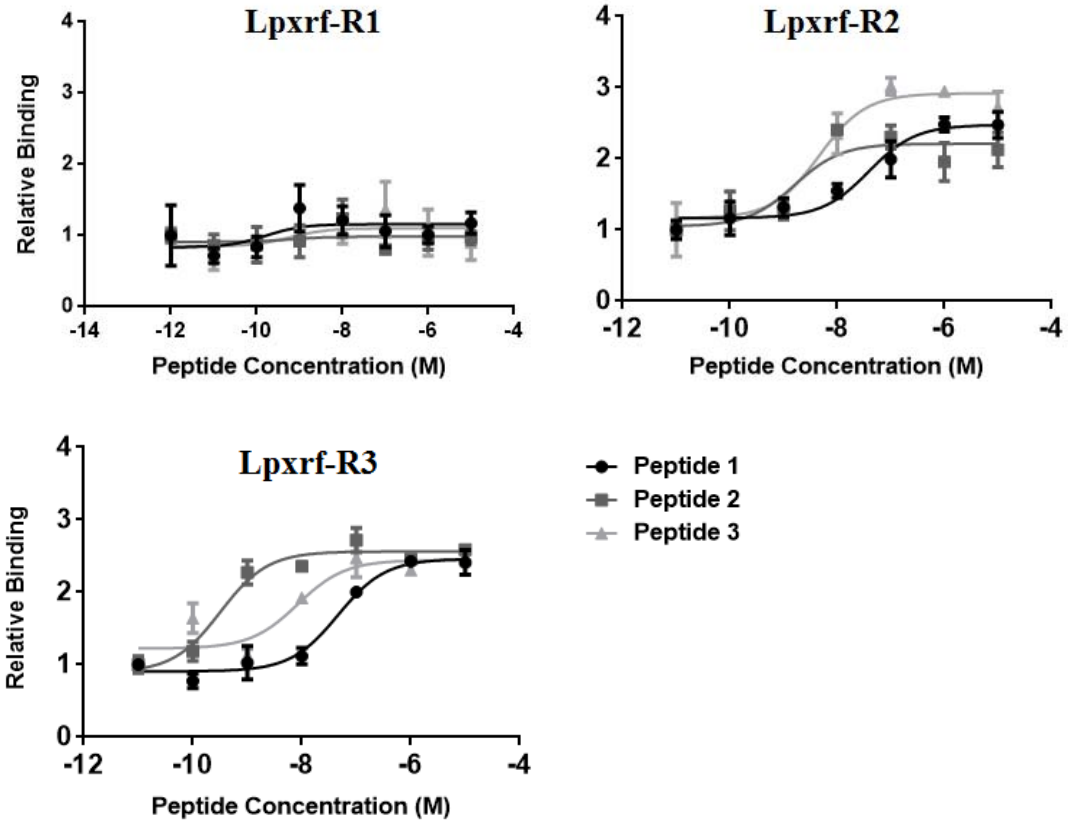

Figure 2.8. The activation of the PKA/cAMP pathway (CRE) in all three zebrafish Lpxrf receptors by all three zebrafish Lpxrfa peptides. The zebrafish Lpxrf-R1, Lpxrf-R2, and Lpxrf-R3 were exposed to either Lpxrfa-1 (black circles), Lpxrfa-2 (dark grey squares), or Lpxrfa-3 (light grey triangles), using a dual luciferase reporter assay. All of the peptides activated Lpxrf-R2 and Lpxrf-R3 through the PKA/cAMP pathway. The EC_{50}s for each peptide's activation in Lpxrf-R2 and Lpxrf-R3 are illustrated by the dashed lines and are color-coded as mentioned above. However, Lpxrf-R1 was not activated by any of the peptides through this pathway.

10^{-8} M (± 3.78 x 10^{-8} M), 6.91 x 10^{-10} M (± 3.20 x 10^{-10} M), and 4.75 x 10^{-9} M (± 4.28 x 10^{-9} M) for peptides Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3, respectively. After multiple experiments, Lpxrf-R1, on the other hand, did not demonstrate any response of the CRE pathway by any of the Lpxrfa peptides (Figure 2.8).

An alignment of the amino acid sequences of all three zebrafish Lpxrf receptors, conducted by Zhang et al. (2010), is presented in Figure 2.9, along with quail GNIHR and human NPFF1 receptor. When analyzing the homologies of the three major regions of the three zebrafish Lpxrf receptors, we found the following: The N-terminus is 27-30% conserved among the three receptors, while the transmembrane domains are highly conserved (62-72%), which is typical of GPCRs (Parhar et al. 2004, Nocillado et al 2007). For the C-terminus, however, there is 11-40% homology among the three Lpxrf receptors. Focusing solely on the C-terminus, the region that tends to exhibit the most variation (Parhar et al. 2004, Nocillado et al 2007), there is 40% homology between Lpxrf-R2 and -R3. However, Lpxrf-R1 has 11% and 24% homology with Lpxrf-R2 and Lpxrf-R3, respectively.

Lpxrfa Activation of Kiss Receptors and Inhibition of Kiss-Activated Kiss Receptors

Because none of the three zebrafish Lpxrf receptors could be detected histologically in the zebrafish brain/pituitary in this study, 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 2.10). However, Kiss2 and Kiss1 activated Kiss1ra and Kiss1rb, respectively, in a dose-dependent manner (Figure 2.10), as seen in Zmora et

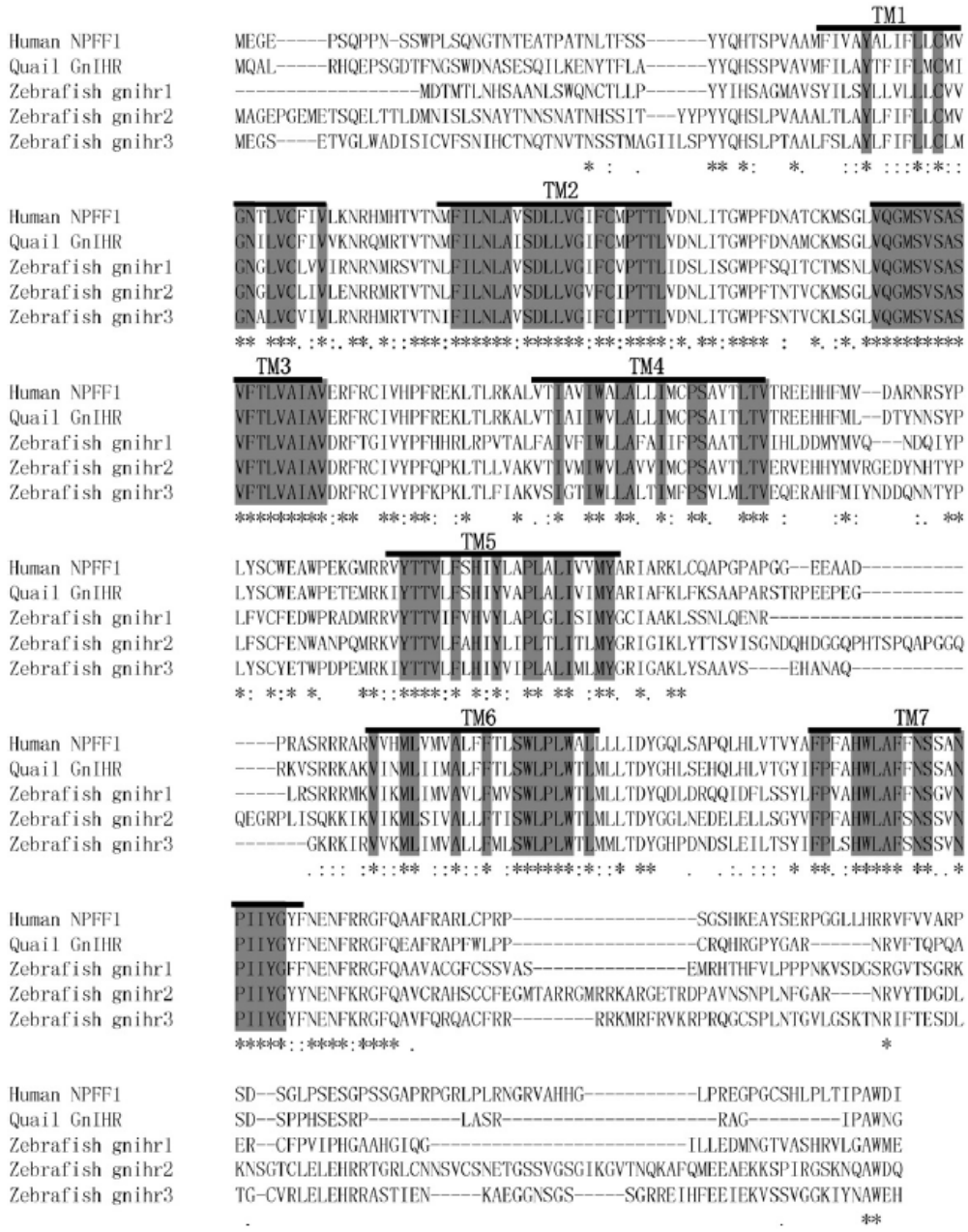

Figure 2.9. Amino acid sequence alignment of zebrafish Lpxrf receptors (gnihr1-3), alongside quail GNIHR and human NPFF1 receptor from Zhang et al. (2010). Caption from Zhang et al. (2010): Multiple alignment of the deduced aa sequences of the zebrafish gnihrs with the human and the quail sequences. The putative transmembrane regions are indicated by horizontal bars. The identical aa in the transmembrane regions are shaded in gray.

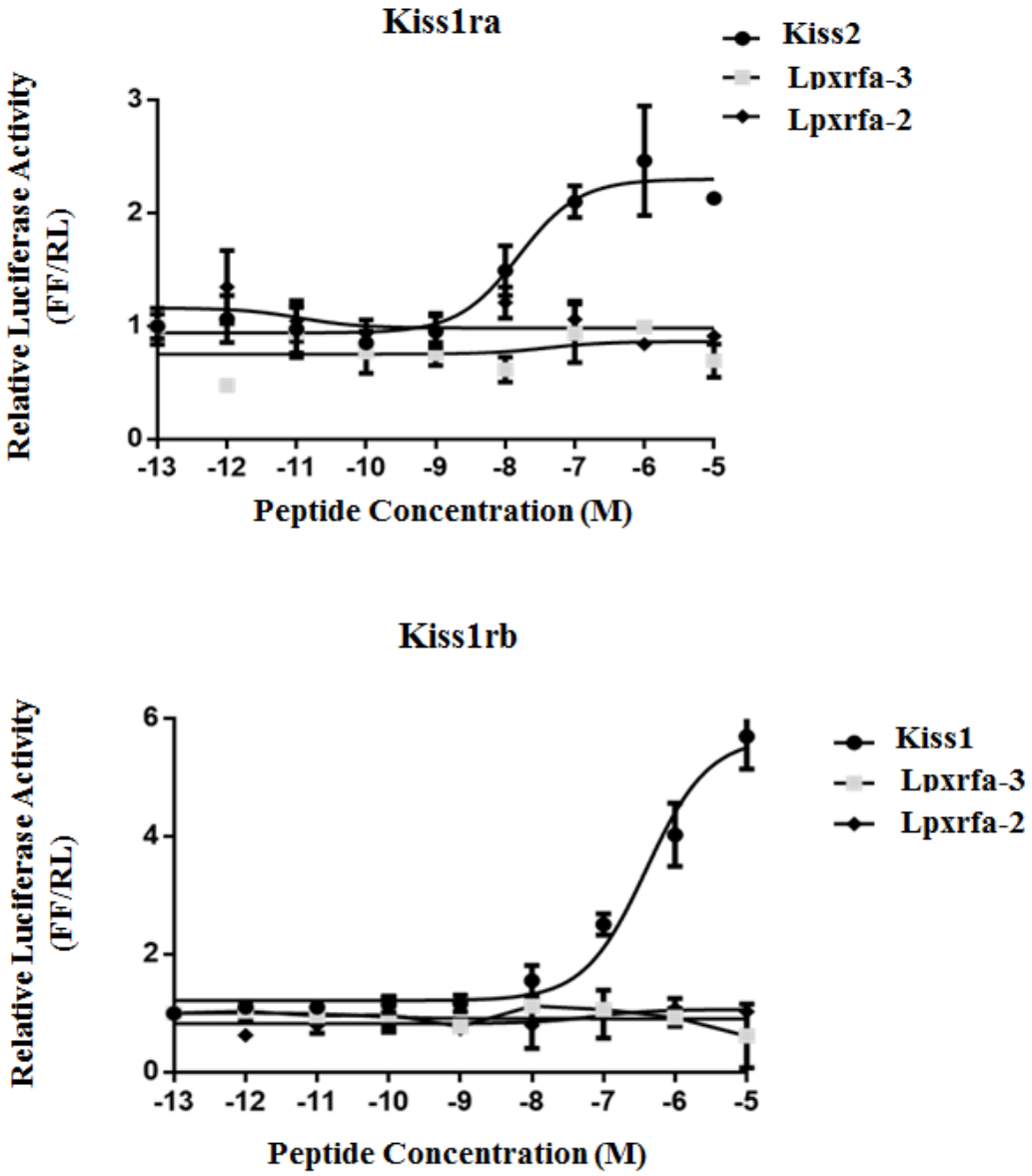

Figure 2.10. The activation of the PKC/Ca^{2+} pathway (SRE) in zebrafish Kiss1ra and Kiss1rb by Kiss1/Kiss2, Lpxrfa-2, and Lpxrfa-3. Lpxrfa-2 and Lpxrfa-3 did not activate either Kiss1ra or Kiss1rb; however, Kiss2 and Kiss1 activated Kiss1ra and Kiss1rb, respectively. Values are presented as mean ± standard deviation. black circles, Kiss1/2. grey squares, Lpxrfa-3. black diamonds, Lpxrfa-2.

al. (2015). Therefore, Lpxrfa-2 and Lpxrfa-3 were subsequently used to determine if they could inhibit Kiss1's activation and Kiss2's activation of Kiss1rb and Kiss1ra, respectively. For Kiss1rb, none of the concentrations of Lpxrfa-3 inhibited Kiss1's activation, while only the highest concentrations (10^{-7} and 10^{-6} M) of Lpxrfa-2 slightly inhibited Kiss1's activation (Figure 2.11). Regarding Kiss1ra, most of the lower concentrations of Lpxrfa-3 inhibited Kiss2's activation, while almost all of the Lpxrfa-2 concentrations inhibited Kiss2's activation (IC_{50} = 5.915 x 10^{-13} M; Figure 2.11).

Neuroanatomical Localization of Lpxrfa-ir Fibers and* kiss1ra-*Expressing Cells

Because Lpxrfa-2 and Lpxrfa-3 had significant effects on inhibiting Kiss2's 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 were widespread throughout the zebrafish brain, as seen in the European sea bass (Escobar et al. 2013) and striped bass (Zmora et al. 2012). In the pre-optic area and hypothalamus, we found several *kiss1ra*-expressing cells that were innervated by Lpxrfa-ir fibers in the slides treated with the anti-sense riboprobe and anti-zebrafish Lpxrfa (Figure 2.12A, D). Slides of the preoptic area and the hypothalamus treated with the sense riboprobe revealed no *kiss1ra*-expressing cells (Figure 2.12C, F).

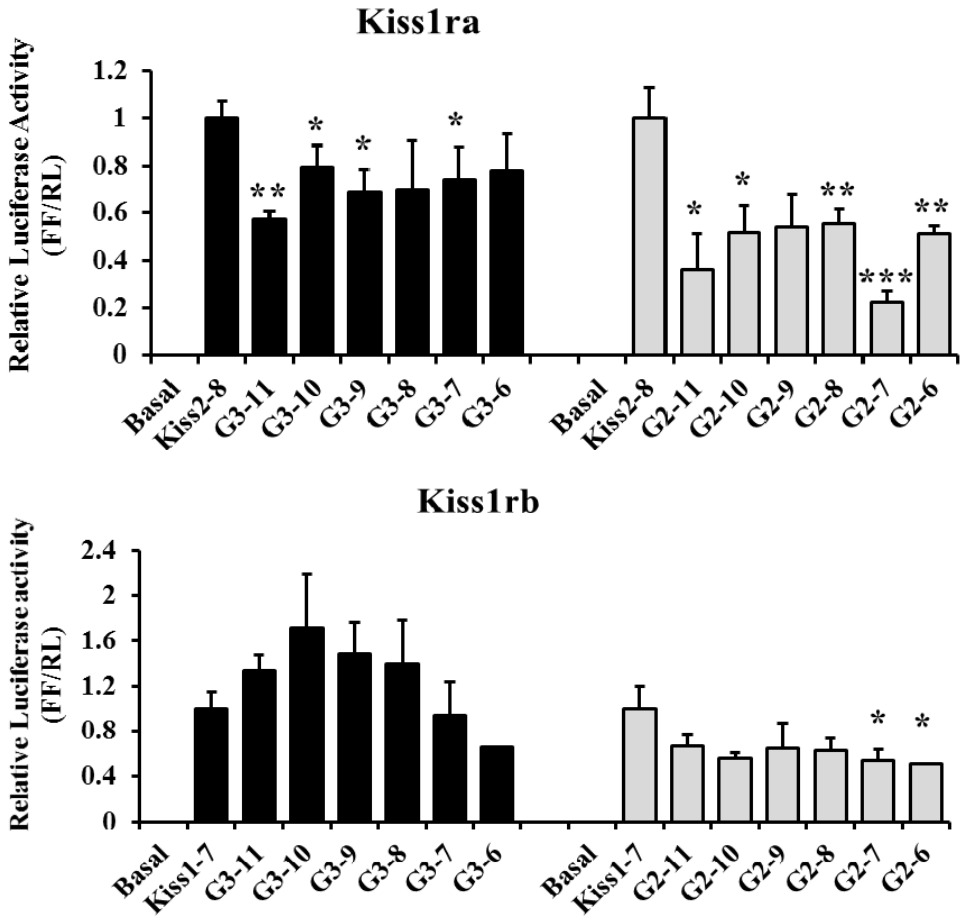

Figure 2.11. Inhibition of Kiss-stimulated activation of Kiss receptors by Lpxrfa peptides in the PKC/Ca^{2+} (SRE) pathway. High concentrations of Lpxrfa-2 (G2) slightly inhibited Kiss1's activation of Kiss1rb, while most concentrations of Lpxrfa-2 inhibited Kiss2's activation of Kiss1ra (IC_{50} = 5.915 x 10^{-13} M). Lpxrfa-3 (G3) also inhibited Kiss2's activation of Kiss1ra at most concentrations but had no effect on Kiss1's activation of Kiss1rb. Values are presented as mean ± standard deviation. Differences between control (activation by Kiss) and each treatment were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. Numbers after G2 or G3 represent the molarity concentrations (e.g., "G3-11" is Lpxrfa-3 at 10^{-11} M).

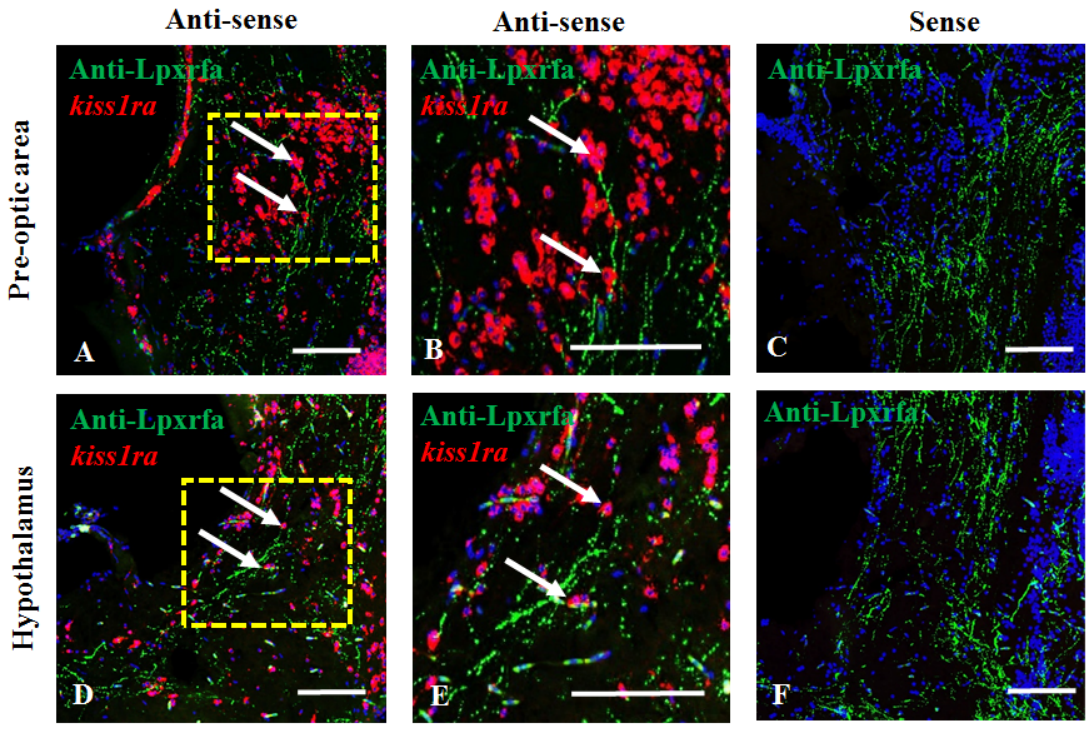

Figure 2.12. Zebrafish *kiss1ra*-expressing cells are innervated by Lpxrfa-ir fibers in the forebrain. The anti-sense *kiss1ra* riboprobe stained cells (red) in many regions of the brain, especially the preoptic area (A) and the hypothalamus (D), and these cells were innervated (white arrows) by Lpxrfa-ir 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 in any regions of the brain (C, F). Scale bars = 100 µm.

DISCUSSION

In this chapter, valuable information is presented on the characterization of the Lpxrfa/Lpxrf-R system in the zebrafish by providing neuroanatomical distribution, functional effects on gonadotropin synthesis, receptor activation pathways, and

potential utilization of additional receptor pathways. This study demonstrates the first localization of Lpxrfa soma in the zebrafish brain in the ventral zone of the periventricular hypothalamus with fibers extending throughout the fore-, mid-, and hind-brains and into the pituitary. We have also demonstrated that Lpxrfa plays a negative role on two of the pituitary gonadotropin genes, at least in an *in vitro* setting. In addition, receptor activation assays have revealed that Lpxrf-R2 and Lpxrf-R3 in zebrafish are activated by all three zebrafish Lpxrfa peptides through the PKA/cAMP pathway. Lastly, zebrafish Lpxrfa-2 and Lpxrfa-3 peptides have the ability to antagonize Kiss2-activated Kiss1ra receptors. An indication that this pathway can actually occur in the zebrafish brain comes from the observation of the innervation of *kiss1ra*-expressing neurons by Lpxrfa-ir fibers.

In order to visually detect Lpxrfa, we originally planned to generate a *lpxrfa:eGFP* transgenic line through the modification of BAC DNA so that the activation of the *lpxrfa* promoter drives the expression of eGFP. While embryos injected with the transgene and *tol2* mRNA exhibited transient eGFP expression in the hypothalamus, which was verified by *lpxrfa in situ* hybridization and Lpxrfa immunohistochemistry, neither the germ cells nor the somatic cells of the F0 fish possessed the transgene, once the embryos reached adulthood. Consequently, we could not establish a *lpxrfa:eGFP* line, in which the transgene was inherited. However, the same BAC recombination methods used in this study were used to generate a *gnrh2:eGFP* transgenic line in zebrafish (Xia et al. 2014); therefore, the ability to produce a transgenic line can be gene-specific. In addition, it is possible that the BAC DNA did not contain enough of the upstream regulatory elements of the

lpxrfa gene to allow for the activation of the *lpxrfa* promoter, which would determine the expression of eGFP. Because the *lpxrfa:eGFP* transgenic line could not be produced, we, instead, utilized the polyclonal zebrafish Lpxrfa antibody to visualize Lpxrfa in fixed tissues. The anti-zebrafish Lpxrfa demonstrated to be very specific as determined by its ability to detect the same *lpxrfa*-expressing cells detected via *in situ* hybridization and its recognition of COS7 cells expressing recombinant Lpxrfa transfected with the *lpxrfa:*pcDNA3.1 plasmid.

Using the zebrafish Lpxrfa antibody, brain localization of zebrafish Lpxrfa soma in this study revealed a single cluster in the ventral zone of the periventricular hypothalamus, adjacent to the diencephalic ventricle. Similar results were found in the cyprinid, the goldfish, which also possesses Lpxrfa soma in the same region in the hypothalamus and in the nervus terminalis (Sawada et al. 2002). Some fish species possess Lpxrfa soma in different brain regions, including the nucleus reticularis and octaval nucleus in the hindbrain for Indian major carp (Biswas et al. 2015) and the posterior ventricular nucleus of the caudal preoptic area, the nucleus posterioris periventricularis, and the nucleus olfacto-retinalis for cichlids (DiYorio et al. 2016, Ogawa et al. 2016). 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 (Paullada-Salmerón et al. 2016a); however, there are no Lpxrfa-ir soma in the hypothalamus. Therefore, Lpxrfa soma anatomical localization in the brain can be species-specific, with perhaps more evolutionary advanced fish (e.g., perciforms) possessing more extensive soma distribution than less evolved fish

species (e.g., cyprinids). There is also the possibility of sex and reproductive status affecting neuroanatomical localization and expression of neuropeptides in the brain, as seen in the European sea bass (Alvarado et al. 2013) and the striped bass (Zmora et al. 2012). However, no major sex-specific differences in Lpxrfa-ir soma and fiber distribution in the brain and pituitary were noted in this study.

In addition to distribution in the brain, this study demonstrated that zebrafish Lpxrfa can be considered a "hypophysiotropic" neuropeptide due to its innervation of the pituitary (though somewhat weakly), as seen in almost all fish examined to date (Sawada et al. 2002, Biran et al. 2014, Biswas et al. 2015, Paullada-Salmerón et al. 2016a). Thus, it is not surprising that Lpxrfa tends to regulate gonadotropin production in many of these species (Qi et al. 2013, Biran et al. 2014, Paullada-Salmerón et al. 2016b). Because Lpxrfa-ir fibers in this study did not reveal direct contact with gonadotropes in the proximal pars distalis but appeared to be localized to the neurohypophysis, it is possible that Lpxrfa peptides reach the adenohypophysis via blood vasculature, as seen in Gnrh3 in zebrafish (Golan et al. 2015), instead of direct innervation of the gonadotrope cells. In the European sea bass, on the other hand, Lpxrfa-ir fiber innervations in the pituitary are closely associated with Fsh-, Lh-, and Gh-producing cells (Paullada-Salmerón et al. 2016a). It is possible that the sea bass does not utilize a neurosecretory-vasculature system for accessing the gonadotropes, unlike the zebrafish (Golan et al. 2015). Most importantly, this weak 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 seen by the results of the pituitary culture study.

In this study, zebrafish Lpxrfa-3 exhibits a direct inhibitory effect on *lhβ* and *cgα* mRNA levels at low physiological levels (10 pM) in the pituitary explants experiment. These results are supported by Zhang et al. (2010), who demonstrated that goldfish Lh levels can be reduced by zebrafish Lpxrfa-3 administration. Other fish species have also demonstrated an inhibitory effect of Lpxrfa on gonadotropin synthesis/release, including the male European sea bass (Paullada-Salmerón et al. 2016b), common carp (Peng et al. 2016), and the cichlid *Cichlasoma dimerus* (DiYorio et al. 2016). However, some species exhibit stimulatory effects, such as the tilapia (Biran et al. 2014), grass puffer (Shahjahan et al. 2010), and goldfish (Moussavi et al. 2012), which is occasionally influenced by gonadal state (Moussavi et al. 2012). For instance, administration of Lpxrfa peptides to goldfish in early gonadal recrudescence leads to increased *lhβ* levels, while no effect is seen in late recrudescence (Moussavi et al. 2012). It is possible that, because the zebrafish is a daily spawner in which the ovaries exhibit asynchronous oocyte development (Eaton and Farley 1974), Lpxrfa has a mostly negative impact on gonadotropins in this species and does not vary between stimulatory and inhibitory, as seen in some species that display distinct, gradual stages of gonadal development, like the goldfish (Moussavi et al. 2012). Therefore, because teleost Lpxrfa peptides can be stimulatory and/or inhibitory on gonadotropin production, it can be postulated that teleosts present a transitional/intermediate evolutionary stage between Lpxrfa peptides of jawless fish that exhibit stimulatory effects and GNIH/RFRP of birds/mammals that have inhibitory effects on gonadotropins (Osugi et al. 2012, Tsutsui et al. 2012). The

multiplicity of the teleost Lpxrfa peptides and their cognate receptors may be the basis of the variable effects of these peptides.

Although zebrafish Lpxrfa-3 was capable of downregulating *lhβ* and *cgα* mRNA levels in an *in vitro* setting, there were no significant effects of zebrafish Lpxrfa-3 on pituitary gonadotropin expression levels *in vivo*. For *cgα*, at least, there was a decreasing trend; however, this trend was not significant. Thus, the results of our *in vitro* and *in vivo* studies of Lpxrfa-3's effects on the pituitary were not in agreement. In the goldfish Lpxrfa system, *fshβ* and *lhβ* mRNA levels increase when goldfish Lpxrfa is administered *in vivo* during early recrudescence of the fish (Moussavi et al. 2012). However, using pituitaries in culture sampled from goldfish in early recrudescence, *fshβ* and *lhβ* mRNA levels decrease dose-dependently in the presence of goldfish Lpxrfa (Moussavi et al. 2012). Therefore, the differences observed between the effects of teleost Lpxrfa on pituitary gonadotropin expression in *in vitro* and *in vivo* settings is not limited to the zebrafish. It is possible that the lack of an effect in our *in vivo* experiment is due to the interaction of other reproductive and non-reproductive factors that are available in an *in vivo* setting. Unlike our *in vitro* experiment, in which only the pituitary is present, the *in vivo* experiment permits the interaction of other factors, such as neuropeptides from the brain (e.g., Gnrh, Kiss, etc.), hormones from the gonad (e.g., steroids, inhibin, activin, etc.), or other hormones from peripheral tissues (e.g., insulin-like growth factors from liver). For instance, GNIH and its orthologs have been described to be influenced by a number of factors, particularly melatonin (Chowdhury et al. 2010), stress (Kirby et al. 2009), and sex steroids (Molnár et al. 2011). Therefore, it is possible that these

factors or others are interacting with zebrafish Lpxrfa-3 to comprehensively influence its effect on pituitary gonadotropin expression *in vivo*.

Other explanations for this difference between the *in vitro* and *in vivo* results include the following: One possibility is that the systemic administration, which requires transportation of the peptide via the bloodstream, poses a limiting factor – permitting quick degradation and/or lacking the appropriate carrier protein(s). Another explanation is that the peptide does not cross the blood-brain barrier. These two possibilities can be overcome by intracerebroventricular injection of the peptide, which was not performed in this experiment. In addition, more peptide doses and injection/sampling times should also be included to determine if an *in vivo* effect can be observed. However, in this study, we have, at least, demonstrated that zebrafish Lpxrfa-3 is capable of directly inhibiting *lhβ* and *cgα* mRNA levels in the zebrafish pituitary, when the influential factors that are outside of the pituitary are removed.

While zebrafish Lpxrfa-3 has an inhibitory effect on Lh in goldfish (Zhang et al. 2010), the goldfish Lpxrfa precursor peptide has a stimulatory effect on *fshβ* and *lhβ* during certain reproductive stages in the goldfish (Moussavi et al. 2012). Thus, it is reasonable to assume that the effect of Lpxrfa peptides on gonadotropins is related to the sequence of the peptide. However, a comparison of goldfish Lpxrfa-3, which can have a stimulatory effect on gonadotropins (Moussavi et al. 2012) and zebrafish Lpxrfa-3, which has an inhibitory effect on gonadotropins (Zhang et al. 2010), reveals that these two peptides are homologous, with both having the core SATLPQRF-NH_2 sequence at the C-terminus. Regardless, we examined the sequences of deduced Lpxrfa peptides from multiple teleost species and the GNIH/RFRP-3 sequences from

some bird/mammal species in an attempt to identify a sequence motif that can be linked to the effects on gonadotropins. The peptide sequence alignment (Figure 2.13) revealed that the inhibitory or stimulatory effects on the gonadotropins are not related to the amino acid sequences of these peptides. Because GNIH and its orthologs have been demonstrated to interact with a multitude of other reproductive and non-reproductive neuropeptides in the brain (for review, see Ubuka et al. 2012b), it is possible that the ability of Lpxrfa peptides to stimulate or inhibit gonadotropins in teleosts is due to their interactions with one or more of these other neuropeptides, including Gnrh1 or Gnrh3, or with multiple receptors. It is also possible that the combined effects of different forms of Lpxrfa peptides from one species (Moussavi et al. 2012) can have different, additive, synergistic, or neutralizing effects on the gonadotropins compared to the individual Lpxrfa peptide (Zhang et al. 2010).

In the zebrafish, all three Lpxrfa peptides are capable of activating both Lpxrf-R2 and Lpxrf-R3 only through the PKA/cAMP pathway, while none of the peptides could activate Lpxrf-R1 through neither the PKA/cAMP nor the PKC/Ca^{2+} pathways. The lack of activation in 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, we believe that Lpxrf-R1 is a non-functional receptor or is potentially utilizing a pathway other than the PKA/cAMP and PKC/Ca^{2+} signal transduction pathways. The utilization of the CRE (PKA/cAMP) pathway has been seen in other fish Lpxrfa systems, including the tilapia (Biran et al. 2014) and the orange-spotted grouper (Wang et al. 2015). Both of these species only possess one Lpxrf receptor, compared

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zebrafishLpxrfa-3                    SATLPQRF -
tilapiaLpxrfa-2                    PNSSPNLPQRF +
quailGNIH                            PSAYLPLRF -
ratRFRP-3                            PS--LPQRF -
starlingGNIH                         PFANLPLRF -
seabassLpxrfa-2                   SPNSTPNMPQRF --
seabassLpxrfa-1                    PLHLHANMPMRF -
carpLpxrfa-2                    PIIKKPTHLHANLPLRF -
carpLpxrfa-3                       DRASKSTINLPQRF -
pufferLpxrfa-2                LYPPTLQPHHQHVNMPMRF +
pufferLpxrfa-3               DGVQGGDHVPNLNPNMPQRF +
goldfishLpxrfa-1                    PTHLHANLPLRF +-
goldfishLpxrfa-2                     AKSNINLPQRF +-
goldfishLpxrfa-3                  SGTGLSATLPQRF +-
clownfishLpxrfa                   SGTGLSATLPQRF +
cdLpxrfa-1                         TPNSSPNLPQRF -
cdLpxrfa-2                           APNQVLPQRF +
lampreyLpxrfa-2                   SEPFWHRTRPQRF +
hagfishLpqrfa-1                         ALPQRF +
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Figure 2.13. Amino acid alignment of GNIH/RFRP-3/Lpxrfa peptides from multiple vertebrate species. There is no conserved GNIH/RFRP-3/Lpxrfa peptide motif that can be connected to the reported inhibitory (-) or stimulatory (+) effects of GNIH and its orthologs. cd = *Cichlasoma dimerus*.

to the three of the cyprinids, the zebrafish (Zhang et al. 2010) and goldfish (Qi et al. 2013). Thus, it is possible that teleosts could have lost the other two Lpxrf receptors (possibly due to lack of functionality like zebrafish Lpxrf-R1) before the emergence of perciforms (e.g., tilapia and grouper) and birds/mammals, which possess only one receptor and also utilize the PKA/cAMP pathway (Yin et al. 2005, Ubuka et al. 2009a, Biran et al. 2014, Wang et al. 2015). In addition, the orange-spotted grouper and tilapia Lpxrf receptors are also capable of utilizing the SRE (PKC/Ca^{2+}) pathway (Biran et al. 2014, Wang et al. 2015). Consequently, the perciform utilization of SRE

and CRE pathways for Lpxrf receptors may explain why the Lpxrfa peptides of these species are flexible and can exert stimulatory (Biran et al. 2014) and inhibitory (Wang et al. 2015) effects on gonadotropins.

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 detected using end-point PCR and QPCR in this study and using end-point PCR in Zhang et al. (2010). 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 with was tested, alongside the non-amplified procedure. 3) We selected samples in which the *lpxrfr2* and *lpxrfr3* mRNA levels determined via QPCR were highest (adult vs juvenile, male vs female, and morning vs evening). 4) A positive control using an anti-sense riboprobe against *lpxrfa* was employed at each run. However, none of these methods allowed the *lpxrfr2* and *lpxrfr3* mRNA signals to be visualized histologically (Figure 2.14).

There is only one report that demonstrates the histological localization of Lpxrf receptor mRNA in the teleost brain (Qi et al. 2013), although the expression is still detectable through PCR in Zhang et al. (2010). The results of this goldfish report, however, are questionable because of the widespread, non-specific distribution of the signals, the lack of proper controls, and the staggering similarity between the localization of two of the receptors (Qi et al. 2013). Similarly, in the tilapia,

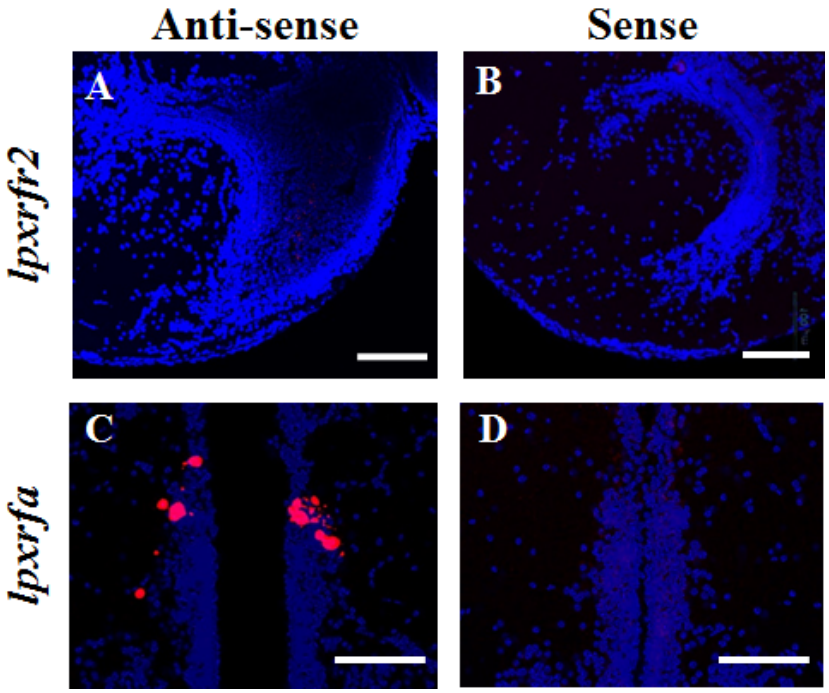

Figure 2.14. Histological detection of Lpxrf receptor mRNA was not successful in the zebrafish. *In situ* hybridization was conducted on *lpxrfr2* because its expression was much greater than *lpxrfr3* in the brain (data not shown) and because Lpxrf-R1 appears to be a non-functional receptor. The *in situ* hybridization protocol utilized the TSA Plus Kit to amplify the signal, and the results revealed no detectable expression of *lpxrfr2* in adult female brains sampled in the morning (hypothalamus shown in A, B). However, the positive control of *lpxrfa in situ* hybridization yielded *lpxrfa*-expressing soma (red) in the expected region of the hypothalamus (C, D). Scale bars = 100 µm.

exhausting efforts to detect the Lpxrf receptor mRNA in the brain have failed, which forced the authors to generate an antibody against the C-terminus of the receptor to detect it by immunohistochemistry (Ogawa et al. 2016). 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. This is in stark contrast to the expression of *kiss1ra*, which tends to be highly expressed and is distributed in multiple, yet distinct, brain regions in teleosts (Servili et al. 2011, Zmora et al. 2012, Escobar et al. 2013). In addition, after consulting with other researchers who have focused on Lpxrf receptors, it appears that the difficulty in histologically detecting these transcripts is most likely due to their expression being highly dependent on the physiology of the organism (J. A. Muñoz-Cueto, personal communication).

Due to the inability to localize Lpxrf receptors in the zebrafish brain-pituitary axis, we were unable to determine the direct target sites of Lpxrfa peptides. We, therefore, shifted our attention to another important neuropeptide: kisspeptin. Kisspeptins, like the Lpxrfa peptides, belong to the RFamide peptide family. Kisspeptin, known for its positive effect on the BPG axis, is a direct stimulator of GNRH expression and secretion (Irwig et al. 2005), and Kiss receptors are widely expressed throughout the teleost brain (Servili et al. 2011). Together with the common knowledge that neuropeptide receptors are promiscuous, we set forth to test the possibility that Lpxrfa peptides utilize an additional pathway (i.e., the kisspeptin receptor pathway) to exert their effects on reproduction. While zebrafish Lpxrfa-2 and Lpxrfa-3 peptides do not directly activate zebrafish Kiss1ra or Kiss1rb, they do

antagonize Kiss2's activation of Kiss1ra, the reproductively related Kiss receptor (Servili et al. 2011, Zmora et al. 2014). Because Lpxrfa-ir fibers also innervate *kiss1ra*-expressing cells in the forebrain, it is reasonable to assume that zebrafish Lpxrfa-2 and Lpxrfa-3 might elicit their functions also through Kiss1ra. Thus, in addition to 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 actions.

Interestingly, the antagonistic effects of Lpxrfa-2 and Lpxrfa-3 on Kiss2's activation of Kiss1ra are obvious at the low dose of 10 pM, which is much lower than the calculated EC_{50}s of Lpxrf-R2 ($EC_{50} \geq 1880$ pM) and Lpxrf-R3 ($EC_{50} \geq 691$ pM). This suggests that very low levels of Lpxrfa peptides are needed to exert this antagonistic effect and that the inhibition of Kiss1ra is more sensitive than Lpxrfa-2's and Lpxrfa-3's activation of Lpxrf-R2 and Lpxrfa-R3. Therefore, it is possible that Lpxrfa peptides utilize the antagonistic pathway of Kiss1ra more readily than the activation pathways of the Lpxrf receptors to exert their functions. Further studies utilizing Kiss receptor knockout lines and/or Kiss2 antagonists are needed to determine whether this pathway is indeed being utilized. In addition, GNIH and its orthologs are capable of interacting with other types of reproductive neuropeptides in the brain, including GNRH neurons (Smith et al. 2008). Therefore, there are potential additional routes in the brain/pituitary axis by which Lpxrfa can exert its effects on the pituitary.

In summary, the zebrafish Lpxrfa/Lpxrf-R system has been characterized in this chapter in terms of descriptive and functional assays. Using a polyclonal

antibody developed for this purpose, we have localized zebrafish Lpxrfa soma to the ventral zone of the periventricular hypothalamus of the brain with Lpxrfa fibers extending throughout the fore-, mid-, hind-brains, and the pituitary, similar to other cyprinids. The function of zebrafish Lpxrfa has also been better elucidated in Lpxrfa-3's ability to reduce *lhβ* and *cgα* mRNA levels at low physiological concentrations in the pituitary *in vitro* but not necessarily *in vivo*. Therefore, zebrafish are one of the teleost species that most likely exhibit an inhibitory effect of Lpxrfa on gonadotropins. In addition, we have shown that zebrafish Lpxrf-R2 and Lpxrf-R3 utilize the PKA/cAMP pathway when activated by any of the three peptides encoded in the zebrafish Lpxrfa precursor, while Lpxrf-R1 is not activated. Lastly, Lpxrfa-2 and Lpxrfa-3 are capable of inhibiting Kiss2's activation of the Kiss1ra receptor, which coincides with the innervation of *kiss1ra*-expressing cells by Lpxrfa-ir fibers. Thereby, Lpxrfa might also utilize the Kiss2/Kiss1ra signaling pathway to elicit its effects in the brain/pituitary axis. In general, zebrafish Lpxrfa appears to be a functional reproductive neuropeptide in the brain, inhibiting gonadotropin synthesis in the pituitary, while potentially utilizing pathways of other reproductive neuropeptides to exert its functions.

Chapter 3: Neuroanatomical and Functional Relationships between the Lpxrfa and Gnrh3 Systems in the Zebrafish Brain during Development and Adulthood

ABSTRACT

GNIH and its orthologs have been demonstrated to interact with GNRH neurons in the brain to exert their effects on reproduction in a variety of species, including birds and mammals. However, there is little information on the interactions between Lpxrfa and Gnrh and on the direct, functional effects of Lpxrfa on Gnrh expression in the teleost brain. Focusing on Lpxrfa-Gnrh3 relationships, the goal of this chapter was to determine the modes by which these two important reproductive neuropeptides interact with and affect one another. Therefore, the relationships of the two systems were examined via neuroanatomical, functional, and loss-of-function studies during both adulthood and ontogeny. During early development, expression profiles of *lpxrfa* and *gnrh3* in WT embryos/larvae revealed no correlation between the two transcripts, and knockdown of *lpxrfa* had no effects on *gnrh3* expression. However, in the *gnrh3*$^{-/-}$ knockout line, *lpxrfa* expression is significantly lower than in *gnrh3*$^{+/+}$ fish during early development, indicating that Gnrh3 may participate in the regulation of *lpxrfa* by upregulating this transcript during this time period. Conversely, in adults, double-labeled immunohistochemistry revealed that Lpxrfa-ir fibers interact with and project to Gnrh3-ir soma in the pre-optic area of the brain, and zebrafish Lpxrfa-3 consistently reduced the expression of *gnrh3* in the adult brain *in vitro*. However, this effect of Lpxrfa-3 was not observed with systemic injections in adults, potentially because of the involvement of other unknown factors. Regardless, the overall results indicate that Lpxrfa negatively regulates Gnrh3 in zebrafish. An

indication that Lpxrfa and Gnrh3 may be involved in the regulation of reproductive cyclicity and that Gnrh3 modulates *lpxrfa* expression arose from the finding that, in the adult *gnrh3*$^{-/-}$ knockout line, *lpxrfa* mRNA levels were lower than WT levels only in the female brain in the evening (when Gnrh3 levels increase in WT females). This difference was not evident in the morning when Gnrh3 levels decrease in preparation for spawning. Efforts to detect and localize Lpxrf receptors in the brain, including in Gnrh3 neurons, were unsuccessful. However, because zebrafish Lpxrfa-2 and Lpxrfa-3 antagonize Kiss2's activation of Kiss1ra, because Kiss2 neurons express *kiss1ra*, and because Kiss2 neurons project to Gnrh3 neurons, it is possible that Lpxrfa in zebrafish may utilize the Kiss2/Kiss1ra system to exert an inhibitory effect on Gnrh3. Altogether, it appears that, during ontogeny, Gnrh3 partakes in a one-way regulation of *lpxrfa* and that this relationship evolves to become mutual by adulthood. Based on the neuroanatomical and functional findings in adults, zebrafish Lpxrfa and Gnrh3 mutually interact within the adult brain, providing an additional pathway, other than direct pituitary contact, for Lpxrfa to exert its neuroendocrine regulation of reproduction.

INTRODUCTION

Other than direct contact with the median eminence/pituitary, GNIH and its orthologs have been implicated in interacting with GNRH neurons in the brain to elicit their effects on the gonadotropes. Multiple sources in birds and mammals have documented anatomical interactions between GNIH/RFRP and GNRH neurons in the brain (for review, see Bentley et al. 2006). While most of these interactions are between GNIH/RFRP and hypophysiotropic GNRH1 neurons (Bentley et al. 2003,

Kriegsfeld et al. 2006, Ubuka et al. 2008), GNIH neurons also interact with GNRH2 neurons (Ubuka et al. 2008). These interactions between GNIH/RFRP and GNRH neurons are reinforced by expression of the GNIH/RFRP receptor (GPR147) on GNRH neurons (Smith et al. 2008, Ubuka et al. 2008). In addition, this information is supported by the ability of RFRP-3 to inhibit GNRH-stimulated increases in gonadotropin expression in sheep pituitary cultures *in vitro* (Clarke et al. 2008, Sari et al. 2009), suggesting that RFRP-3 and GNRH axon terminals possibly interact in the median eminence before affecting the gonadotropes. Therefore, it has been implied, in many species studied so far, that GNIH/RFRP in birds/mammals is capable of influencing gonadotropin synthesis/release not only via the median eminence but also by modulating GNRH neurons (particularly GNRH1 neurons) in the brain.

In addition to neuroanatomical relationships between GNIH/RFRFP and GNRH, there is also evidence for functional relationships between these neuropeptides, particularly with GNIH/RFRP-3 effects on GNRH. RFRP-3 administration decreases the firing rate of GNRH1 neurons in mouse brain slices (Ducret et al. 2009), thereby demonstrating a direct effect of RFRP-3 on GNRH. As mentioned previously, RFRP-3 has also repeatedly been shown to inhibit the GNRH-stimulated increases in gonadotropin expression both *in vivo* and *in vitro* (Clarke et al. 2008, Sari et al. 2009, Pineda et al. 2010a). GNIH exerts this effect on gonadotropes by inhibiting the AC/PKA/cAMP-dependent pathway, as shown by research in LβT2 cells (Son et al. 2012). Therefore, GNIH/RFRP may interact directly with the pituitary via the median eminence and/or indirectly with GNRH neurons to elicit its functions on gonadotropins.

While the information about the relationships between RFRP-3 and GNRH is abundant in mammals, this topic is not as well explored in teleosts. Structural information on Lpxrfa and Gnrh in teleosts is mostly limited to neuroanatomical studies that demonstrate the signals of such proteins in similar regions of the brain, such as in the Indian major carp (Biswas et al. 2015). However, in the tilapia, Lpxrfa-ir soma are not closely associated nor in direct contact with Gnrh1 or Gnrh3 neurons (Ogawa et al. 2016). Functionally, on the other hand, Qi et al. (2013) demonstrated that intraperitoneal injections of goldfish Lpxrfa-2 and Lpxrfa-3 each reduce hypophysiotropic *gnrh3* mRNA levels *in vivo*, while Lpxrfa-3 inhibits Gnrh-stimulated *fshβ* and *lhβ* expression in pituitary cells *in vitro*. In addition, European sea bass and the orange-spotted grouper both exhibit reduced *gnrh1* expression levels in response to sea bass Lpxrfa-1 and all three of the grouper Lpxrfa peptides, respectively (Wang et al. 2015, Paullada-Salmerón et al. 2016b). Therefore, in most teleosts studied thus far, Lpxrfa peptides seem to have a mostly negative impact on the gene expression of Gnrhs.

In order to better clarify the relationship between Lpxrfa and Gnrh in teleosts and to elucidate the molecular mechanisms by which Lpxrfa elicits its effects on the brain/pituitary, the goal of this study was to characterize the neuroanatomical and functional interactions between Lpxrfa and Gnrh3 (the hypophysiotropic form in zebrafish) in the zebrafish brain during adulthood and development. Furthermore, the research on GNIH/RFRP/Lpxrfa loss-of-function is limited to one study, which demonstrated that RNAi of *GNIH* in songbirds leads to sexual arousal (Ubuka et al. 2012a). Therefore, another goal of this study was to achieve *lpxrfa* loss-of-function

through the development of an *lpxrfa*$^{-/-}$ knockout line (see Chapter 4 for more details). However, because such a line could not be generated, we conducted *lpxrfa* knockdown in the zebrafish embryo/larva and determined its effects on *gnrh3* expression. The specific objectives of this chapter included the following:

A) Lpxrfa-Gnrh3 Relationships During Development:
    1) Characterize the WT developmental expression profiles of *lpxrfa* and *gnrh3*,
    2) Achieve *lpxrfa* knockdown in early development with *lpxrfa*-targeting anti-sense MO oligonucleotides and determine its effects on *gnrh* expression, and
    3) Determine the effects of the inherited functional loss of Gnrh3 on *lpxrfa* expression during early development.

B) Lpxrfa-Gnrh3 Relationships During Adulthood:
    1) Localize the neuroanatomical relationships between Lpxrfa and Gnrh3 in the adult zebrafish brain by immunohistochemistry,
    2) Determine the *in vitro* and *in vivo* effects of Lpxrfa on *gnrh* expression in the adult brain, and
    3) Determine the effects of the inherited functional loss of Gnrh3 on *lpxrfa* expression in adult male and female brains.

METHODS

Animals

All zebrafish used in this study originated from the in-house colony at 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 monitored and fed twice daily with a commercial flake food or pellets *ad libitum*. Zebrafish embryos and larvae were raised in individual containers of freshwater until 30 dpf, before being transferred to the recirculating system. Starting at 5 dpf, larval zebrafish were fed *Paramecium* twice daily, until 14 dpf, when *Artemia* nauplii was introduced to their diet. Prior to tissue collections, adult fish were euthanized by immersion in an ice bath followed by quick decapitation. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland Baltimore School Of Medicine.

Peptides

The zebrafish Lpxrfa peptide (Lpxrfa-3: SGTGPSATLPQRF-NH_2) was synthesized at a 95% purity by Genscript.

***Developmental Expression Profiles of* lpxrfa *and* gnrh3**

To begin to characterize the relationship between Gnrh3 and Lpxrfa in zebrafish development, we measured the temporal expression profiles of *lpxrfa* and *gnrh3* during the first 30 days of life. Wild-type embryos/larvae were sampled at 8 developmental time points (1, 2, 3, 8, 12, 18, 24, and 30 dpf), and all samplings were done in triplicate. Ten embryos or larvae were pooled and collected at each sampling point from 1 dpf to 18 dpf, whereas 8 larvae and 6 larvae were pooled and collected at 24 dpf and 30 dpf, respectively. Embryos/larvae were frozen on dry ice and stored at -80 °C until RNA extraction. QPCR for *lpxrfa* ($R^2 \geq 0.995$) and *gnrh3* ($R^2 \geq 0.984$)

was then conducted as described below to determine how the expression of these two genes are correlated during early development.

lpxrfa ***Loss-of-Function Effects on*** **gnrh3** ***Expression During Ontogeny***

Since loss-of-function data is mostly missing for GNIH orthologs, we next sought to examine whether *lpxrfa* has a role in the development of the Gnrh3 system by utilizing loss-of-function techniques. Because an *lpxrfa*$^{-/-}$ knockout line could not be generated, we, therefore, used *lpxrfa*-targeting (splice-blocking) anti-sense MO oligonucleotides (Gene Tools) to knockdown *lpxrfa* expression during early development (MO: 5' – ACTTTATCAGAATGTTTACCTTTCT – 3'). The *lpxrfa* MO oligonucleotides (0.5 mM) were injected into one- to two-cell stage WT embryos, along with a control group that received a standard control MO oligonucleotide (0.5 mM; Gene Tools). At 24, 48, 72, and 96 hpf, embryos/larvae (n = 10) were sampled in triplicate for RT-PCR to measure *lpxrfa* expression in order to validate the efficiency of the MO oligonucleotide's knockdown effects. Embryos/larvae were frozen on dry ice and stored at -80 °C until RNA extraction. RT-PCR primers for *lpxrfa* were positioned on either side of the exon-intron boundary where splicing was disrupted by the MO oligonucleotide: For: 5' – CCACCCTCCCGCAGCGGTTT – 3' and Rev: 5' – CTGTTTCAAACATGTAGTCGTGG – 3'. Controls in the RT-PCR included the following templates: no RT, cDNA, genomic DNA, and water. These samples of embryos were also used to quantify the expression of *gnrh3* to determine the effects of *lpxrfa* knockdown on *gnrh3* expression. RNA extraction, cDNA synthesis, and *gnrh3* QPCR for embryos/larvae ($R^2 = 0.998$) was conducted as described below.

Expression of lpxrfa *in Development of* gnrh3$^{-/-}$ *Fish*

Even though we were able to obtain *lpxrfa* knockdown using anti-sense MO oligonucleotides, we were not able to produce a functional *lpxrfa*$^{-/-}$ knockout line. We did, however, succeed in generating and validating a *gnrh3*$^{-/-}$ knockout line in order to determine how *lpxrfa* differs in development and adulthood when an important neuropeptide, like Gnrh3, is completely absent. Thus, using the *gnrh3*$^{-/-}$ knockout line, which is characterized thoroughly in Chapter 4, we wanted to determine how an inherited loss of Gnrh3 affects expression of *lpxrfa* in early development of the zebrafish. The *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ embryos/larvae were sampled at 8 developmental time points (1, 2, 3, 8, 12, 18, 24, and 30 dpf). All samplings were done in triplicate. Ten embryos or larvae were pooled and collected at each sampling point from 1 dpf to 18 dpf, whereas 8 larvae and 6 larvae were pooled and collected at 24 dpf and 30 dpf, respectively. Embryos/larvae were frozen on dry ice and stored at -80 °C until RNA extraction. QPCR for *lpxrfa* ($R^2 \geq 0.995$) was then conducted as described below.

Generation of Antibody against Zebrafish Gnrh3 Gap

In order to generate an antibody to recognize zebrafish Gnrh3, the cDNA encoding the zebrafish Gnrh3 Gap (from 136 bp to 309 bp of NCBI Reference Sequence NM_182887.2) 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 from the insoluble fraction were prepared according to Brent (1997). These proteins were dissolved in 8M urea, purified with nickel-nitrolotriacetic acid columns (Promega), and de-salted on sephadex G-15

columns (Pharmacia). The purified proteins were used for the production of antiserum in rabbits (ProteinTech). The final bleed antiserum was used as the primary polyclonal antibody in all zebrafish Gnrh3 Gap immunohistochemistry.

Verification of Anti-Zebrafish Gnrh3 Gap Antibody

The specificity of the zebrafish Gnrh3 Gap antibody was verified by confirming the recognition and specificity of the antibody to Gnrh3 expressed in a heterologous cell line and by co-localizing Gnrh3 immunoreactivity in *gnrh3* transgenic adult brain sections, expressing tdTomato in *gnrh3* neurons (Abraham et al. 2008, Xia et al. 2014). The entire zebrafish *gnrh3* coding region (from 28 bp to 312 bp of NCBI Reference Sequence NM_182887.2) was cloned into the pcDNA3.1 mammalian expression vector (Life Technologies) under the control of the CMV promoter. The zebrafish *gnrh3*-pcDNA3.1 plasmid and the control pcDNA3.1 plasmid were transfected into COS7 cells with FuGENE 6.0 (Promega). As an additional control to check for cross-reactivity, cells were also transfected with the zebrafish *gnrh2*-pcDNA3.1 plasmid. The cells were grown in 25 cm^2 sterile cell culture flasks in DMEM supplemented with 10% FBS, 1% glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Biological Industries) at 37 °C and 5% CO_2. After 48 hours of incubation, cells were transferred to chamber slides. After 24 hours, the cells were fixed with 4% PFA in PBS for 1 hour at room temperature and were washed with PBS. Blocking and immunostaining for zebrafish Gnrh3 Gap was then conducted as briefly described: Sections were blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Sections were incubated overnight at 4 °C with rabbit anti-zebrafish Gnrh3 Gap (1:5000) diluted in 1% BSA

and 0.3% Triton X-100 in PBS. Sections were incubated for 1 hour at room temperature with goat anti-rabbit secondary antibody Cy3 (KPL) diluted 1:500. For Gnrh3 Gap immunohistochemistry on *gnrh3* transgenic fish, the brains were sampled, fixed, cryopreserved, and sectioned as described in the next section, and after dried slides were fixed with pre-chilled acetone for 2 minutes, sections were blocked and immunostained with anti-Gnrh3 Gap in the same manner as the COS7 cells, with the exception that the secondary antibody was goat anti-rabbit Alexa 488 (Life Technologies).

Lpxrfa and Gnrh3 Immunohistochemistry in Adult Brain

For determining the neuroanatomical interactions between zebrafish Lpxrfa and Gnrh3 in the brain, we conducted double immunohistochemistry on WT adult brains. Brains were dissected from adult fish and fixed overnight with 4% PFA (in PBS) at 4 °C. Before cryopreservation, brains were transferred to 30% sucrose in PB overnight at 4 °C. Tissues were frozen in OCT, sectioned coronally at 15 µm thickness, transferred to charged slides, and stored at -20 °C until processed. Briefly, dried sections were fixed in pre-chilled acetone for 2 minutes and allowed to dry. Sections were incubated with 0.5% H_2O_2 in PBS for 30 minutes at room temperature to quench endogenous HRPs. Sections were blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Sections were incubated overnight at 4 °C with anti-zebrafish Lpxrfa (1:5000) diluted in 1% BSA and 0.3% Triton X-100 in PBS. Sections were incubated for 1 hour at room temperature with goat anti-rabbit HRP (1:1000). The signal was detected using the TSA Plus kit, and fluorescence was obtained via the Cy3 dye from the kit. After quenching HRPs with

0.02N HCl for 10 minutes, blocking and immunostaining for Gnrh3 with anti-zebrafish Gnrh3 Gap (1:1000) were conducted in the same manner, except that fluorescence was obtained via the FITC dye from the TSA Plus kit.

Effects of Lpxrfa-3 on Brain Expression **in vitro**

In order to determine the effects of zebrafish Lpxrfa-3 on adult brains *in vitro*, we modified a protocol from Klenke (2006). Brains of adult male zebrafish were dissected and immediately placed into pre-cooled media (DMEM with 25 mM D-glucose and HEPES (Life Technologies), supplemented with 50 µM bacitracin, 50 µM ascorbic acid, and 0.1% (w/v) BSA (pH 7.4)). After dissection of all samples, brains were sliced at 200-300 µm thickness with a McIlwain mechanical tissue chopper (The Vibratome Company) and returned to the original culture media. Tissues were held in sterile inserts in 12-well sterile cell culture plates with 2 mL of media. Brains were washed with media 3 x 1 hour with slight agitation (45 rpm) at 28 °C. Brain slices were incubated for six hours 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 treatment). As mentioned previously, zebrafish Lpxrfa-3 was chosen because of its high homology to goldfish Lpxrfa-3, which was demonstrated to be a mature peptide detected by mass spectrometry (Sawada et al. 2002), and because of its ability to reduce serum Lh levels in the goldfish *in vivo* (Zhang et al. 2010). After six hours, which was pre-determined to be sufficient to prevent degradation of brain RNA, 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 as described below.

Effects of Lpxrfa-3 on Brain Expression in vivo

In order to determine if the effects of zebrafish Lpxrfa-3 on *gnrh3* expression occurs *in vivo* in the same manner as *in vitro*, female and male adult zebrafish were administered zebrafish Lpxrfa-3 and subsequently sampled for QPCR. Males and females were intraperitoneally injected with 0.5 µg/fish and 1 µg/fish zebrafish Lpxrfa-3, respectively, in PBS with 1% Ethan's blue dye. A separate group was injected with PBS with 1% Ethan's blue dye. Injections were administered twice approximately 4 hours apart: first injection at 9:40 – 11:15 and second injection at 14:00 – 15:20. Three hours after the second injection, fish (n = 3-4) were sampled for brains, which were frozen on dry ice and stored at -80 °C until RNA extraction. This protocol was modified from Zhang et al. (2010), in which two intraperitoneal injections (3 hours apart) of zebrafish Lpxrfa-3 into adult goldfish resulted in an inhibitory effect on serum Lh levels at 1 and 3 hours after the second injection. QPCR was then conducted for *gnrh3* ($R^2 = 0.998$), as described below.

Expression of lpxrfa *in* gnrh3$^{-/-}$ *Adult Brains*

Since we determined how an inherited functional loss of Gnrh3 affects *lpxrfa* expression in early development, we wanted to determine how this loss also affects expression of *lpxrfa* in male and female adult brains. The brains of male and female adult *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish were used to quantify *lpxrfa* mRNA levels for each sex for each genotype. We also wanted to determine if time of day influences this difference, because zebrafish reproductive cycles are daily (Eaton and Farley 1974) and because GNIH and its orthologs can be influenced by melatonin (Chowdhury et al. 2010). Therefore, we tested, for each sex, whether *lpxrfa* mRNA expression in the

brain differs between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish in the morning (9:00 immediately prior to spawning) and in the evening (18:00). Six fish per sex per genotype were sacrificed in the morning and in the evening for brain tissues, and the experiment was conducted twice for females. Brains were frozen on dry ice and stored at -80 °C for RNA extraction, and *lpxrfa* QPCR ($R^2 \geq 0.994$) was conducted as described below.

Quantification of Gene Transcripts

Total RNA was extracted from brains and embryos/larvae using the manufacturer's protocol for the TRIzol® reagent (Invitrogen) and was quantified with a Nanodrop (Thermo Scientific). Total RNA (1 µg) was treated with genomic DNA wipeout buffer for 9 minutes at 42 °C and synthesized into first-strand cDNA with the Qiagen QuantiTect RT Kit. The final reaction volume for all cDNA synthesis reactions was 20 µL. The mRNA levels of the target genes in the brains and embryos/larvae were measured via QPCR. Gene-specific QPCR primers for each of the target genes are listed in Table 3.1. Specificity of each primer set's amplification was confirmed by a dissociation curve. Each QPCR reaction was carried out in duplicate with a final volume of 10 µL: 2x DyNAmo Flash SYBR Green QPCR mix (Life Technologies), 200 nM primer mix, 0.3x ROX (Life Technologies), 20 ng cDNA, and sterile MilliQ water, in a 7500 Fast Real-Time PCR System (Applied Biosystems). The cycle conditions were 95 °C for 7 minutes, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. Each plate included a standard curve using the reverse-transcribed RNA of the specific clone as a template. Absolute copy number was calculated for the tested reactions and normalized against *ef1α* levels. Each plate included two no RT controls and two no template controls.

Table 3.1. QPCR primers used to quantify gene transcripts in Chapter 3.

Gene	Type	Sequence (5' → 3')	T_m (°C)	GC%	Amplicon Size (bp)
gnrh3	For	TGGAGGCAACATTCAGGATGT	56.6	47.6	104
gnrh3	Rev	CCACCTCATTCACTATGTGTATTGG	55.4	44.0	
gnrh2	For	CAGAGGTTTCAGAGGAAGTGAAGC	57.5	50.0	101
gnrh2	Rev	TGAGGGCATCCAGCAGTATTG	57.2	52.4	
lpxrfa	For	TGTCCTACTTCGCTCTTCTTTC	54.3	45.5	80
lpxrfa	Rev	GCAATCTGAGAGCCGTAACT	54.7	50.0	
ef1α	For	AAGACAACCCCAAGGCTCTCA	58.6	52.4	255
ef1α	Rev	CCTTTGGAACGGTGTGATTGA	55.5	47.6	

Microscopy

All sections were mounted with either anti-fading solution with DAPI or Vectashield with DAPI (Vector Labs) and imaged with one of two 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 at a resolution of 1360 x 1024 and a magnification of 20x. Brain sections were imaged with a Leica Microsystems DMi8 confocal microscope with a resolution of 1024 x 1024, a magnification of 20x or 40x, and a z-step size of 0.10 or with a Zeiss Axioplan 2 microscope with an Attoarc HBO100 W power source, equipped with a CCD Olympus DP70 camera, with a resolution of 1360 x 1024 and a magnification of 20x. All images were analyzed with Image J and/or Adobe Photoshop.

Statistics

All data are represented as mean values ± SEM, unless otherwise specified. To determine whether the developmental expression profiles of *lpxrfa* and *gnrh3* were connected, a correlation analysis was conducted with the Statistical Analysis Software (SAS). When comparing *gnrh3* mRNA levels in control MO-injected versus *lpxrfa* MO-injected embryos and when comparing *lpxrfa* mRNA levels of *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish during early development, a one-tailed homoscedastic Student *t*-test was used at each sampling time point. For QPCR values in the *in vitro* and *in vivo* assays, each concentration/dose was compared to the control (0 nM Lpxrfa-3 or PBS) by a one-tailed, homoscedastic Student *t*-test. In observing differences between *lpxrfa* mRNA levels in *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ adult brains, a one-tailed, homoscedastic Student *t*-test was also used for each sex at each time of day. Statistical significance was established if $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$.

RESULTS

Lpxrfa and Gnrh3 Relationships during Development

Developmental Expression Profiles of lpxrfa *and* gnrh3

To initially determine whether Lpxrfa and Gnrh3 have a relationship during development, we measured the expression profiles of *lpxrfa* and *gnrh3* during the first 30 days of life. When examining mRNA levels of *lpxrfa* and *gnrh3*, we found that *lpxrfa* levels peak earlier (2 dpf) than *gnrh3* levels (3 dpf; Figure 3.1). Both genes exhibited increases in expression very early in development (approximately 2-3 dpf) with decreases to less than 500 copy numbers/ng by 30 dpf (Figure 3.1). However,

the expression levels of *lpxrfa* and *gnrh3* were not correlated, as demonstrated by a correlation level of 0.47213 ($P = 0.2385$).

lpxrfa *Knockdown Effects on* gnrh3 *Expression During Early Development*

Because of the inability to establish an *lpxrfa*$^{-/-}$ knockout line in the zebrafish, our *lpxrfa* loss-of-function experiment was changed to the use of *lpxrfa*-targeting anti-sense MO oligonucleotides to induce *lpxrfa* knockdown in early development. In order to first validate that the *lpxrfa*-targeting (splice-blocking) MO oligonucleotides were working properly, samples of embryos/larvae collected at 24, 48, 72, and 96 hpf were used for RT-PCR of *lpxrfa*, with primers strategically flanked on either side of the MO oligonucleotide's target site. End-point PCR revealed the amplification of a larger, un-spliced portion (473 bp) of *lpxrfa* in only the embryos/larvae that were administered the *lpxrfa*-targeting anti-sense MO oligonucleotide, and this effect was attenuated over time (Figure 3.2A). Therefore, the *lpxrfa*-targeting MO oligonucleotide was validated as knocking down *lpxrfa* expression. Next, QPCR of *gnrh3* was conducted on the same embryos/larvae to determine how *lpxrfa* knockdown affects *gnrh3* expression. Surprisingly, there was no significant effect on *gnrh3* expression at any of the four time points assessed (Figure 3.2B).

Expression of lpxrfa *in Development of* gnrh3$^{-/-}$ *Fish*

In order to further test the interactions between zebrafish Lpxrfa and Gnrh3, we utilized the new platform of the TALEN technology by incorporating the use of

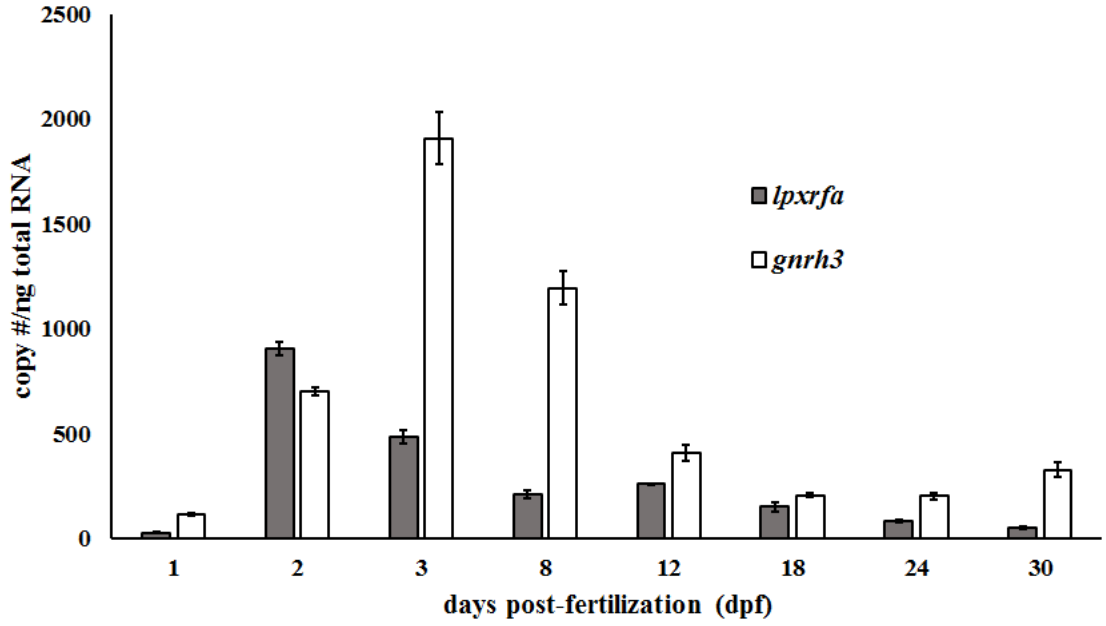

Figure 3.1. Expressions of *lpxrfa* and *gnrh3* during the first 30 days of life are not correlated. Wild-type developmental expression profiles of *lpxrfa* and *gnrh3* during development. Using QPCR, *lpxrfa* and *gnrh3* levels were determined for embryos/larvae at each of the eight sampling time points. Correlation analysis revealed no correlation between the expressions of the two genes. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. *lpxrfa*, grey bars. *gnrh3*, white bars.

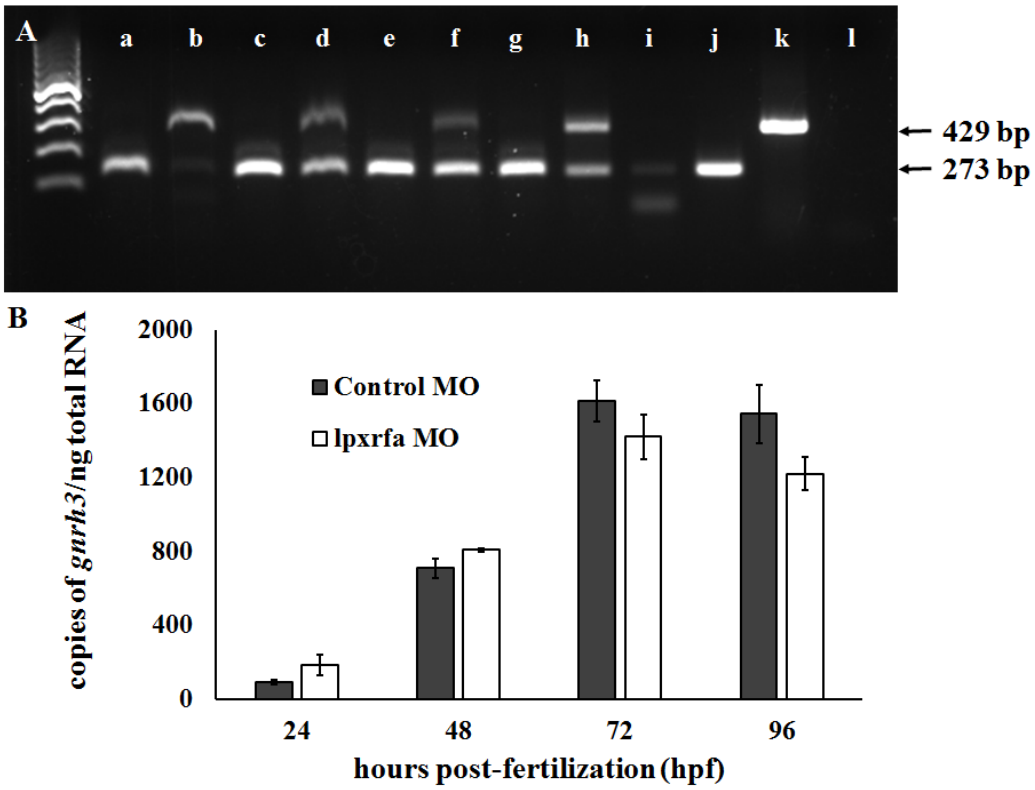

Figure 3.2. *lpxrfa* knockdown does not affect *gnrh3* expression during early development. (A) RT-PCR of *lpxrfa* on embryo/larva samples: control MO 24 hpf (a), *lpxrfa* MO 24 hpf (b), control MO 48 hpf (c), *lpxrfa* MO 48 hpf (d), control MO 72 hpf (e), *lpxrfa* MO 72 hpf (f), control MO 96 hpf (g), *lpxrfa* MO 96 hpf (h), no RT control (i), cDNA control (j), gDNA control (k), and NT control (l). The *lpxrfa* MO resulted in amplification of a larger, un-spliced portion of *lpxrfa* (429 bp; compared to the expected 273 bp), which was attenuated over time. (B) *lpxrfa* knockdown did not affect *gnrh3* expression at any of the 4 times points assessed. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. Differences between values at each time point were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. control MO, grey bars. *lpxrfa*-targeting MO, white bars.

knockout lines (see Chapter 4 for details). Although an *lpxrfa*$^{-/-}$ knockout line could not be established, a *gnrh3*$^{-/-}$ knockout line was generated and validated in order to determine how *lpxrfa* differs in the complete absence of Gnrh3 during both development and adulthood. After sampling *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ embryos/larvae at eight developmental time points, QPCR of *lpxrfa* was conducted to determine any differences between the two genotypes. At all of the time points sampled, except 24 and 30 dpf, *lpxrfa* mRNA levels were significantly higher in *gnrh3*$^{+/+}$ embryos/larvae than in *gnrh3*$^{-/-}$ embryos/larvae (Figure 3.3). At 24 and 30 dpf, however, there were no differences in *lpxrfa* expression between the two genotypes (Figure 3.3).

Lpxrfa and Gnrh3 Relationships during Adulthood

Verification of Anti-Zebrafish Gnrh3 Gap Antibody

The anti-zebrafish Gnrh3 Gap antibody stained soma and fibers in the *gnrh3*$^{+/+}$ forebrain (Figure 3.4Aa), while the negative control pre-immune serum did not yield any signal in the same region (Figure 3.4Ab). We also conducted Gnrh3 immunohistochemistry on brain sections of adult *gnrh3:tdTomato* fish to look for co-localization between the two signals. In the forebrain, positive Gnrh3 immunostaining of soma (Figure 3.4Ba) was observed coinciding with Gnrh3 neurons expressing Gnrh3-tdTomato (Figure 3.4Bb), indicating that the cells in which activation of the *gnrh3* promoter occurs are also recognized by the anti-Gnrh3 Gap antibody (Figure 3.4Bc). The antibody also specifically recognized Gnrh3 peptide expressed in COS7 cells (Figure 3.4Cc) *in vitro*, while Gnrh2-expressing cells (Figure 3.4Cb) or cells carrying the control pcDNA3.1 plasmid (Figure 3.4Ca) were not stained. Therefore, our polyclonal antibody specifically recognizes the zebrafish

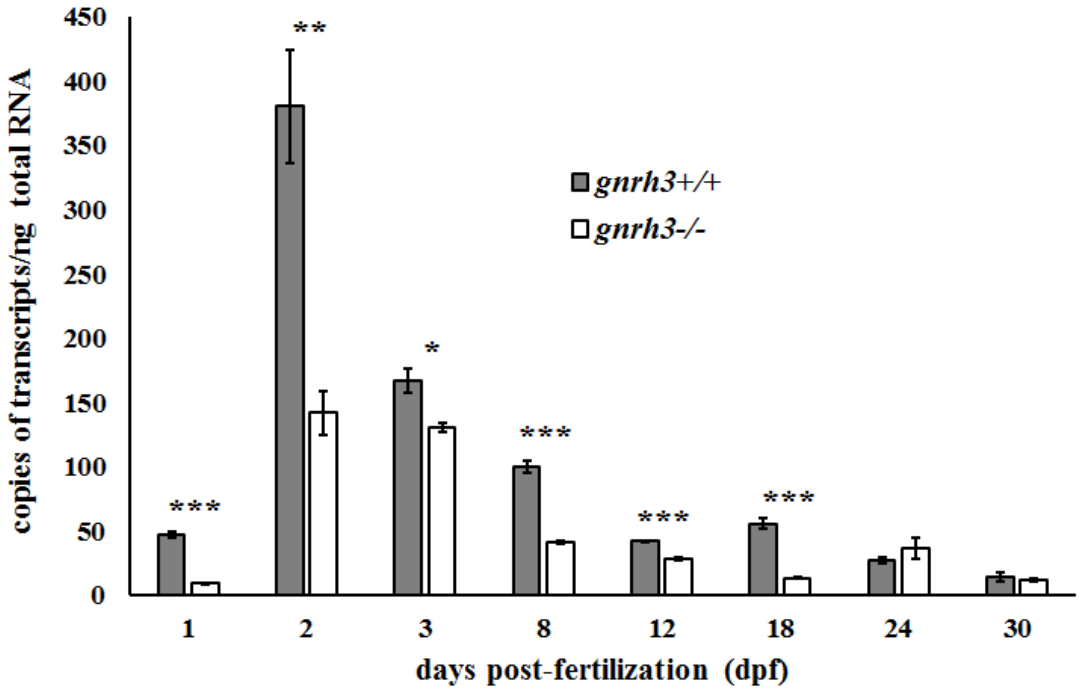

Figure 3.3. Developmental mRNA levels of *lpxrfa* are higher in *gnrh3*$^{+/+}$ fish than in *gnrh3*$^{-/-}$ fish during the majority of development. The mRNA levels of *lpxrfa* were measured in pooled samples of whole *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ embryos/larvae at different time points. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. Differences between genotypes at a specific time point were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. *gnrh3*$^{+/+}$, grey bars. *gnrh3*$^{-/-}$, white bars.

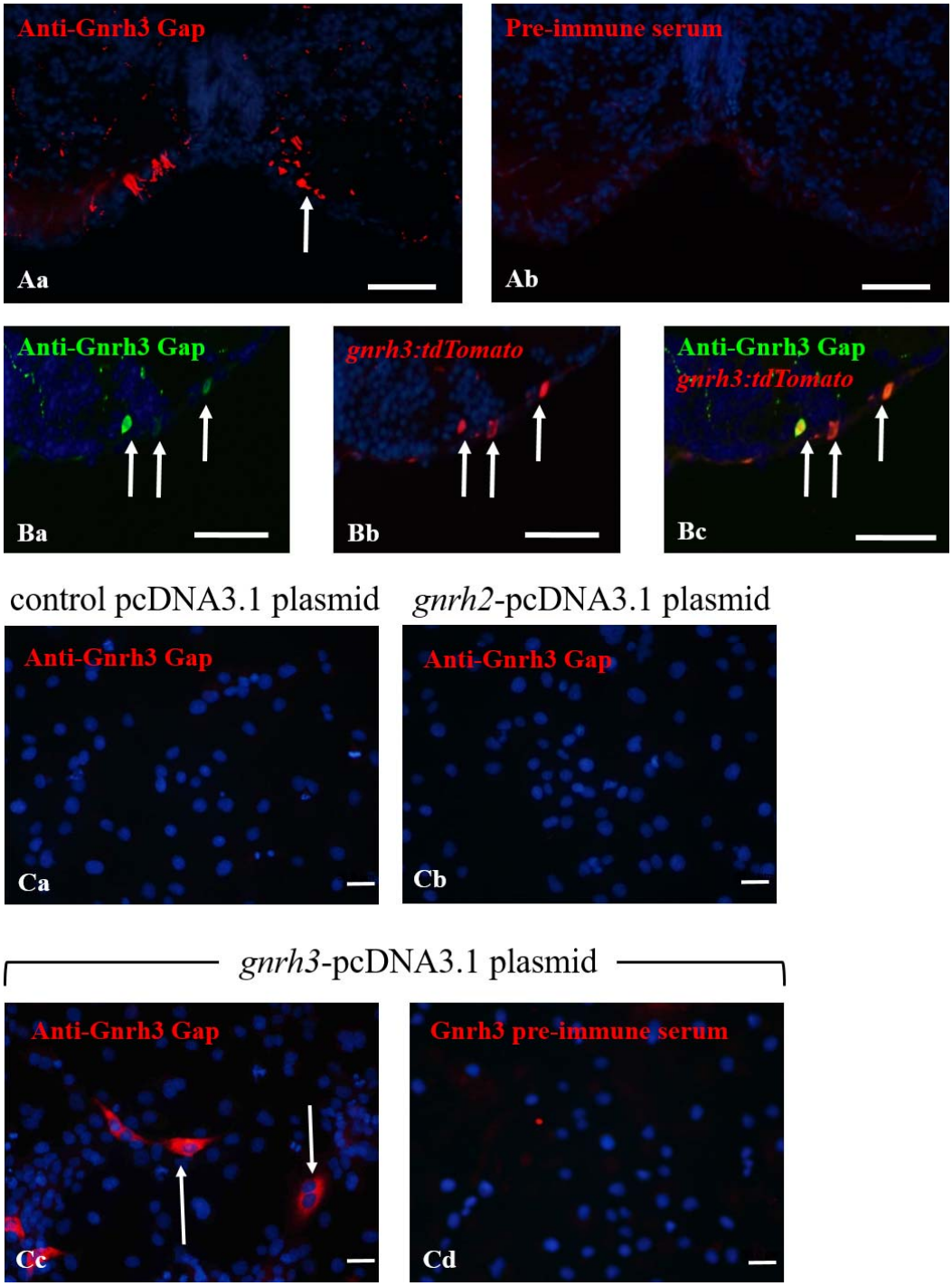

Figure 3.4. Anti-zebrafish Gnrh3 Gap polyclonal antibody is specific to zebrafish Gnrh3. (A) The immunostaining of Gnrh3-ir soma and fibers (white arrow; Aa) in the brain with anti-zebrafish Gnrh3 Gap was eliminated when the

antibody was substituted with pre-immune serum (Ab). (B) Immunostaining with anti-Gnrh3 Gap (green; Ba) on brain sections of *gnrh3:tdTomato* adults (red; Bb) shows co-localization between Gnrh3-ir soma and *gnrh3:tdTomato*-labeled soma (Bc), indicating the specificity of the antibody to Gnrh3. White arrows indicate Gnrh3-tdTomato-expressing soma that were positively stained by anti-Gnrh3 Gap. (C) Immunostaining with anti-zebrafish Gnrh3 Gap (Ca-Cc) or pre-immune serum (Cd) in COS7 cells transfected with control pcDNA3.1 plasmid (Ca), *gnrh2*-pcDNA3.1 plasmid (Cb), or *gnrh3*-pcDNA3.1 plasmid (Cc, d). The cells that express zebrafish Gnrh3 and are immunostained with anti-zebrafish Gnrh3 Gap (red) are indicated by white arrows (Cc). Scale bars = 50 μm.

Gnrh3 Gap, does not cross-react with the Gnrh2 Gap, and can be used for identifying Gnrh3-expressing neurons in the zebrafish brain.

Lpxrfa and Gnrh3 Immunohistochemistry in Adult Brain

Double Lpxrfa and Gnrh3 immunohistochemistry in adult brain sections was used to determine Lpxrfa's neuroanatomical interactions with Gnrh3 neurons. 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 fore-, mid-, and hind-brains (see Chapter 2). Gnrh3 soma were found within the ventral telencephalon/terminal nerve and preoptic area/hypothalamus, and Gnrh3-ir fibers projected posteriorly through the hypothalamus and then ventrally to the pituitary, as reported previously (Steven et al. 2003, Abraham et al. 2008). Overall, Lpxrfa-ir and Gnrh3-ir fibers were frequently 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 interacted with and projected to Gnrh3-ir soma (Figure 3.5B-D). This innervations and projections were very prevalent in Gnrh3-ir soma.

***Effects of Lpxrfa-3 on Brain Expression* in vitro**

As a first step 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 hours of incubation, 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* mRNA levels (Figure 3.6), incubation with all of the zebrafish Lpxrfa-3 concentrations resulted in significant reductions (approximately 50%) in *gnrh3* mRNA levels (Figure 3.6). Therefore, the inhibitory effect of Lpxrfa-3 on the brain is more specific to *gnrh3* than to *gnrh2*.

***Effects of Lpxrfa-3 on Brain Expression* in vivo**

Because zebrafish Lpxrfa-3 was capable of inhibiting *gnrh3* expression in the brain *in vitro*, we tested the administration of zebrafish Lpxrfa-3 to adult zebrafish to determine if the same effect could occur *in vivo*. Three hours after the second injection of zebrafish Lpxrfa-3, zebrafish were sampled for brains, which were used to conduct *gnrh3* QPCR. Unlike what was observed *in vitro*, the brains of fish that were injected with zebrafish Lpxrfa-3 did not have significantly different *gnrh3* levels than those injected with the vehicle (PBS; Figure 3.7). Therefore, the effects of

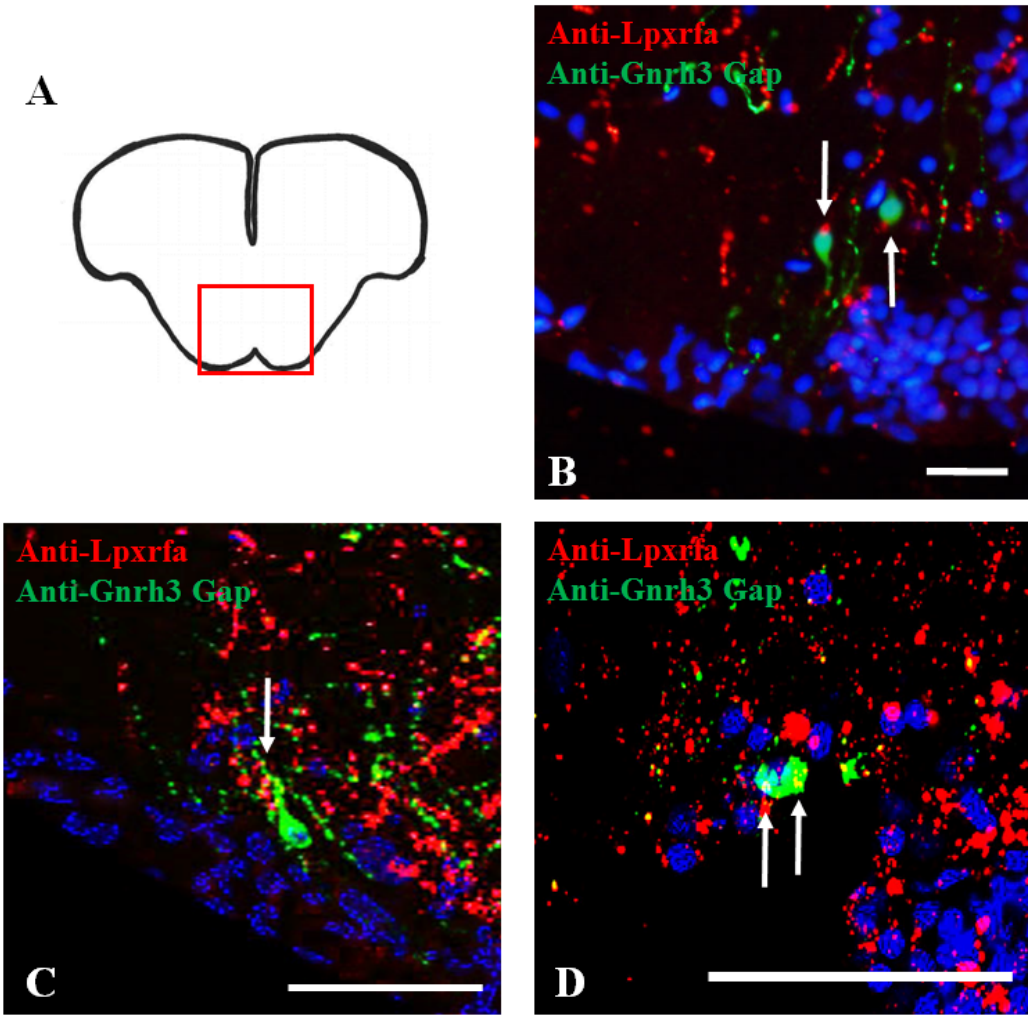

Figure 3.5. Zebrafish Lpxrfa-ir fibers interact with and project to Gnrh3-ir soma. Coronal sections of adult WT brains immunostained with anti-zebrafish Lpxrfa (red) and anti-zebrafish Gnrh3 Gap (green). In sections of the preoptic area (red square in A), Lpxrfa-ir neuron fibers (red) interact with and make direct contact with Gnrh3-ir soma (green), as indicated by the white arrows (B-D). Scale bars = 100 µm.

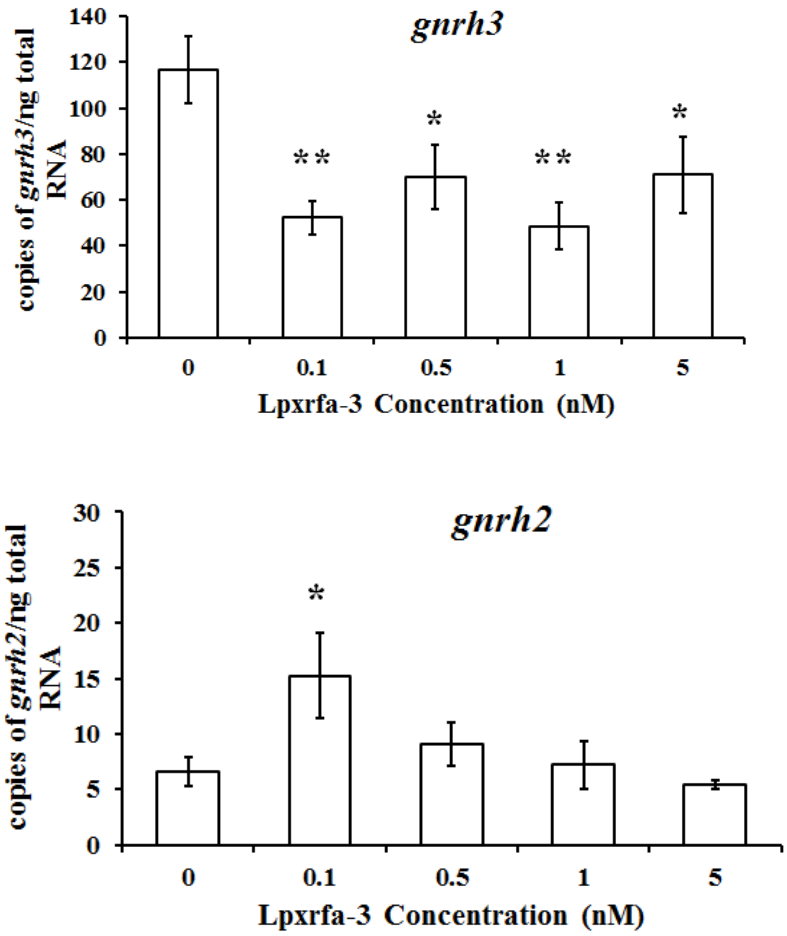

Figure 3.6. Zebrafish Lpxrfa-3 administration reduces *gnrh3* gene expression in the male adult brain *in vitro*. After incubation with zebrafish Lpxrfa-3, brain slices from adult males had reduced *gnrh3* mRNA levels at all of the concentrations tested *in vitro*. Zebrafish Lpxrfa-3 had a slight stimulatory effect on *gnrh2* at the lowest concentration tested. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. Differences between each treatment and the control (0 nM) were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$.

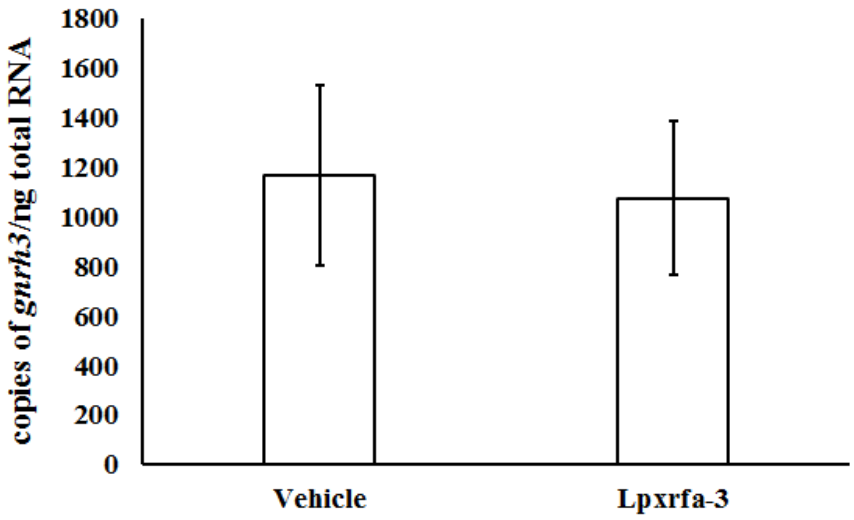

Figure 3.7. Zebrafish Lpxrfa-3 *in vivo* administration does not affect the expression of *gnrh3* in adults. After two consecutive injections of 0.5 µg/fish and 1 µg/fish zebrafish Lpxrfa-3 for males and females, respectively, *gnrh3* mRNA levels were determined via QPCR. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. Differences between the effects of the vehicle and the treatment were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$.

zebrafish Lpxrfa-3 on *gnrh3* expression in the brain differs depending on whether the experiment is conducted *in vitro* or *in vivo*.

Expression of* lpxrfa *in* gnrh3$^{-/-}$ *Adult Brains

With the *gnrh3*$^{-/-}$ knockout line available as an important tool, we assessed differences in *lpxrfa* expression between both sexes, incorporating different sampling times during the day to assess differences in the reproductive cycle of this species. In order to determine whether *lpxrfa* expression in the brain differs between *gnrh3*$^{+/+}$

and *gnrh3*$^{-/-}$ adult fish for each sex at different times of the day (morning and evening), we conducted *lpxrfa* QPCR on brain samples collected from each group (n = 6). When observing males, there were no differences between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ brain *lpxrfa* expression levels in the morning immediately prior to spawning or in the evening in preparation for spawning the following morning (Figure 3.8). Females, on the other hand, consistently and reliably exhibited differences between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ brain *lpxrfa* expression levels in the evening but not in the morning. In the evening, adult female *gnrh3*$^{-/-}$ brains had significantly lower (approximately 50%) mRNA levels of *lpxrfa* than adult female *gnrh3*$^{+/+}$ brains (Figure 3.8).

DISCUSSION

Throughout this chapter, the neuroanatomical and/or functional relationships between two important neuropeptides in the adult and developing zebrafish brain, Lpxrfa and Gnrh3, have been explored. Firstly, the developmental expression profiles of *lpxrfa* and *gnrh3* were provided, demonstrating no observable correlation between the two genes during the first 30 days of life. With loss-of-function techniques, we revealed that the knockdown of *lpxrfa* does not affect *gnrh3* expression in the developing zebrafish embryo/larva. However, in the *gnrh3*$^{-/-}$ knockout line, *lpxrfa* mRNA levels were significantly lower throughout development than in *gnrh3*$^{+/+}$ fish. Using double-labeling immunohistochemistry, we demonstrated that Lpxrfa-ir neuron fibers project to and innervate Gnrh3-ir soma in the pre-optic area of the adult brain. This neuroanatomical relationship was supported by the demonstration that zebrafish Lpxrfa-3 significantly reduces *gnrh3* expression in the male brain *in vitro*. In the *gnrh3*$^{-/-}$ adult female brain, we also

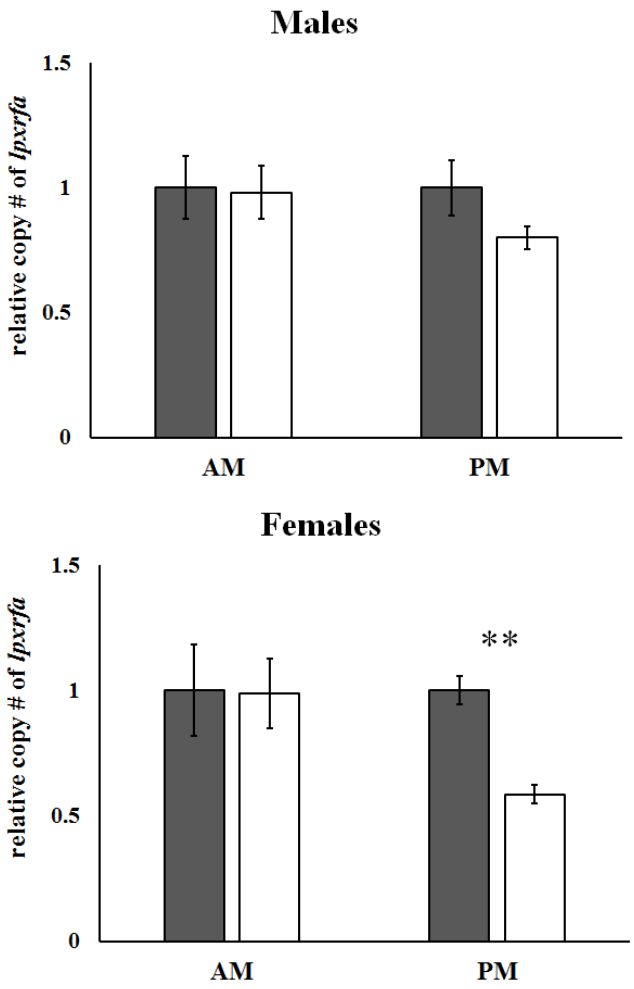

Figure 3.8. Female *lpxrfa* mRNA levels in the adult brain are lower in *gnrh3*$^{-/-}$ fish than in *gnrh3*$^{+/+}$ fish only in the evening, while males exhibit no differences between the two genotypes at either time of day. Using QPCR, brain *lpxrfa* levels were determined for each genotype for each sex at each sampling time (morning immediately prior to spawning and evening). Absolute mRNA levels were normalized to *ef1α* levels and are presented as relative mean of control ± SEM. Differences between genotypes for each sex at each sampling time were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. *gnrh3*$^{+/+}$, grey bars. *gnrh3*$^{-/-}$, white bars.

demonstrated that *lpxrfa* mRNA levels are significantly lower than in the *gnrh3*$^{+/+}$ adult female brain in the evening but not in the morning immediately prior to spawning. Overall, these results demonstrate a potential one-way regulation of *lpxrfa* by Gnrh3 during early development but a mutual regulation between Lpxrfa and Gnrh3 in the adult zebrafish brain, indicating that, in addition to a direct control on the pituitary, Lpxrfa regulates reproduction also at the level of the brain.

In order to better characterize the Lpxrfa/Gnrh3 relationship during development, the expression profiles of *lpxrfa* and *gnrh3* were generated throughout the first 30 days of life. Although *lpxrfa* was assessed through RT-PCR in Zhang et al. (2010) during early zebrafish development, the mRNA levels were never quantified. Based on our results, there is no obvious correlation between the expressional trends of *lpxrfa* and *gnrh3* in the first 30 days of development. Expression of RFamide peptides, including GNIH and its orthologs, in early development has been noted in many vertebrates, particularly within the central nervous system (for review, see Sandvik et al. 2014). Because reproductive peptides in the brain are often pleiotropic, it is possible that *lpxrfa* and *gnrh3* participate in additional functions that are most likely related to early neuronal development, organization, and growth functions. Accordingly, Abraham et al. (2008) demonstrated an autocrine neuromodulator/neurotransmitter role for Gnrh3 in the migration and development of Gnrh3 neurons. In addition, because GNIH/RFRP has been associated with feeding and behavior in birds and mammals (Tachibana et al. 2005, Clarke et al. 2012, Ubuka et al. 2012a), it is possible that increased *lpxrfa*

expression in zebrafish development is associated with these two physiological processes.

As thoroughly explained in Chapter 4, we were not able to generate a true *lpxrfa*$^{-/-}$ knockout line in zebrafish. While fish that were homozygous for a 7 bp deletion in the *lpxrfa* coding region were obtained, immunohistochemistry with anti-zebrafish Lpxrfa revealed that the Lpxrfa peptide was still present in these fish. Therefore, the 7 bp deletion was not sufficient to prevent translation of the Lpxrfa peptide, and our *lpxrfa*$^{-/-}$ line was not a true knockout line. Since we were unable to obtain this useful loss-of-function tool, we exploited transient *lpxrfa* loss-of-function (knockdown via anti-sense MO oligonucleotides) to examine the Lpxrfa/Gnrh3 relationship during the ontogeny of the Gnrh3 and Lpxrfa neuronal systems.

Based on previous literature in teleosts demonstrating an inhibitory effect of Lpxrfa on Gnrh (Wang et al. 2015; Paullada-Salmerón et al 2016b), we hypothesized that *lpxrfa* knockdown would result in increases in *gnrh3* levels. As mentioned above, Abraham et al. (2008) has shown that *gnrh3* is needed for the proper migration and organization of the Gnrh3 neuronal system at this early stage. Thus, we questioned whether Lpxrfa is also involved in regulating *gnrh3* levels and, in turn, Gnrh3 neuronal migration and organization. While RT-PCR demonstrated a proper knockdown and amplified a larger, un-spliced variant of *lpxrfa* in the embryos administered the *lpxrfa*-targeting anti-sense MO oligonucleotide, knocking down *lpxrfa* surprisingly did not affect *gnrh3* mRNA levels in 24 – 96 hpf zebrafish embryos/larvae. The lack of an effect in this experiment differs from the only other example of GNIH/RFRP/Lpxrfa loss-of-function experiment, which demonstrated a

strong induction of arousal in adult male songbirds that were administered small-interfering RNAs against *GNIH* (Ubuka et al. 2012a), reinforcing the observation in zebrafish that Lprxfa most likely has a reproductive regulatory role in adulthood.

On the other hand, the results from the effects of the inherited functional loss of Gnrh3 on *lpxrfa* during development were quite different and indicated a potential regulation of Lpxrfa by Gnrh3 in early development. While our efforts to establish an *lpxrfa*$^{-/-}$ knockout zebrafish line were unsuccessful, we did generate and validate a *gnrh3*$^{-/-}$ knockout line in zebrafish, which is thoroughly characterized in Chapter 4. In the *gnrh3*$^{-/-}$ embryos/larvae, *lpxrfa* mRNA levels were significantly lower than in *gnrh3*$^{+/+}$ embryos/larvae throughout most of early development, indicating a potential one-way regulation of Lpxrfa by Gnrh3. It is possible that, during this time period, Gnrh3 is regulating *lpxrfa* expression, while, as demonstrated by *lpxrfa* knockdown, *lpxrfa* does not regulate *gnrh3*. Therefore, although *lpxrfa* is not necessarily essential to the development of the Gnrh3 system, Gnrh3 is essential to the development of the Gnrh3 system (Abraham et al. 2008) and most likely participates in the development of the Lpxrfa system. In addition, because *gnrh3*$^{-/-}$ embryos/larvae also exhibit increased levels of *fshβ*, *lhβ*, and *cgα* mRNA at some of the same time points during development (see Chapter 4), it is possible that these increases are due to the decreased levels of the gonadotropin-inhibiting *lpxrfa*. Overall, it is likely that the relationship between Lpxrfa and Gnrh3 in zebrafish is a one-way regulation during early development.

In the adult zebrafish brain, axons of Lpxrfa and Gnrh3 neurons mutually project and end in close contact of each other, and projections of Lpxrfa fibers toward

Gnrh3 soma are particularly evident in the preoptic area, the major brain region in which hypophysiotropic Gnrh3 soma reside (Steven et al. 2003). This finding is also found in birds and mammals, including rats/mice (Kriegsfeld et al. 2006), sheep (Clarke et al. 2008), and starlings (Ubuka et al. 2008), and between Lpxrfa and Gnrh in the Indian major carp (Biswas et al. 2015). However, in another teleost, the tilapia, it was found that Lpxrfa does not associate with neither Gnrh3 nor hypophysiotropic Gnrh1 neurons in the brain (Ogawa et al. 2016). Because tilapia, unlike the zebrafish, exhibit a stimulatory effect of Lpxrfa on gonadotropins rather than inhibitory (Biran et al. 2014), it is possible that this species does not utilize an Lpxrfa-Gnrh relationship and that tilapia Lpxrfa may influence gonadotropin secretion solely by direct pituitary contact. Another possibility for tilapia and other teleost species is that Lpxrfa's actions alternate between stimulatory and inhibitory and between the use of Gnrh as a mediator and direct pituitary effect, depending on the reproductive stage and/or sex as seen with Lpxrfa in goldfish (Moussavi et al. 2012) and with Kiss2 in the striped bass (Zmora et al. 2012).

The neuroanatomical interaction between zebrafish Lpxrfa and Gnrh3 is supported by the evidence that one of the zebrafish Lpxrfa peptides (Lpxrfa-3) consistently reduces *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 studied to date, including the goldfish Lpxrfa-2 and Lpxrfa-3 peptides on *gnrh3* (Qi et al. 2013) and sea bass Lpxrfa-1 on *gnrh1* (Paullada-Salmerón et al. 2016b). This result supports the reports in sheep that RFRP-3 can reduce GNRH-stimulated gonadotropin release (Clarke et al. 2008, Sari

et al. 2009). This finding is also in agreement with that of Ducret et al. (2009), who demonstrated that the application of RFRP-3 to mouse brain slices reduces GNRH1 neuron firing rates; therefore, this negative impact of RFRP-3/Lpxrfa on hypophysiotropic Gnrh neurons seems to be conserved across vertebrate classes.

Although zebrafish Lpxrfa-3 was capable of inhibiting *gnrh3* mRNA levels in an *in vitro* setting, there were no significant effects of zebrafish Lpxrfa-3 on brain *gnrh3* mRNA levels *in vivo*. Thus, the results of our *in vitro* and *in vivo* studies of Lpxrfa-3's effects on the brain were not in agreement. In the goldfish, injections of goldfish Lpxrfa-2 and Lpxrfa-3 reduced *fshβ* mRNA levels significantly, while the administration of each peptide to pituitary cultures did not change the expression levels of either *fshβ* or *lhβ* (Qi et al. 2013), unlike what was shown in Chapter 2 of this study. Only when goldfish Lpxrfa-3 was paired with an LHRH analogue (LHRH-A) could this peptide reduce the LHRH-A-stimulated increases in *fshβ* expression *in vitro* (Qi et al. 2013). Therefore, it is not uncommon for the results of *in vitro* and *in vivo* experiments to disagree and is observed in the Lpxrfa system of another cyprinid. Because this study utilized one dose of Lpxrfa-3 and one schedule of injection/sampling times, the inclusion of additional doses and injection/sampling times in the adult zebrafish should be explored to determine if an *in vivo* effect of Lpxrfa on *gnrh3* can be observed. In addition, the use of intracerebroventricular injections would avoid the obstacles of rapid peptide degradation, lack of appropriate carrier protein(s) in bloodstream, and possible inability to cross the blood-brain barrier that can be observed in intraperitoneal injections.

It is also possible that the lack of an effect in our *in vivo* experiment is due to the interactions of other reproductive and non-reproductive factors that are available in an *in vivo* setting. Unlike our *in vitro* experiment, in which only the brain is present and the neural interactions are neutralized, the *in vivo* experiment permits the interaction of other factors. Therefore, it is possible that these factors are interacting with zebrafish Lpxrfa-3 to comprehensively influence its effect on *gnrh3* in the brain *in vivo*. However, in this study, we have demonstrated that zebrafish Lpxrfa-3 is capable of directly inhibiting *gnrh3* mRNA levels in the zebrafish brain when factors that are outside of the brain are removed. It is also possible that, because the results of our assays reveal both no changes (*in vivo*) and reductions in *lhβ*, *cgα*, and *gnrh3* mRNA levels (*in vitro*), the effect (stimulatory vs inhibitory vs no effect) of Lpxrfa on Gnrh and gonadotropins is changing, as seen in some teleosts (Moussavi et al. 2012, Qi et al. 2013). This supports the idea that the teleosts are an intermediate group, in which Lpxrfa peptides can have stimulatory and/or inhibitory effects on reproduction (Osugi et al. 2012, Tsutsui et al. 2012).

Our efforts to localize Lpxrf receptors in the brains of zebrafish at various stages of life and reproduction failed. This prevented us from determining whether zebrafish Gnrh3 neurons express Lpxrf receptors, which has been shown with GNIH/RFRP and GPR147 in mice (Rizwan et al. 2012), sheep (Smith et al. 2008), and starlings (Ubuka et al. 2008). Therefore, we, instead, shifted our focus to Lpxrfa's impact on Kiss receptors (see Chapter 2). Kiss ligands stimulate Gnrh neurons (Irwig et al. 2005, Filby et al. 2008), and zebrafish Lpxrfa-2 and Lpxrfa-3 are capable of antagonizing Kiss2's activation of Kiss1ra (see Chapter 2). Therefore,

because 1) of the widespread localization of *kiss1ra*-expressing cells in the zebrafish brain (see Chapter 2), 2) Kiss2 neurons interact with Gnrh3 neurons in the zebrafish brain (Servili et al. 2011), and 3) Kiss2 (but not Gnrh3) neurons express *kiss1ra* in the zebrafish brain (Servili et al. 2011), it is possible that zebrafish Lpxrfa exerts its effects on Gnrh3 neurons through Kiss2 and Kiss1ra (in addition to direct Gnrh3 neuron contact). Since Gnrh3 neurons in zebrafish innervate the pituitary (Abraham et al. 2008, Xia et al. 2014) and stimulate gonadotropin synthesis and secretion (Lin and Ge 2009), the negative relationship between Lpxrfa and Gnrh3, possibly mediated by Kiss2 and Kiss1ra, offers another pathway by which Lpxrfa can exert its inhibitory effects on the gonadotropes in the pituitary.

In addition to the effects of Lpxrfa on Gnrh3 in adults, this study also examined the effects of the functional loss of Gnrh3 (through the *gnrh3*$^{-/-}$ knockout line) on *lpxrfa* expression in the brains of adult males and females, separately. After first observing significantly lower *lpxrfa* mRNA levels in the female *gnrh3*$^{-/-}$ adult brain but not the male *gnrh3*$^{-/-}$ adult brain compared to their WT counterparts, we expanded the experiment to include samples taken in the morning immediately before spawning and in the evening prior to final gamete maturation. Based on the results, male brains do not exhibit any differences in *lpxrfa* expression in response to the inherited functional loss of Gnrh3, in neither the morning nor in the evening. Female brains, on the other hand, exhibit significantly lower *lpxrfa* mRNA levels in the complete absence of Gnrh3, and this effect is only seen in the evening and not in the morning. This sex-specific phenomenon can be explained by the following:

First, it is possible that Gnrh3 plays a role in regulating the daily cycle of *lpxrfa* expression in the female brain. In fish, like the zebrafish, that spawn daily (Eaton and Farley 1974), females tend to show more pronounced daily cycles in important reproductive factors than males (Jensen et al. 2001), such as daily cycles of final oocyte maturation, ovulation, and spawning, supported by circadian hormonal cycles (Gothilf et al. 1997). In addition, GNIH has been shown to be regulated by the hormone melatonin in birds, in which seasonal and daily increases in melatonin stimulate the production of GNIH, thereby shutting down the reproductive axis (Ubuka et al. 2005, Chowdhury et al. 2010). These data, however, are lacking in teleosts. Therefore, it is possible that Gnrh3, at least partially, regulates the daily, reproductive cycle of *lpxrfa* in the more reproductively cyclic females than in males, perhaps mediating between melatonin and Lpxrfa. This is supported by the innervation of the retina by Gnrh3 neurons (Abraham et al. 2008), offering a potential connection between the light-receiving retina and Lpxrfa neurons. Consequently, the relationship between zebrafish Lpxrfa and Gnrh3 might be mutual in adults, in which Gnrh3 could potentially mediate photoperiodic signals to Lpxrfa neurons (Figure 3.9).

Second, because Lpxrfa has an inhibitory effect on *gnrh3* in the adult brain, it is possible that in females, where there are more apparent daily cycles in important reproductive factors than males (Jensen et al. 2001), *lpxrfa* levels decrease in the evening because Gnrh3 is completely absent in the *gnrh3*$^{-/-}$ fish. Naturally, Gnrh levels are high in the evening for morning daily spawners (Karigo et al. 2012) like the zebrafish, prior to increases in gonadotropin expression (that characterize oocyte maturation and ovulation) in the middle of the night and spawning at “lights on” the

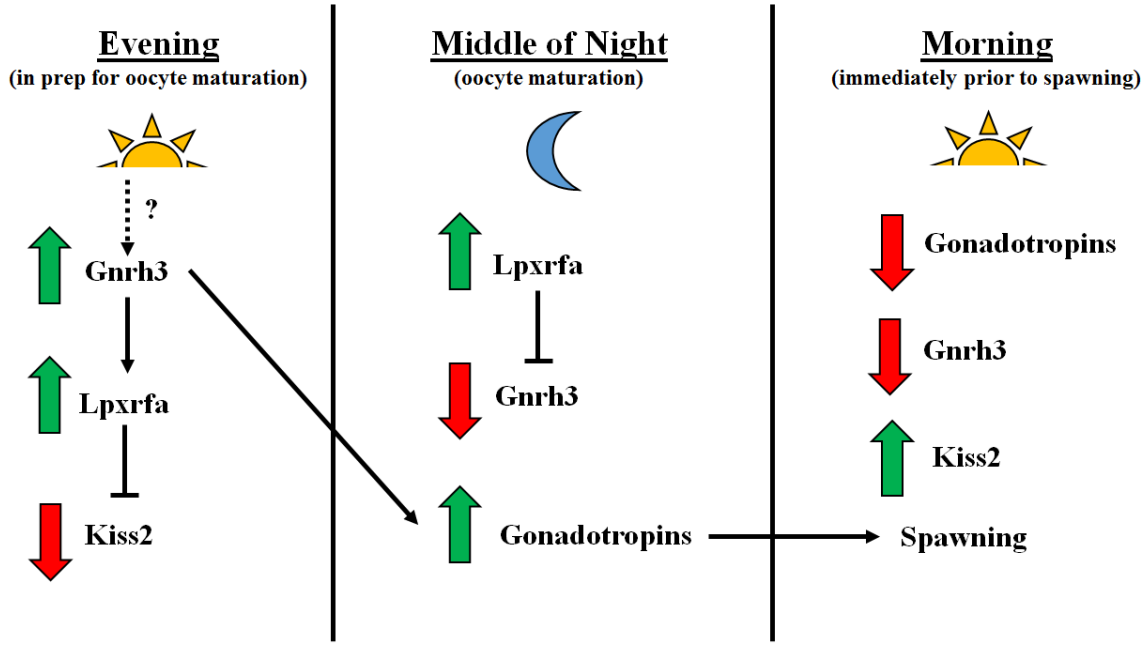

Figure 3.9. Potential regulation of reproduction in the daily cycle of the zebrafish by Lpxrfa, Gnrh3, Kiss2, and gonadotropins. In the evening, Gnrh levels are normally high in morning daily spawners (Karigo et al. 2012), like the zebrafish. We suggest that the elevation of Gnrh3 levels in the evening allows for the expression of *lpxrfa*, which, in turn, might be responsible for the low levels of *kiss2* seen in the zebrafish at this time (Zmora et al. 2014). It is also possible that Gnrh3 is mediating photoperiodic signals to Lpxrfa in the evening. In the middle of the night, there are increases in expression levels of the gonadotropins (So et al. 2005, Karigo et al. 2012), caused by the elevation of Gnrh3 in the evening. At this time, Gnrh3 levels have decreased, most likely in response to the *lpxrfa* expression in the evening (Karigo et al. 2012). In the morning at dawn, zebrafish spawn and exhibit high levels of *kiss2* (Zmora et al. 2014), along with lower levels of Gnrh3 and gonadotropins.

following morning (Figure 3.9; So et al. 2005, Selvaraj et al. 2012). Thus, because we have demonstrated an inhibitory effect of zebrafish Lpxrfa on *gnrh3*, we suggest that *lpxrfa* is expressed in the evening, in response to the increased *gnrh3* levels, in order to start the subsequent decrease in *gnrh3* levels that is usually observed at the times of oocyte maturation, ovulation, and spawning (Figure 3.9). In this case, Gnrh3 and Lpxrfa probably cross-talk with each other: Gnrh3 allows for expression of *lpxrfa* in the evening, which, in turn, downregulates *gnrh3* levels (Figure 3.9). Therefore, according to this potential scenario, the decrease in *lpxrfa* levels in the *gnrh3*$^{-/-}$ female brain in the evening can be explained by the complete lack of Gnrh3 peptide that is needed to keep *lpxrfa* mRNA levels elevated.

In addition, Lpxrfa is not the only zebrafish neuropeptide that exhibits differences during the daily, reproductive cycle, as Kiss2 neurons display diurnal plasticity in both males and females (Zmora et al. 2014): At 18 hours before spawning, no *kiss2*-expressing soma are present in the medio-basal hypothalamus in the vicinity of Lpxrfa-ir somas; however, at the time of spawning, there are multiple *kiss2*-expressing soma in this region (Zmora et al. 2014; Figure 3.10). In the striped bass, medio-basal hypothalamus Kiss2 neurons are essential to the execution of spawning (Zmora et al. 2015). Since Kiss2 neurons (but not Gnrh3 neurons) express *kiss1ra* in the zebrafish (Servili et al. 2011) and interact with Gnrh neurons to stimulate the secretion of Gnrh/GNRH in fish and mammals (Irwig et al. 2005, Zmora et al. 2014), our finding that Lpxrfa modulates Kiss2's action via Kiss1ra offers another avenue by which Lpxrfa may affect Gnrh3. Taken together, the levels of *lpxrfa* observed in adult zebrafish in the evening, which are likely regulated by Gnrh3

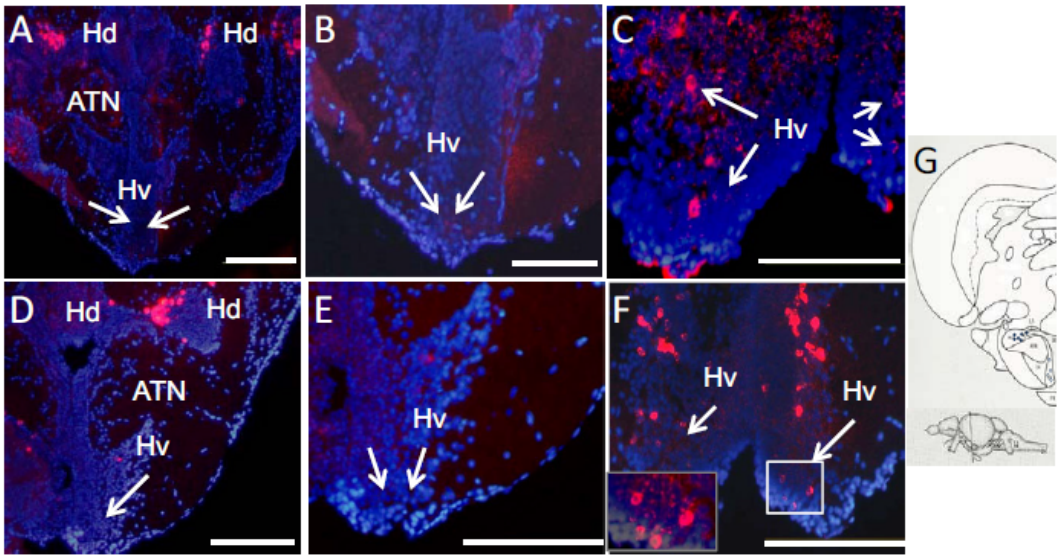

Figure 3.10. Figure from Zmora et al. (2014) that demonstrates the differences in zebrafish *kiss2* expression in the medio-basal hypothalamus in the evening and morning. Caption from Zmora et al. (2014):

Kiss2 neuronal distribution in male and female zebrafish before and at spawning. ISH using kiss2 anti-sense riboprobe on females (A–C) and males (D and E) medio-basal hypothalamic sections. A, Female brain 18 hours before spawning. B, Higher magnification of A focusing on the Hv region. C, Female brain at spawning. D, Male brain 18 hours before spawning. E, Higher magnification of D focusing on the Hv region. F, Male brain at spawning. G, Upper panel illustrates the relevant brain cross-section demonstrating the organization of the regions, black dots represent kiss2-expressing neuronal cell bodies exist at all times, and gray dots represent those observed only at spawning; lower panel illustrates sagittal whole brain demonstrating the anatomical location of the presented cross-section (illustrations were adopted from Ref. 45). Hv, ventral zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; ATN, anterior tuberal nucleus. Scale bars, 100 μm.

neurons and are low in the complete absence of Gnrh3 in females, may downregulate the expression of *kiss2* in the medio-basal hypothalamus (Figure 3.9). However, in the morning, *kiss2*-expressing cells appear at the same time that spawning occurs (Figure 3.9).

In conclusion, this chapter has demonstrated that zebrafish Lpxrfa and Gnrh3 are two important neuropeptides that closely interact within the zebrafish brain. Developmental expression profiles of *lpxrfa* and *gnrh3* during the first 30 days of development revealed no correlation between the two transcripts. Knockdown of *lpxrfa* via anti-sense MO oligonucleotides was accomplished but, surprisingly, revealed no effects on *gnrh3* expression in early development. It is possible that Lpxrfa and Gnrh3 exert additional functions (e.g., neuromodulator or neurotransmitter) in early development that are not related to reproduction. However, the inherited functional loss of Gnrh3 (via the *gnrh3*$^{-/-}$ knockout line) demonstrated significantly lower *lpxrfa* mRNA levels during most of development. Therefore, the relationship between these two neuropeptides seems to be a one-way regulation (Gnrh3 regulating Lpxrfa) during development. In adults, while Lpxrfa fibers project to and interact with Gnrh3 neurons in the adult pre-optic area, zebrafish Lpxrfa-3 also consistently and reliably reduces the expression of *gnrh3* in the adult brain *in vitro*, with little to no effect on *gnrh2* expression, thereby reinforcing the reproductive functions played by Lpxrfa and Gnrh3. In the *gnrh3*$^{-/-}$ adult brain, females exhibit lower levels of *lpxrfa* compared to their WT counterparts in the evening but not in the morning immediately prior to spawning, suggesting roles for Lpxrfa and Gnrh3 in the daily, reproductive cycles of the female. Overall, during development, Gnrh3 appears

to regulate *lpxrfa* expression, while the relationship between these two neuropeptides evolves to become mutual in the adult brain due to their neuroanatomical and functional interactions. This mutual relationship between zebrafish Lpxrfa and Gnrh3, possibly mediated by Kiss2/Kiss1ra, offers an additional pathway (i.e., other than direct contact with the pituitary) for Lpxrfa to exert its inhibitory effects on the pituitary and enables a more sophisticated regulation of gametogenesis.

Chapter 4: Generation, Validation, and Characterization of a *gnrh3*$^{-/-}$ Line

ABSTRACT

The increased availability of efficient loss-of-function techniques in recent years has enabled more research to understand the exact functions that genes exert within an organism, with many of these studies utilizing the zebrafish. Previous research in mice and humans has demonstrated that *Gnrh*/*GNRH* null mutations result in hypogonadotropic hypogonadism and sterility. The goal of this study was to eliminate *gnrh3* functions in zebrafish to determine how ontogeny and reproductive performance are affected and how this effect is translated in factors downstream of Gnrh3 along the reproductive axis. Using the TALEN technology, we developed a *gnrh3*$^{-/-}$ zebrafish line that harbors a 62 bp deletion in the *gnrh3* gene. Our *gnrh3*$^{-/-}$ zebrafish line represents the first targeted and heritable mutation of a Gnrh isoform in any organism. Using immunohistochemistry, we verified that *gnrh3*$^{-/-}$ fish do not possess Gnrh3 peptide in any region of the brain or pituitary. However, other than changes in mRNA levels of pituitary gonadotropin genes (*fshβ*, *lhβ*, and *cgα*) during early development, which are corrected by adulthood, there were no changes in ontogeny and reproduction in *gnrh3*$^{-/-}$ fish. The *gnrh3*$^{-/-}$ zebrafish exhibit normal Gnrh3 neuronal migration during ontogeny and are fertile as adults, displaying normal gametogenesis and reproductive performance in males and females. Together with previous results that zebrafish Gnrh3 cell ablation causes sterility, these results indicate that a compensatory mechanism is being activated, which is probably primed early on upon Gnrh3 neuron differentiation and possibly confined to Gnrh3 neurons.

INTRODUCTION

In order to truly understand the role that a particular gene plays, loss-of-function techniques are excellent methods for determining how an organism responds when that gene is absent. The traditional methodology for mice has been through genetic manipulation of embryonic stem cells (Chemelli et al. 1999, Kanatsu-Shinohara et al. 2006), while zinc finger-nucleases have been the dominant tool in fish for years (for review, see Urnov et al. 2010). Zinc finger-nucleases are composed of zinc finger proteins (DNA recognition site) combined with the non-specific nuclease FokI, which, working as a dimer, induce double-stranded breaks in genomic DNA to generate fish with heritable mutations. Zinc finger-nucleases have been successful for knocking out genes in zebrafish (Doyon et al. 2008, Meng et al. 2008, Foley et al. 2009) but often exhibit off-site targeting (Pattanayak et al. 2011, Sander et al. 2013). Within the past five to six years, new techniques have come forward (i.e., TALENs and later CRISPR/Cas9) that are highly efficient at allowing targeted and heritable mutations in zebrafish lines, with a lower incidence of off-site targeting and a higher specificity for target DNA sequences.

The TALEN technology involves the use of naturally occurring proteins found in the plant pathogen *Xanthomonas* spp. that regulate plant genes during pathogenesis. These TALE proteins consist of a central repeat domain that is responsible for DNA targeting. Each repeat unit consists of 33-35 amino acids that recognizes a specific target base, which is determined by two important amino acids that constitute the "repeat variable di-residue" (RVD). This connection is so specific that a code has been generated to determine which RVD recognizes which target

base. Thus, TALE proteins can be engineered to target a specific DNA sequence (Miller et al. 2011). When combined to FokI, a TALEN can be designed to specifically induce a double-stranded break in DNA at a desired location. The result is a mutation in the gene of interest due to the error-prone nature of non-homologous end-joining (Miller et al. 2011). Additionally, the insertion of a sequence of interest can also be accomplished with TALENs via homology-directed repair (Miller et al. 2011). The TALEN technology is quite effective and allows for the production of "knockout" zebrafish lines, if the mutated gene is not essential for the survival of the embryo to maturity. In addition to the TALEN technology, the new CRISPR/Cas9 technology has also allowed the production of multiple knockout lines. CRISPR/Cas9 works by utilizing a system in bacteria with adaptive immunity to viruses (Doudna and Charpentier 2014). However, this methodology has high incidents of reported off-site targeting (Cradick et al. 2013, Fu et al. 2013).

The original goal of this chapter was to generate and validate *lpxrfa*$^{-/-}$ and *gnrh3*$^{-/-}$ knockout zebrafish lines with the TALEN technology to understand how Gnrh3 and Lpxrfa are influenced, respectively, by the inherited loss of the functional proteins and how they influence each other. The availability of these knockout lines would prove to be invaluable tools for understanding the molecular mechanisms that these neuropeptides utilize in their relationship with one another. However, despite a thorough effort described in the Discussion, technical difficulties did not allow the production of a functional *lpxrfa*$^{-/-}$ knockout line. On the other hand, this study did succeed in producing a true, validated knockout line of *gnrh3*$^{-/-}$ fish, which was used in Chapter 3 to understand how an inherited functional loss of Gnrh3 affects *lpxrfa*,

further elucidating the Lpxrfa-Gnrh3 relationship in zebrafish. Therefore, in this study, we focused on the generation, validation, and basic characterization of the *gnrh3*$^{-/-}$ knockout line, which indeed lacked Gnrh3 protein as observed with immunohistochemistry. Most importantly, because this study represents the first targeted, heritable mutation in a GNRH isoform in any organism, the findings of this study have the potential to shed light on how the inherited loss of Gnrh3 contributes to changes downstream in the reproductive axis and to reproductive performance.

The early development of zebrafish Gnrh3 begins with its expression in two cell clusters within the olfactory epithelium at approximately 24 hpf (Abraham et al. 2008). Shortly thereafter, fibers begin to extend from these cells that guide the Gnrh3 soma on a migration pattern that eventually leads to innervation of the pituitary at 4-5 dpf (Abraham et al. 2008, Xia et al. 2014). In adult zebrafish, Gnrh3 soma are found along the terminal nerve/ventral telencephalon and in the pre-optic area/hypothalamus (Abraham et al. 2008) with several projections throughout the brain and into the pituitary (Xia et al. 2014), where they interact with vasculature leading to the gonadotropes (Golan et al. 2015). Earlier studies investigating the roles of Gnrh3 in the ontogeny of its neurons and in reproduction have established the importance of Gnrh3 in both processes. The knockdown of *gnrh3* by anti-sense MO oligonucleotides resulted in misguided migration of Gnrh3 soma during early development, suggesting that Gnrh3 itself is needed for proper migration of its neurons (Abraham et al. 2008). In addition, successful laser ablation of Gnrh3-expressing cells in the olfactory region during early development (4-6 dpf) has led to the production of all females, in which oocyte development is arrested at stage II

(cortical alveolus/pre-vitellogenic follicles), and sterility (Abraham et al. 2010). Therefore, it is believed that Gnrh3 neurons originating in the olfactory region during early development are essential to full gamete maturation and spawning in zebrafish, at least in females.

In 1977, Cattanach et al. (1977) reported the discovery of a natural *Gnrh1* mutant mouse (*hpg*), in which individuals possess a 33.5 kb deletion that includes the latter half of the *Gnrh1* gene (Mason et al. 1986). These *hpg* mice exhibit reduced pituitary content and circulating levels of FSH and LH and display hypogonadotropic hypogonadism, in which all individuals are sterile (Cattanach et al. 1977). In addition, some humans with hypogonadotropic hypogonadism are characterized by a failure to undergo puberty and have been described to possess one of approximately six different forms of mutations in *GNRH1* (Chan et al. 2009), in which one of the mutational "hot spots" tends to be in the region encoding the decapeptide (Mengen et al. 2015). Because highly efficient loss-of-function knockout techniques were not available in fish until recently, comparative experiments could not be conducted on teleosts to determine how the inherited loss of Gnrh function manifests in teleost physiological processes.

Because humans with *GNRH1* mutations (Chan et al. 2009), *hpg* mutant mice (Cattanach et al. 1977), and Gnrh3 cell-ablated zebrafish (Abraham et al. 2010) are all sterile individuals with arrested gonad development, we hypothesized that knocking out the *gnrh3* gene in zebrafish would also lead to disrupted gametogenesis and the production of sterile fish. In addition, because knockdown of *gnrh3* resulted in misguided migration of the Gnrh3 neuronal system in early development, we

hypothesized that knocking out *gnrh3* would also affect reproductive ontogeny. The goal of this study was to establish a *gnrh3*$^{-/-}$ knockout line in zebrafish to determine the mechanisms by which Gnrh3 exerts its regulatory functions. Specifically, we aimed at identifying the downstream BPG components that are impacted by the inherited loss of Gnrh3. The effects of *gnrh3* knockout on reproductive ontogeny, gametogenesis, and reproductive performance were also determined. We also used the *gnrh3*$^{-/-}$ knockout line to understand how Lpxrfa is affected by the complete absence of Gnrh3, which was thoroughly described in Chapter 3. The specific objectives of this chapter were to:

1) Generate and validate a *gnrh3*$^{-/-}$ line in the zebrafish with the TALEN technology,
2) Quantify important reproductive genes/peptides downstream of Gnrh3 in the reproductive axis during development and adulthood in the *gnrh3*$^{-/-}$ line,
3) Determine if the ontogeny and migration of Gnrh3 neurons during development are affected by the complete absence of Gnrh3, and
4) Determine how gametogenesis and reproductive performance are affected by the inherited loss of functional Gnrh3.

METHODS

Animals

All zebrafish used in this study originated from the in-house colony at 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 monitored and fed twice daily with a commercial flake food

or pellets *ad libitum*. Zebrafish embryos and larvae were raised in individual containers of freshwater until 30 dpf, before being transferred to the recirculating system. Starting at 5 dpf, larval zebrafish were fed *Paramecium* twice daily, until 14 dpf, when *Artemia* nauplii was introduced to their diet. Prior to tissue collections, adult fish were euthanized by immersion in an ice bath followed by quick decapitation. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland Baltimore School Of Medicine.

Preparation and Micro-Injection of TALEN mRNAs

A pair of zebrafish *gnrh3*-targeting TALENs were designed to induce a double-stranded DNA break at base pair 3700 in *gnrh3* genomic DNA (Ensembl ENSDARG00000056214) or base pair 71 in the *gnrh3* cDNA coding region (NCBI Reference Sequence NM_182887.2) by an NIH-sponsored initiative (NIH R01 GM088040). This TALEN target site is the second base pair of the codon that encodes for the first amino acid (glutamine) in the zebrafish Gnrh3 decapeptide, which is located within the recognition site of the Cac8i restriction enzyme, allowing a useful way to screen for mutated genomic DNA. Each *gnrh3*-targeting TALEN sequence was cloned into the JDS71 vector under the control of the T7 promoter (Addgene). The TALEN plasmids were linearized with PmeI, and one μg of each linearized plasmid was transcribed into mRNA with the Ambion mMessage mMachine® Transcription Kit, using the T7 RNA polymerase. After DNase treatment, a polyA tail was added to the mRNA samples (Ambion PolyA Tailing Kit).

Approximately 50-75 ng/µL of both TALEN mRNAs (left and right) were simultaneously micro-injected into one- to two-cell stage WT zebrafish embryos (F0), which were grown to maturity. To identify fish that carry a mutation, sperm (~0.5-1 µL) was collected by stripping each male individual, resuspended in 10 µL HBSS, and used as a PCR screening template with TALEN-targeted primers. PCR products were digested with Cac8I and run on a 2% agarose gel to distinguish between mutant and WT DNA. Genomic DNA PCR products that were not digested by Cac8I were assumed to be mutated. The undigested (mutant DNA) products were gel-extracted, purified, and sequenced.

Generation of* gnrh3$^{-/-}$ *Line

F0 males that contained a *gnrh3*-specific mutation were crossed with WT females to obtain F1 offspring. Once mature, F1 fish were fin-clipped for gDNA extraction. Briefly, caudal fin tissue was immersed in gDNA extraction buffer (50 mM KCl, 10 mM Tris-HCl (pH = 8.0), 150 mM $MgCl_2$, 0.3% Tween-20, and 0.3% NP40 in sterile MilliQ water), boiled at 95-100 °C for 15 minutes, cooled on ice, and digested with proteinase K (New England BioLabs) at 55 °C for at least 1-3 hours. After digestion, lysates were boiled at 95-100 °C for 15 minutes to inactivate proteinase K and centrifuged for 3 minutes at 12,000 rpm. The resulting gDNA was genotyped by PCR, Cac8I digestion, and sequencing, as described above.

F1 adults that contained a *gnrh3* mutation were crossed with WT fish to obtain heterozygous fish (F2). Heterozygous adults (F2) were in-crossed to obtain the F3 generation, of which ¼ were WT (*gnrh3*$^{+/+}$), ½ were heterozygous for the *gnrh3* mutation (*gnrh3*$^{+/-}$), and ¼ were homozygous for the *gnrh3* mutation (*gnrh3*$^{-/-}$).

All F3 *gnrh3*$^{-/-}$ fish were verified by a two-step PCR and sequencing of fin-clip gDNA. The first step involves a double PCR reaction, in which two different forward primers (Step1mutantFor and Step1WTFor), along with the same reverse primer (StepRev), compete for the mutated or WT gDNA, respectively. The results from step 1 distinguish between *gnrh3*$^{+/+}$, *gnrh3*$^{+/-}$, and *gnrh3*$^{-/-}$ gDNA. The second step PCR (primers Step2For and StepRev) is conducted on the *gnrh3*$^{-/-}$ gDNA and amplifies a larger product for sequencing. The primers for the two-step PCR can be found in Table 4.1.

Gnrh3 Immunohistochemistry

Brains and whole heads (for pituitary visualization) were dissected from adult fish and fixed overnight with 4% PFA (in PBS) at 4 °C. Whole heads were fixed after the removal of the eyes, jaw, gills, and other soft tissues, and before cryopreservation, fixed whole heads were decalcified with 0.5 M EDTA (pH = 8.0) for 5-7 days at 4 °C. All tissues were transferred to 30% sucrose (in PB) overnight at 4 °C before frozen in OCT the next day. Brain and whole heads were sectioned in OCT at 15 μm thickness, transferred to charged slides, and stored at -20 °C until immunohistochemistry. Briefly, dried sections were fixed in pre-chilled acetone for 2 minutes. Sections were blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Sections were incubated overnight at 4 °C with rabbit anti-zebrafish Gnrh3 Gap (1:1000 – 1:3000) diluted in 1% BSA and 0.3% Triton X-100 in PBS. After PBS washes with 0.3% Triton X-100, sections were incubated for 1 hour at room temperature with goat anti-rabbit secondary antibody Alexa 488 (Life Technologies) or Cy3 (KPL, Inc.) diluted 1:300 – 1:500 in 1% BSA

Table 4.1. PCR primers for a two-step PCR process used to screen F3 fish fin-clip gDNA as either *gnrh3*$^{+/+}$, *gnrh3*$^{+/-}$, or *gnrh3*$^{-/-}$ and to submit for sequencing.

Primer	PCR Step	Type	Sequence (5' → 3')	T_m (°C)	GC %	Amplicon size (bp)*
Step1mutantFor	1	Mutant For	TTAGTTTAGATTTCAGCAGTTT TAGCATCATA	55.3	28.1	544
Step1WTFor	1	WT For	GAAGGTTGCTGGTCCAGTTGTT GCTG	62.2	53.8	582
Step2For	2	For	TTGAGATGGAAGACAATCCTTT	51.9	36.4	800
StepRev	1/2	Rev	TGCACATGTACTTGCTGAATTA	52.6	36.4	

*when paired with StepRev primer.

and 0.3% Triton X-100 in PBS.

To provide more verification of the lack of Gnrh3 protein in the *gnrh3*$^{-/-}$ fish, we also immunostained *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ adult brain sections with an additional polyclonal primary antibody (anti-Gnrh3 decapeptide, kindly provided by the late Dr. Judy King). Before blocking and overnight incubation with the primary antibody (1:700), slides were quenched with 0.3% H_2O_2 in PBS for 30 minutes. Goat anti-rabbit-HRP (1:1000) was used to detect the primary antibody, and visualization of the protein signal required the TSA Plus kit, according to the manufacturer's protocol. Fluorescence was obtained via the Cy3 dye from the TSA Plus kit.

gnrh3 in situ *Hybridization*

Brains were dissected from adult *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish and fixed overnight with 4% PFA (in PBS) at 4 °C. Before cryopreservation, tissues were transferred to 30% sucrose (in PB) overnight at 4 °C. Brain tissues were frozen in OCT, sagitally sectioned at 10 µm thickness, transferred to charged slides, and stored at -80 °C until *in situ* hybridization. Anti-sense and sense DIG-labeled riboprobes were synthesized from the cDNA clone of the entire *gnrh3* coding region (from 28 bp

to 312 bp of NCBI Reference Sequence NM_182887.2), using RNA polymerase (Roche Diagnostics). The *in situ* hybridization protocol was followed as according to Zmora et al. (2012) with a riboprobe concentration of 500 ng/mL. The signal was detected using the TSA Plus kit, according to the manufacturer's protocol and using anti-DIG HRP (1:200; Roche Diagnostics). Fluorescence was obtained via the Cy3 dye from the TSA kit. Negative control slides were similarly treated with sense riboprobes.

Developmental and Adult Gene Expression Profiles

In order to characterize the *gnrh3*$^{-/-}$ line during development, *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ embryos/larvae were sampled at 8 developmental time points (1, 2, 3, 8, 12, 18, 24, and 30 dpf). All samplings were done in triplicate. Ten *gnrh3*$^{+/+}$ and ten *gnrh3*$^{-/-}$ embryos or larvae each were pooled and collected at each sampling point from 1 dpf to 18 dpf, whereas 8 larvae and 6 larvae were pooled and collected at 24 dpf and 30 dpf, respectively. Embryos/larvae were frozen on dry ice and stored at -80 °C until RNA extraction. During adulthood, four *gnrh3*$^{+/+}$ and four *gnrh3*$^{-/-}$ fish of reproductive age were selected from each sex for dissection of the brains, pituitaries, and gonads. Tissues were frozen on dry ice and stored at -80 °C until RNA extraction. QPCR was conducted for the following genes for the developmental samples: brain: *gnrh2*, *gnrhr1*, *gnrhr2*, *gnrhr3*, *gnrhr4*, *cyp19a2*; pituitary: *fshβ*, *lhβ*, *cgα*, *gnrhr1*, *gnrhr2*, *gnrhr3*, *gnrhr4*; and gonad: *fshr* and *lhr*. Genes that showed many significant differences between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ levels during development (*fshβ*, *lhβ*, and *cgα*) were also assessed in the adult tissues.

Rescue of* cgα *mRNA Levels in 12 dpf* gnrh3$^{-/-}$ *Larvae

As determined by the developmental expression profile above, at 12 dpf, *gnrh3*$^{-/-}$ larvae had significantly and consistently higher *cgα* mRNA levels than *gnrh3*$^{+/+}$ larvae. If this difference is indeed due to the loss of Gnrh3, we hypothesized that the *gnrh3*$^{-/-}$ levels could be reduced to *gnrh3*$^{+/+}$ levels by exposure to the synthetic Gnrh3 decapeptide. Therefore, we exposed 12 dpf *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ larvae to different concentrations (5 and 50 µM dissolved in fish water) of the Gnrh3 decapeptide with different exposure times (0, 6, 12, and 24 hours) in a 24-well plate. For both genotypes, there were three replicates (n = 5) for each exposure time of each concentration of the Gnrh3 decapeptide. When the exposures began for the 6 and 12 hr larvae, the peptide/water was replenished in the wells that had already received treatment. All of the exposures ended at the same time on the evening in which the larvae were 12 dpf. Embryos were frozen on dry ice and stored at -80 °C until RNA extraction. QPCR for *cgα* was then conducted as described below.

Quantification of Gene Transcripts

Total RNA was extracted from tissues using TRIzol® reagent (Invitrogen), according to the manufacturer's protocol, and total RNA was quantified with a Nanodrop (Thermo Scientific). Total RNA (1 µg for embryos/larvae and 100 ng for adult pituitaries) was treated with gDNA wipeout buffer for 9 min at 42 °C and synthesized into first-strand cDNA with the Qiagen QuantiTect RT Kit in a 20 (embryos/larvae) or 10 (adult pituitaries) µL reaction. Gene-specific QPCR primers (for genes that showed many significant differences during development) are listed in Table 2.1 in Chapter 2, with *ef1α* as an internal control. Specificity of each primer

set's amplification was confirmed by a dissociation curve. The efficiencies of the QPCR primer sets were $R^2 \geq 0.951$ for the developmental time series, $R^2 \geq 0.988$ for adult pituitaries, and $R^2 \geq 0.947$ for the rescue experiment. Each QPCR reaction was carried out in duplicate with a final volume of 10 µL: 2x DyNAmo Flash SYBR Green QPCR mix (Life Technologies), 200 nM primer mix, 0.3x ROX (Life Technologies), 20 ng cDNA, and sterile MilliQ water, in a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc.). The cycle conditions were 95 °C for 7 minutes, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. Each plate included a standard curve using the reverse-transcribed RNA of the specific clone as a template. Absolute copy number was calculated for the tested reactions and normalized against the housekeeping gene *ef1α*. Each plate included two no RT controls and two no template controls.

Lh ELISA

Pituitaries from adult *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ males and females (n = 5 - 8) were pooled and analyzed by ELISA for Lh content. The protocol was originally developed for carp Lh (Aizen et al. 2012), based on the procedure described for tilapia Lh (Aizen et al. 2007), and adopted for zebrafish Lh (Biran et al. 2012). For the carp ELISA, which was used for the current study, the intra-assay and inter-assay coefficients of variation were 7.6% and 11.3%, respectively, while the sensitivity of the assay was 32 pg/mL (Aizen et al. 2012).

Imaging of Gnrh3 Fibers in* gnrh3$^{-/-}$ gnrh3:tdTomato *Juveniles

We crossed our *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish with the *gnrh3:tdTomato* transgenic line previously developed in our lab (Abraham et al. 2008, Xia et al. 2014). Fish that

were heterozygous for both the *gnrh3* mutation and the *gnrh3:tdTomato* transgene were in-crossed to obtain fish that were homozygous for both the mutation and the transgene. Importantly, *gnrh3*$^{-/-}$ fish that express *gnrh3:tdTomato* do not express the Gnrh3 decapeptide, since the *gnrh3:tdTomato* transgene does not include the portion of exon 2 in *gnrh3* that encodes for the Gnrh3 decapeptide. At 34 dpf, *gnrh3*$^{+/+}$ *gnrh3:tdTomato* and *gnrh3*$^{-/-}$ *gnrh3:tdTomato* juveniles were fixed overnight in 4% PFA (in PBS) at 4 °C and then decalcified for 5-7 days in 0.5M EDTA (pH = 8.0) at 4 °C. Before cryopreservation, tissues were transferred to 30% sucrose (in PB) overnight at 4 °C. Fish were frozen in OCT, sagitally sectioned at 30 µm thickness, transferred to charged slides, and stored at -20 °C. After slides were dried, they were washed with PBS and mounted before viewing.

Gonad Histology

Whole gonads were fixed overnight in 4% PFA (in PBS) at 4 °C, dehydrated with an ethanol series, cleared with two xylene washes, embedded in paraffin, sectioned at 5 µm on a HM 340 microtome (Thermo Scientific), and allowed to dry overnight at 42 °C. Sections were stored at 4 °C until paraffin removal and tissue hydration with two xylene washes and an ethanol series. Sections were stained with hematoxylin and eosin and mounted with Permount.

Fecundity, Fertility, and Offspring Survival Assessments

In order to determine fecundity, fertility, and offspring survival of *gnrh3*$^{-/-}$ fish, three to six spawning pairs of each of the following were used to obtain offspring: a) *gnrh3*$^{+/+}$ male x *gnrh3*$^{+/+}$ female, b) *gnrh3*$^{+/+}$ male x *gnrh3*$^{-/-}$ female, c) *gnrh3*$^{-/-}$ male x *gnrh3*$^{+/+}$ female, and d) *gnrh3*$^{-/-}$ male x *gnrh3*$^{-/-}$ female. A male and

female fish were placed in a spawning container the night before spawning and were kept separated until the divider was removed immediately after "lights on" in the morning. One hour after removing the divider, embryos were collected and counted for each pair to determine fecundity. At 4-5 hpf, viable embryos were counted for each pair to determine the percentage of fertilization. Embryos were then assessed for percentage of survival at 2 dpf.

Generation, Validation, and Assessment of Reproduction in the* gnrh3$^{-/-}$ gnrh2$^{-/-}$ *Line

Because the *gnrh3*$^{-/-}$ line did not exhibit any major changes in reproduction, we hypothesized that some factor may be compensating for the lack of a major reproductive phenotype. The first candidate considered as a potential compensation factor was the only other identified Gnrh isoform in the zebrafish: Gnrh2. A *gnrh2*$^{-/-}$ line developed in the Zohar Lab (unpublished results) was crossed with the *gnrh3*$^{-/-}$ line to eventually obtain a double knockout *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ line. The double knockout fish were assessed for gametogenesis (via gonad histology) and for fecundity and fertilization rate as already mentioned.

In order to validate that the *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ fish are true double knockouts, brain sections were obtained as previously described and subjected to a double immunohistochemistry protocol for both Gnrh3 and Gnrh2. Briefly, dried sections were fixed in pre-chilled acetone for 2 minutes and allowed to dry. Sections were incubated with 0.5% H_2O_2 in PBS for 30 minutes at room temperature to quench endogenous HRPs. Sections were blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Sections were incubated overnight at 4 °C with rabbit anti-zebrafish Gnrh3 Gap (1:1000) diluted in 1% BSA and 0.3% Triton

X-100 in PBS. Sections were incubated for 1 hour at room temperature with goat anti-rabbit HRP (1:1000). The signal was detected using the TSA Plus kit, and fluorescence was obtained via the FITC dye from the kit. After quenching HRPs with 0.02N HCl for 10 minutes, blocking and immunostaining for Gnrh2 with the anti-Gnrh2 Gap antibody (1:750; previously developed in our lab) were conducted in the same manner, except that fluorescence was obtained via the Cy3 dye from the TSA Plus kit.

Microscopy

All slides were mounted with anti-fading solution with DAPI or Vectashield with DAPI (Vector Labs) and imaged with one of two microscopes. For brains, pituitaries, and hematoxylin and eosin staining of gonads, a Zeiss Axioplan 2 microscope with a 20x (or 5x for female ovaries) magnification and a resolution of 1360 x 1024 was used with an Attoarc HBO100 W power source, equipped with a CCD Olympus DP70 camera, or a Leica Microsystems DMi8 confocal microscope with a resolution of 1024 x 1024, a magnification of 20x, and a z-step size of 0.10. For *gnrh3:tdTomato* juvenile sections, a Leica Microsystems DMi8 confocal microscope was used with a resolution of 1024 x 1024, a magnification of 20x, and a z-step size of 0.10. All images were analyzed with Image J and/or Adobe Photoshop.

Statistics

All data are represented as mean values ± SEM, unless otherwise specified. For differences between genotypes at each developmental sampling time point or between the sexes for the QPCR and ELISA assays, a one-tailed, homoscedastic Student *t*-test was used. For analysis of fecundity, fertility, and offspring survival

data for the *gnrh3*$^{-/-}$ fish, a one-way ANOVA was conducted with GraphPad Instat 3. For analysis of fecundity and fertility for the *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ fish, a one-tailed, homoscedastic Student *t*-test was conducted. Statistical significance was established if $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$.

RESULTS

Generation and Validation of the *gnrh3*$^{-/-}$ Line

Development of gnrh3$^{-/-}$ *Line*

Following PCR screening for the mutated *gnrh3* gene in the F1 generation, two mutated sequences were detected: a 9 bp continuous deletion within the Gnrh3 decapeptide nucleotide sequence (data not shown) and a 62 bp discontinuous deletion, which included the ATG start codon and the beginning of the nucleotide sequence encoding the Gnrh3 decapeptide (Figure 4.1Aa). The 62 bp deletion was restricted to exon 2 and included four single bp substitutions (black arrows in Figure 4.1Ab). F1 adults with the 62 bp deletion were crossed to WT fish to obtain heterozygous F2 fish (*gnrh3*$^{+/-}$). Heterozygotes were in-crossed, and one-quarter of their offspring were determined to be *gnrh3*$^{-/-}$ by PCR and sequencing (Figure 4.1Ac). The results also revealed the same 62 bp deletion in the *gnrh3*$^{-/-}$ cDNA (Figure 4.1Ba), which was also determined to be homozygous (Figure 4.1Bc). Because the mutation in the gDNA is completely located within exon 2, the sequence of the cDNA mutation was identical to that of the gDNA (Figure 4.1Aa vs Figure 4.1Ba). These results demonstrated no alternative splicing in the *gnrh3*$^{-/-}$ mRNA.

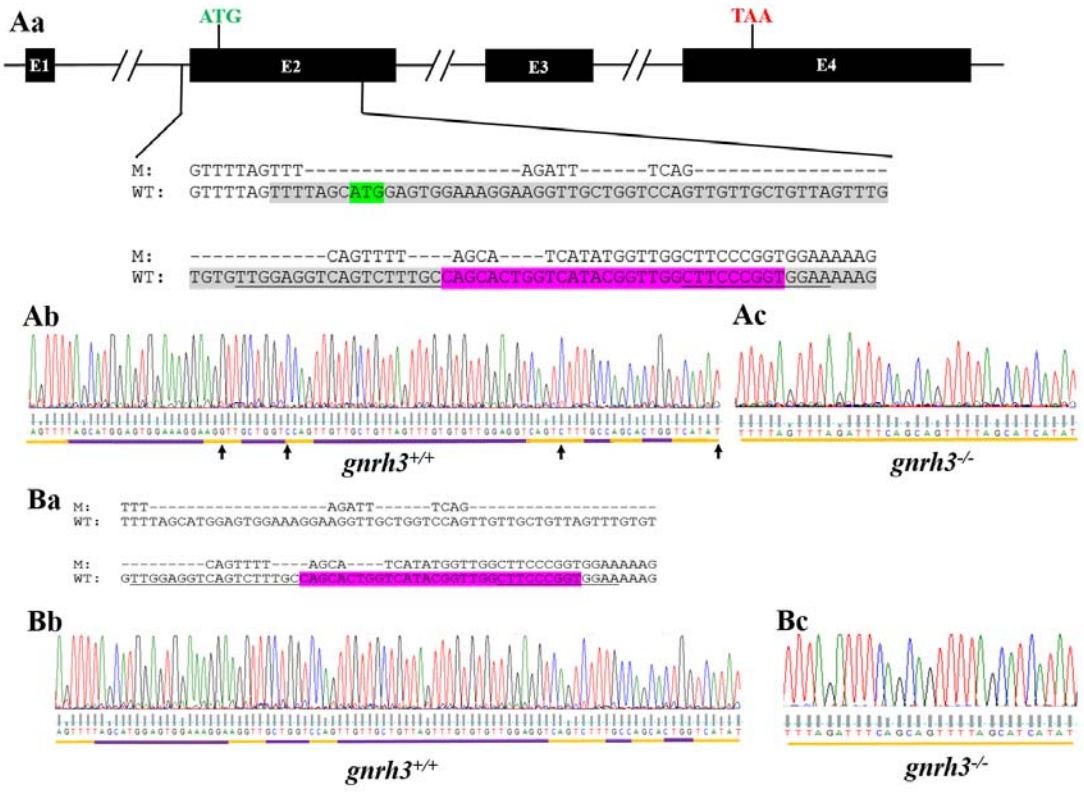

Figure 4.1. Verification of targeted, heritable mutation in *gnrh3* by gene and transcript. (Aa) The *gnrh3*$^{-/-}$ deletion is localized to exon 2 (highlighted in gray) of the *gnrh3* gene and includes the start codon (highlighted in green) and a portion of the nucleotide sequence encoding the Gnrh3 decapeptide (highlighted in pink). The underlined regions represent the TALEN recognition sites. (Ab, c) Sequencing chromatograms of *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ gDNA, demonstrating the homogenous sequence of the *gnrh3*$^{-/-}$ mutation. The purple regions represent the gDNA sequences of *gnrh3*$^{+/+}$ that were deleted in the *gnrh3*$^{-/-}$ gene. Black arrows represent where single base pair substitutions occurred. (Ba) The *gnrh3*$^{-/-}$ cDNA reveals no change in the *gnrh3*$^{-/-}$ mutation after transcription. (Bb, c) Chromatograms of *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ cDNA indicate the homogenous sequence of the *gnrh3*$^{-/-}$ mutation. WT = *gnrh3*$^{+/+}$. M = *gnrh3*$^{-/-}$.

Validation of gnrh3$^{-/-}$ *Line*

After obtaining a specific anti-zebrafish Gnrh3 Gap polyclonal antibody (see Chapter 3), immunohistochemistry was conducted on adult *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ brain and pituitary sections (Figure 4.2A, D). Immunostaining with this primary antibody revealed positive staining of soma and fibers within the pre-optic area (Figure 4.2B) and fibers within the pituitary (Figure 4.2E) of *gnrh3*$^{+/+}$ fish. However, immunostaining of *gnrh3*$^{-/-}$ brain (Figure 4.2C) and pituitary (Figure 4.2F) sections did not reveal any signal. Immunostaining with a polyclonal anti-Gnrh3 decapeptide antibody developed in rabbit also revealed a lack of signal in the *gnrh3*$^{-/-}$ adult brains (Figure 4.2G-I). Thus, the established *gnrh3*$^{-/-}$ line indeed lacks any Gnrh3, rendering it a complete knockout line of *gnrh3*.

Although the *gnrh3*$^{-/-}$ fish demonstrate a lack of Gnrh3, *gnrh3 in situ* hybridization was conducted to determine if *gnrh3* mRNA is still produced in *gnrh3*$^{-/-}$ fish and recognized by a *gnrh3* anti-sense riboprobe. Both *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ brain sections treated with the *gnrh3* anti-sense riboprobe yielded positive signal in soma located in the ventral telencephalon and preoptic area (Figure 4.3A,B). Sections of *gnrh3*$^{+/+}$ fish treated with the *gnrh3* sense riboprobe did not yield any signal (Figure 4.3C). Therefore, *gnrh3*$^{-/-}$ fish are capable of producing *gnrh3* mRNA (though truncated) but not Gnrh3 peptide.

The Effects of Lack of Gnrh3 on the BPG Axis

Developmental and Adult Expression Profiles of Reproductively Relevant Genes

Throughout the first 30 days of development, we sampled *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish at eight time points to assess the mRNA levels of key genes involved in the

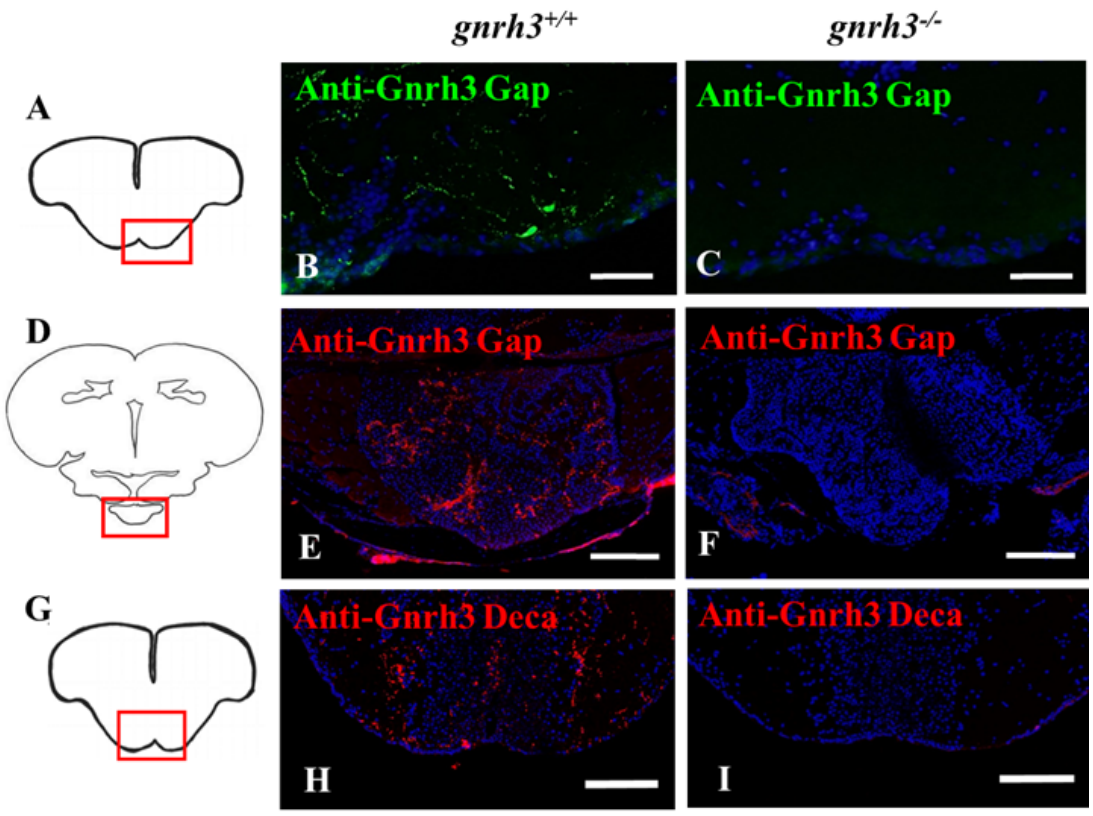

Figure 4.2. Gnrh3 protein not detectable in *gnrh3*$^{-/-}$ fish. (A) Immunohistochemistry on adult coronal brain (A, G) and pituitary (D) sections using anti-zebrafish Gnrh3 Gap (green in B, C; red in E, F) and anti-zebrafish Gnrh3 decapeptide (red in H, I) demonstrates the presence of Gnrh3 signal in the form of somas and fibers in the *gnrh3*$^{+/+}$ pre-optic area of the brain (B, H) and in the form of fibers in the pituitary (E). However, no Gnrh3 signal was found in the *gnrh3*$^{-/-}$ pre-optic area (C, I), pituitary (F), or in any other region of the brain. Scale bars = 50 μm (B, C) and 100 μm (E, F, H, I).

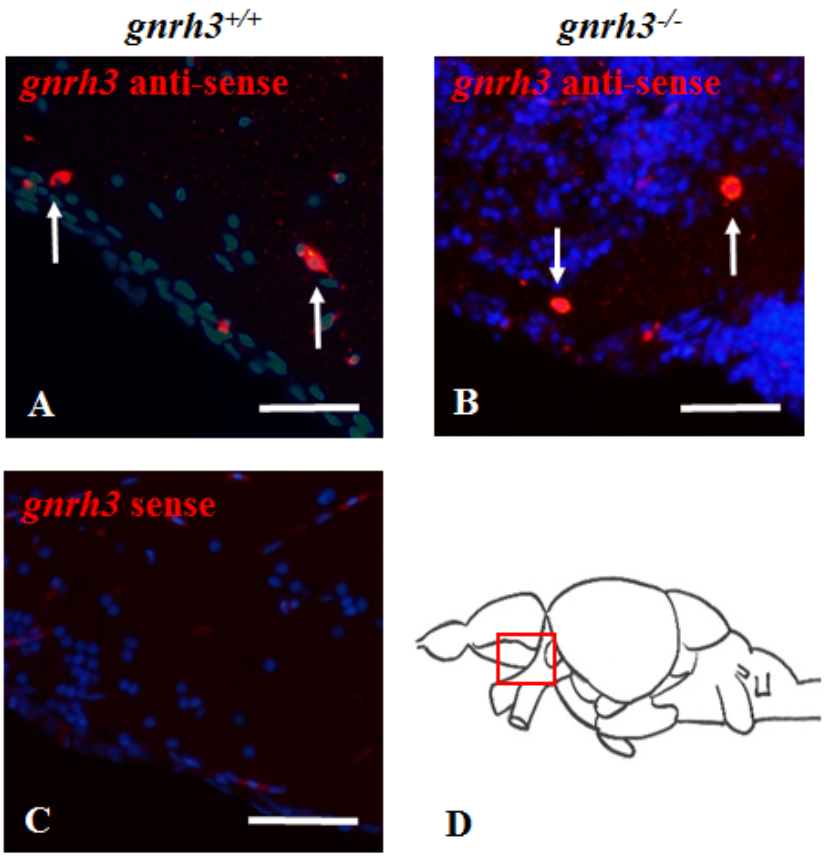

Figure 4.3. *gnrh3* mRNA detectable in *gnrh3*$^{-/-}$ fish. *In situ* hybridization on adult sagittal brain sections (D) using *gnrh3* DIG-labeled riboprobes. The anti-sense *gnrh3* riboprobe demonstrated mRNA (red) in the ventral telencephalon and pre-optic area of both *gnrh3*$^{+/+}$ (A) and *gnrh3*$^{-/-}$ (B) fish. The sense *gnrh3* riboprobe demonstrated no *gnrh3* mRNA signal in the brains of the *gnrh3*$^{+/+}$ fish (C). Scale bars = 50 µm.

reproductive axis. Three genes, in particular, that showed significant differences between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish were the pituitary hormone genes *fshβ* (Figure 4.4A), *lhβ* (Figure 4.4B), and *cgα* (Figure 4.4C). For the Student *t*-test for all three genes, *gnrh3*$^{-/-}$ fish had higher expression levels at 1, 3, and 8 dpf, except for *lhβ* at 8 dpf in which *gnrh3*$^{+/+}$ mRNA levels were higher. At 12 dpf, both *lhβ* and *cgα* levels

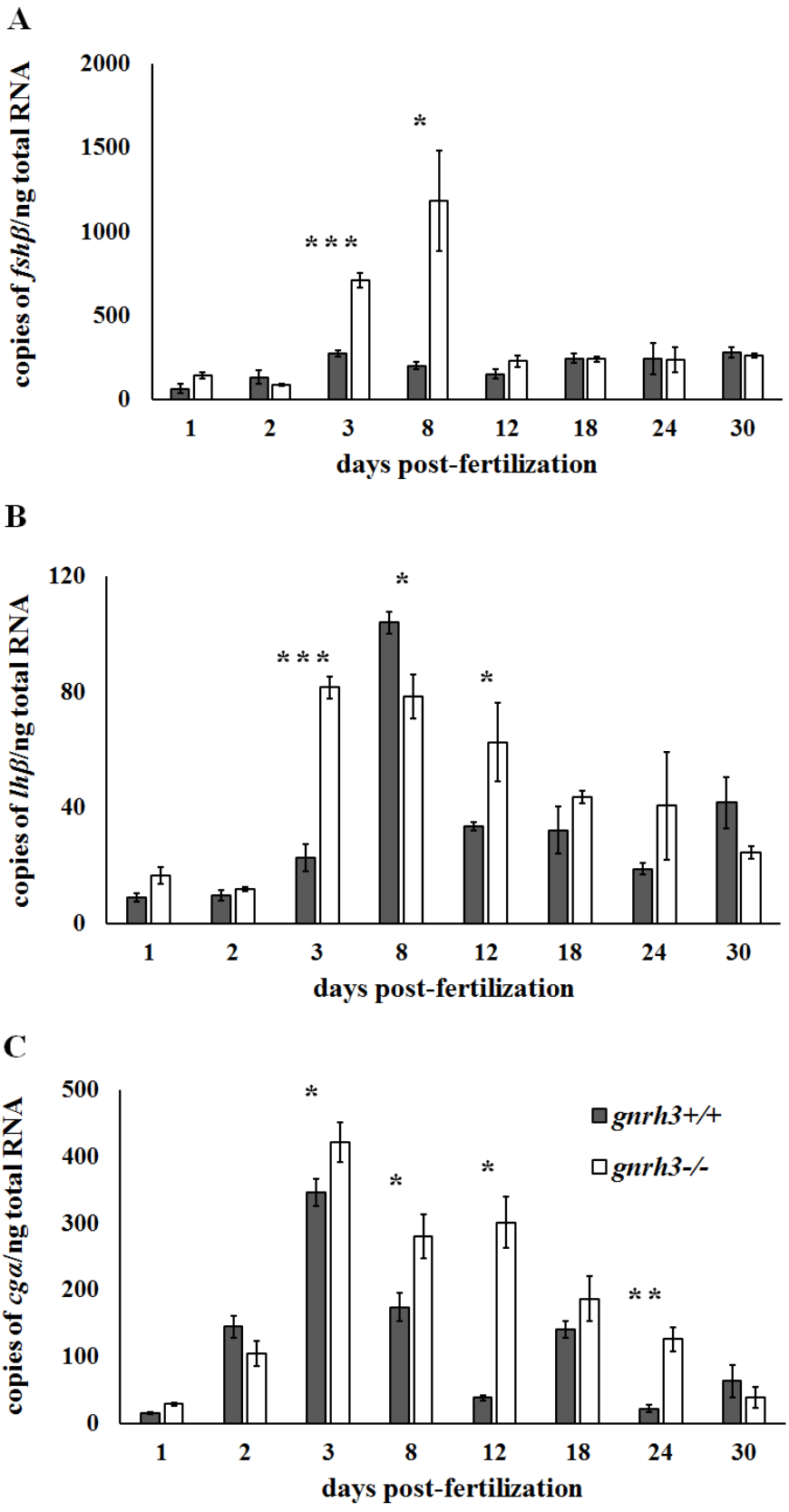
A
copies of fshβ/ng total RNA
2000
1500
1000
500
0
* * *
*
1
2
3
8
12
18
24
30
days post-fertilization
B
copies of lhβ/ng total RNA
120
80
40
0
* * *
*
*
1
2
3
8
12
18
24
30
days post-fertilization
C
copies of cga/ng total RNA
500
400
300
200
100
0
*
*
*
* *
gnrh3+/+
gnrh3-/-
1
2
3
8
12
18
24
30
days post-fertilization

Figure 4.4. Developmental mRNA levels of pituitary gonadotropin genes tend to be higher in *gnrh3*$^{-/-}$ fish than in *gnrh3*$^{+/+}$ fish. At different time points, mRNA levels of *fshβ* (A), *lhβ* (B), and *cgα* (C) were measured in pooled samples of whole embryos/larvae. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. Differences between genotypes at a specific time point were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when *$P \leq 0.05$, **$P \leq 0.005$, and ***$P \leq 0.0005$. *gnrh3*$^{+/+}$, grey bars. *gnrh3*$^{-/-}$, white bars.

were also higher in the *gnrh3*$^{-/-}$ fish, and at 24 dpf, this was also the case for *cgα*. The mRNA levels of *fshβ*, *lhβ*, and *cgα* in *gnrh3*$^{-/-}$ fish were higher by 123 - 492 %, 87 – 268 %, and 21 – 692 %, respectively, over their levels in *gnrh3*$^{+/+}$ fish. Overall, *gnrh3*$^{-/-}$ fish typically had higher mRNA levels of the three pituitary gonadotropin genes during development than *gnrh3*$^{+/+}$ fish. When examined in sexually mature adults, no significant differences in the expression of *fshβ* (Figure 4.5A), *lhβ* (Figure 4.5B), and *cgα* (Figure 4.5C) in male and female *gnrh3*$^{-/-}$ adults were obtained when compared with their *gnrh3*$^{+/+}$ counterparts.

Rescue of* cgα *mRNA levels in 12 dpf* gnrh3$^{-/-}$ *Larvae

Because of the significant and consistent increase in *cgα* mRNA levels in 12 dpf *gnrh3*$^{-/-}$ larvae compared to *gnrh3*$^{+/+}$ larvae, we aimed at rescuing *gnrh3*$^{-/-}$ levels in 12 dpf larvae to *gnrh3*$^{+/+}$ levels by exposure to the Gnrh3 decapeptide. In doing so, we tested two Gnrh3 concentrations (5 and 50 μM) and four exposure times (0, 6, 12, and 24 hours). Importantly, the *gnrh3*$^{-/-}$ mRNA levels of the control (0 hr

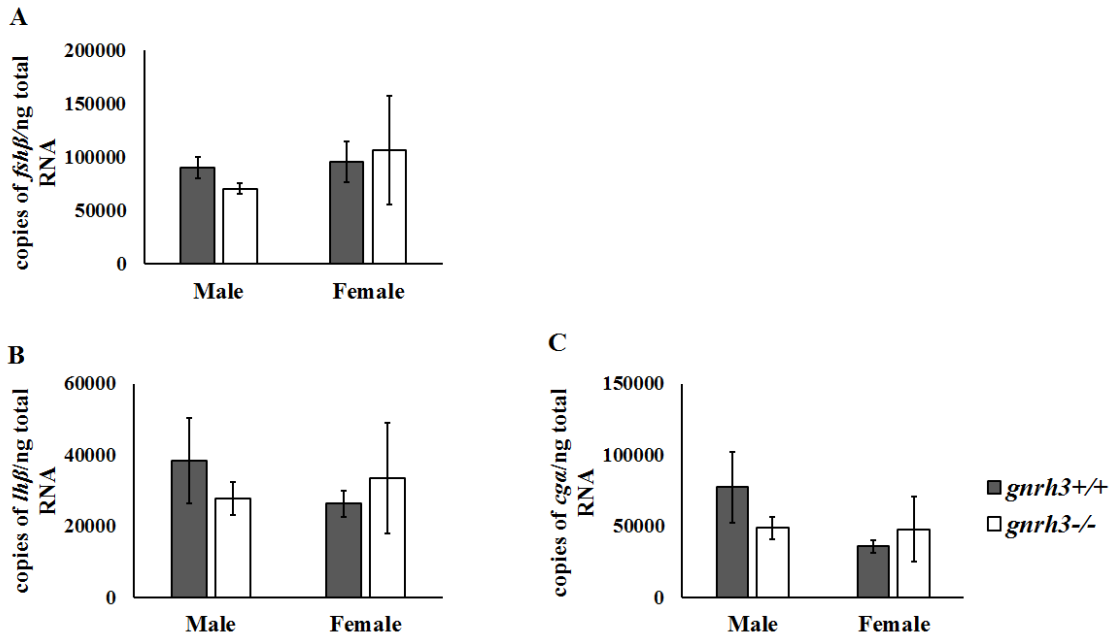

Figure 4.5. Male and female adult mRNA levels of pituitary gonadotropin genes show no differences between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish. Pituitary mRNA levels of *fshβ* (A), *lhβ* (B), and *cgα* (C) were determined using QPCR. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. Differences between genotypes for each sex were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. *gnrh3*$^{+/+}$, grey bars. *gnrh3*$^{-/-}$, white bars.

exposure to Gnrh3) reflected and confirmed the previous results of significantly higher mRNA levels of *cgα* in 12 dpf *gnrh3*$^{-/-}$ larvae compared to *gnrh3*$^{+/+}$ larvae (Figure 4.6 vs Figure 4.4C). However, none of the exposure times of either Gnrh3 concentration (data not shown for 5 µM) were able to reduce *gnrh3*$^{-/-}$ mRNA levels to

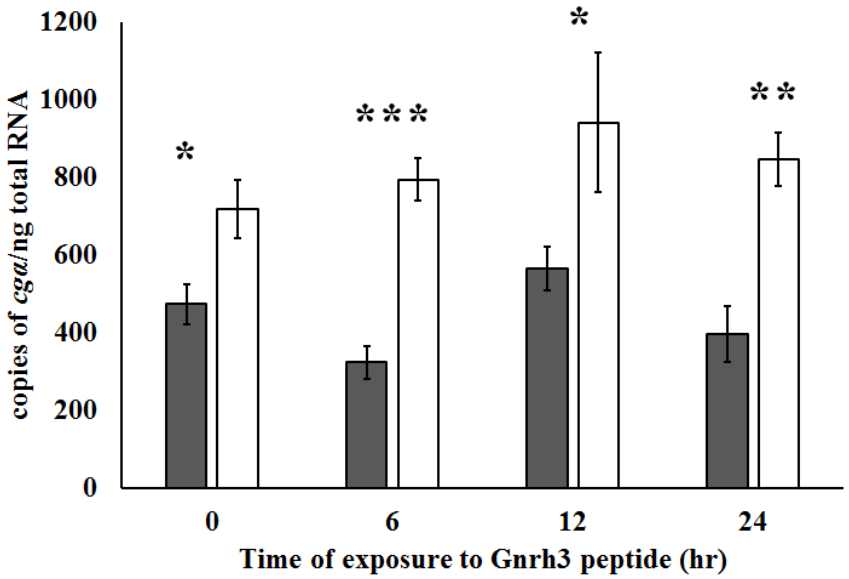

Figure 4.6. The high mRNA levels of *cgα* in 12 dpf *gnrh3*$^{-/-}$ larvae are not rescued to *gnrh3*$^{+/+}$ levels by exposure to 50 µM Gnrh3 decapeptide at any of the exposure times tested. For each genotype, 3 replicate wells (n = 5) were used per exposure time. All larvae were collected on the evening of 12 dpf. Absolute mRNA levels were normalized to *ef1α* levels and are presented as mean ± SEM. Differences between genotypes for each exposure time were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. *gnrh3*$^{+/+}$, grey bars. *gnrh3*$^{-/-}$, white bars.

gnrh3$^{+/+}$ levels (Figure 4.6). Therefore, we did not observe a rescue of *cgα* mRNA levels in *gnrh3*$^{-/-}$ larvae. This could be due to an incorrect dose, exposure time, or the inability of the peptide to penetrate the larvae, which would indicate that our methodology is inconsistent with what is required for a rescue of mRNA levels in *gnrh3*$^{-/-}$ fish to *gnrh3*$^{+/+}$ levels.

Pituitary Lh Content (determined by ELISA)

Pituitary Lh levels in both sexes were similar between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish (Figure 4.7). Therefore, in adults, both mRNA and peptide levels of Lh are not affected by the inherited loss of the *gnrh3* gene.

Imaging of Gnrh3 Fibers in* gnrh3$^{-/-}$ gnrh3:tdTomato *Juveniles

In order to determine whether *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish have different projecting patterns of Gnrh3 fibers during the development of the Gnrh3 system, we analyzed sagittal sections of *gnrh3:tdTomato* brains from both *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ juveniles. Both genotypes revealed Gnrh3-tdTomato-expressing soma in the olfactory bulbs and ventral telencephalon and fibers that extended from the soma to the hypothalamus (Figures 4.8B, C). The appearance of the *gnrh3*$^{+/+}$ *gnrh3:tdTomato* transgenic signal was very similar to that reported previously (Abraham et al. 2008, Xia et al. 2014). Overall, there were no major differences observed between the *gnrh3*$^{+/+}$ *gnrh3:tdTomato* and *gnrh3*$^{-/-}$ *gnrh3:tdTomato* juvenile brain sections. These results indicate that Gnrh3 fibers (that would normally contain Gnrh3 peptide) in *gnrh3*$^{-/-}$ fish do not undergo abnormal projecting patterns or, if they do, are at least corrected by 34 dpf.

The Effects of Lack of Gnrh3 on Reproduction

Gonad Histology

In both males and females, gross morphology of the gonads did not appear different between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish (Figures 4.9A vs 4.9C and Figures 4.9B vs 4.9D). In the histology conducted on males, all stages of advanced spermatogenesis were present in testes from both *gnrh3*$^{+/+}$ (Figure 4.9E) and *gnrh3*$^{-/-}$

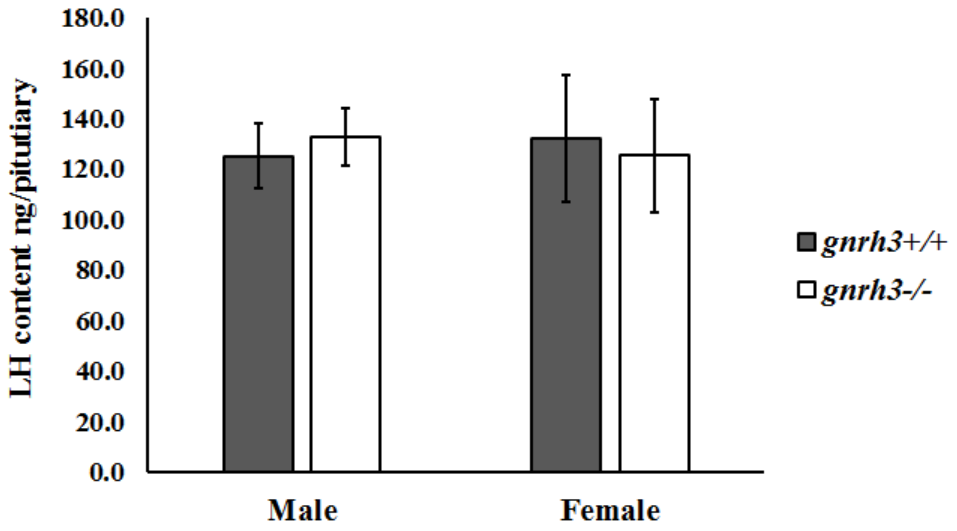

Figure 4.7. Adult male and female Lh levels in the pituitaries of *gnrh3*$^{-/-}$ fish do not differ from *gnrh3*$^{+/+}$ fish. Lh levels in the pituitaries of male and female adults (n = 5 - 8) were measured by ELISA. Values are presented as mean ± SEM. Differences between genotypes for each sex were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. *gnrh3*$^{+/+}$, grey bars. *gnrh3*$^{-/-}$, white bars.

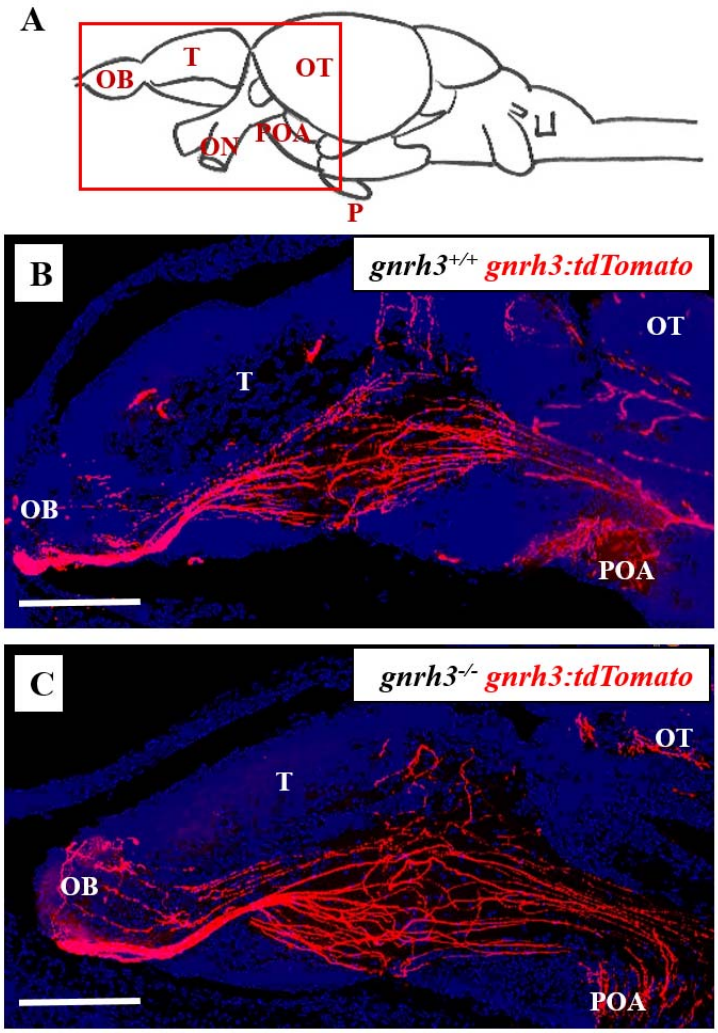

Figure 4.8. *gnrh3*$^{-/-}$ juveniles exhibit normal Gnrh3 neuronal migration. In both *gnrh3*$^{+/+}$ *gnrh3:tdTomato* (B) and *gnrh3*$^{-/-}$ *gnrh3:tdTomato* (C) fish, Gnrh3-tdTomato-expressing soma located in the olfactory region and ventral telencephalon project fibers that extend posteriorly toward the hypothalamus (pituitaries not shown; A). Z-stack images were taken at a 20x magnification on a Leica Microsystems DMi8 confocal microscope with a resolution of 1024 x 1024 and a z-step size of 0.10. All images were analyzed and assembled with Image J and Adobe Photoshop. OB = olfactory bulb. T = telencephalon. OT = optic tectum. ON = optic nerves. POA = pre-optic area. P = pituitary. Scale bars = 100 µm.

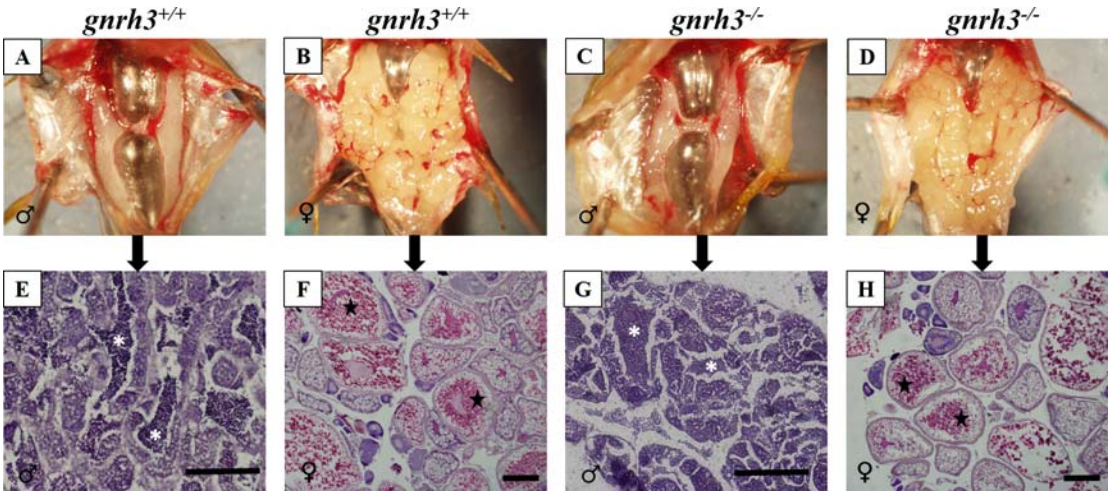

Figure 4.9. Adult gonadal morphology and gametogenesis do not differ between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ fish for both males and females. (A-D) Gross gonadal morphology of adult male (A, C) and female (B, D) fish of both genotypes: *gnrh3*$^{+/+}$ (A, B) and *gnrh3*$^{-/-}$ (C, D). (E-H) Gonadal histology with hematoxylin and eosin staining, demonstrating no differences between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ gametogenesis for both males and females. Male *gnrh3*$^{-/-}$ testes contain spermatozoa in the lumens of spermatocysts (G), similar to *gnrh3*$^{+/+}$ testes (E). Female *gnrh3*$^{-/-}$ ovaries contain all stages of vitellogenesis (H), including late-vitellogenic oocytes, which is similar to *gnrh3*$^{+/+}$ ovaries (F). White asterisks = mature spermatozoa in lumens of spermatocysts. Black stars = mature, late vitellogenic oocytes. Scale bars = 125 µm (testes) and 250 µm (ovaries).

(Figure 4.9G) fish, which included mature spermatozoa in the lumens of the spermatocysts. In females, all stages of oogenesis were observed in ovaries from both *gnrh3*$^{+/+}$ (Figure 4.9F) and *gnrh3*$^{-/-}$ (Figure 4.9H) fish, consistent with the asynchronous oocyte development of zebrafish ovaries. The ovaries from both

gnrh3$^{+/+}$ and *gnrh3*$^{-/-}$ fish included late vitellogenic oocytes that were near to final maturation and ovulation (Figures 4.9F, H).

Fecundity, Fertility, and Offspring Survival

Reproductive competence of *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ adult fish was assessed by measuring fecundity, fertilization rate, and offspring survival to 2 dpf. We in-crossed each genotype (male *gnrh3*$^{+/+}$ x female *gnrh3*$^{+/+}$ and male *gnrh3*$^{-/-}$ x female *gnrh3*$^{-/-}$) and out-crossed each genotype to the other genotype (male *gnrh3*$^{-/-}$ x female *gnrh3*$^{+/+}$ and male *gnrh3*$^{+/+}$ x female *gnrh3*$^{-/-}$). Each of the four spawning combinations included 3 – 6 spawning pairs. In observing the number of eggs produced by each spawning pair in one hour after the dividers were removed in the morning, we demonstrated no differences in fecundity between any of the crosses assessed (Figure 4.10A). Once the embryos were 4-5 hpf, we quantified the number of fertilized embryos to determine fertilization rates for each of the spawning combinations, which again did not differ between any of the four spawning combinations (Figure 4.10B). At 2 dpf, we counted the number of surviving embryos to determine offspring survival, and again, there were no differences between any of the four spawning combinations (Figure 4.10C). Therefore, the reproductive competence and capacity of *gnrh3*$^{+/+}$ fish and *gnrh3*$^{-/-}$ fish does not differ in terms of fecundity, fertility, and offspring survival.

Generation, Validation, and Assessment of Reproduction in* gnrh3$^{-/-}$ gnrh2$^{-/-}$ *Line

The *gnrh2*$^{-/-}$ knockout line (Figure 4.11A) was crossed with the *gnrh3*$^{-/-}$ line to obtain the *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ line. The double knockout fish did not have any Gnrh3 or Gnrh2 signal in any regions of the brain (Figure 4.11C, D), rendering it a true double

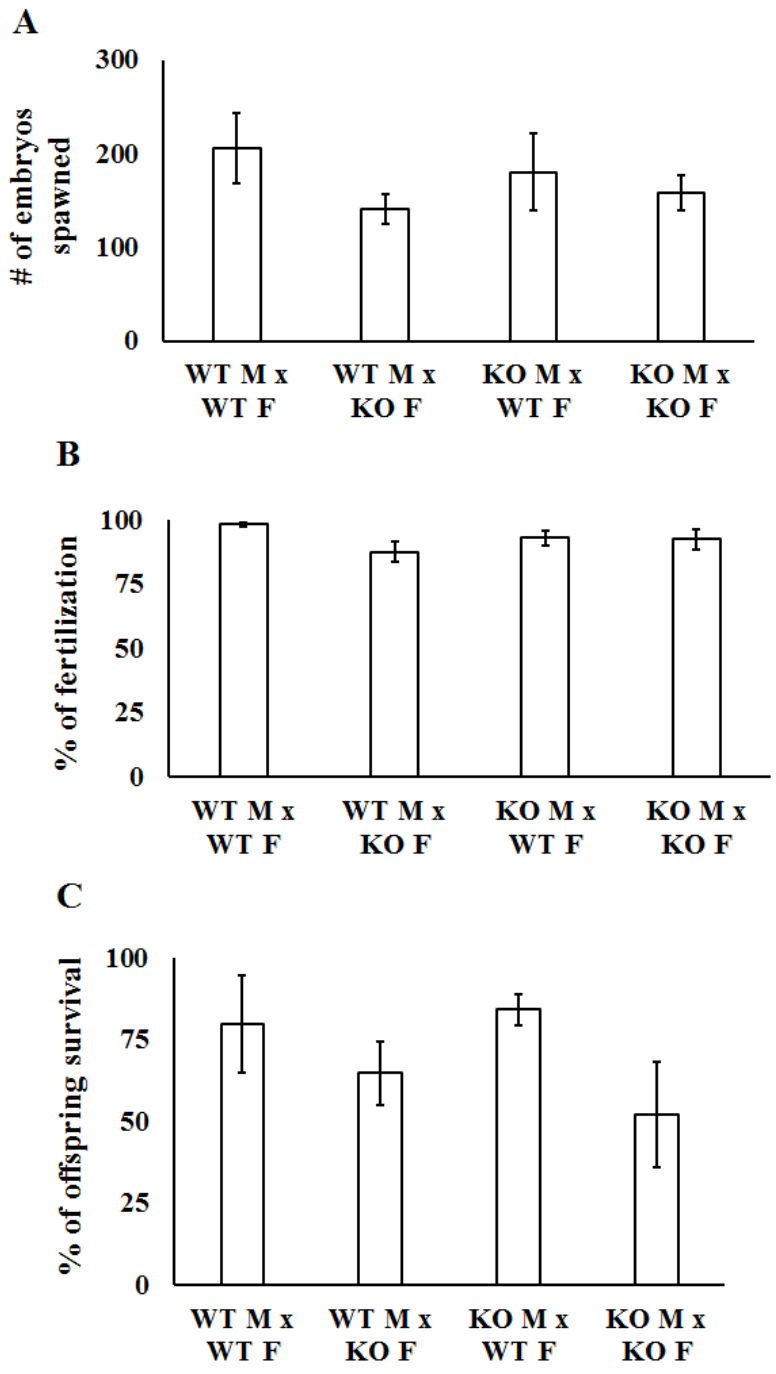

Figure 4.10. Reproductive capacity of *gnrh3*$^{-/-}$ adult fish is not different from that of *gnrh3*$^{+/+}$ fish. Fecundity (A), fertilization rate (B), and offspring survival (C; to 2 dpf) were determined in the spawning of four different crossing combinations: *gnrh3*$^{+/+}$ male x *gnrh3*$^{+/+}$ female, *gnrh3*$^{+/+}$ male x *gnrh3*$^{-/-}$ female, *gnrh3*$^{-/-}$ male x

gnrh3$^{+/+}$ female, and *gnrh3*$^{-/-}$ male x *gnrh3*$^{-/-}$ female. No differences between any of the parameters were observed between any of the spawning combinations. All values are presented as mean ± SEM with 3-6 replicates per group, were subjected to a one-way ANOVA, and were considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. WT = *gnrh3*$^{+/+}$. KO = *gnrh3*$^{-/-}$. M = male. F = female.

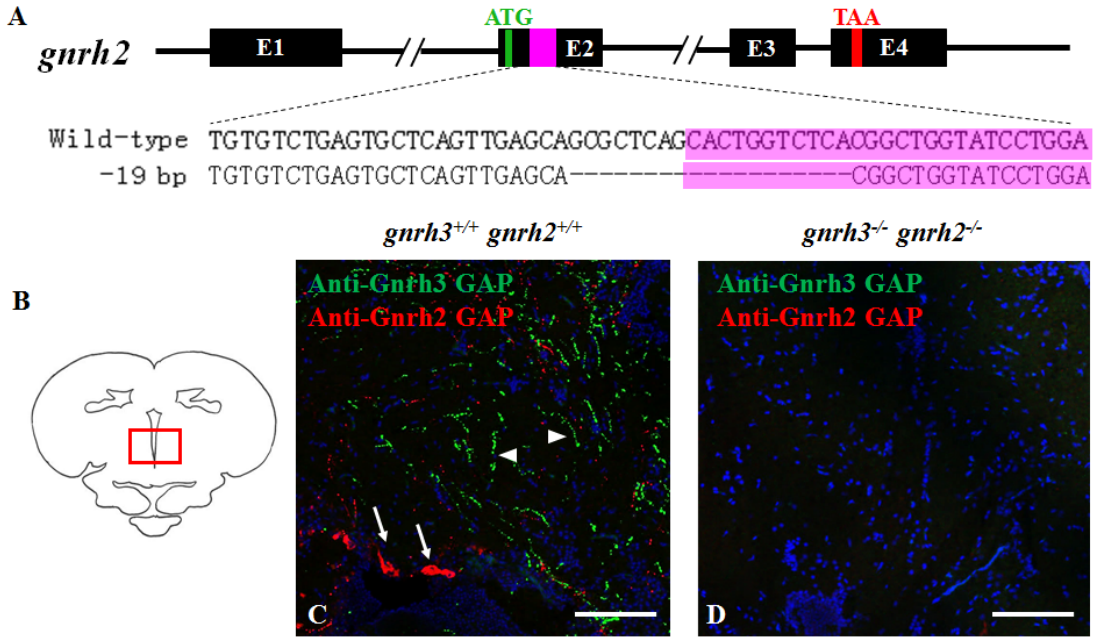

Figure 4.11. Gnrh3 and Gnrh2 proteins not detectable in *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ fish. (A) The 19 bp mutation in the *gnrh2*$^{-/-}$ line that was crossed with the *gnrh3*$^{-/-}$ line to obtain the *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ line. The pink region represents the nucleotide sequence that encodes for the Gnrh2 decapeptide. (B-D) Double immunohistochemistry with anti-Gnrh3 Gap (green) and anti-Gnrh2 Gap (red) in the midbrain (B) of *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ fish. While Gnrh2-ir soma (white arrows) and Gnrh3-ir fibers (white arrowheads) are visible in the midbrain of *gnrh3*$^{+/+}$ *gnrh2*$^{+/+}$ fish (C), neither protein signal is detectable in the *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ midbrain (D). Scale bars = 100 µm.

knockout line. In addition, there were no differences in the gametogenesis of the gonads between *gnrh3*$^{+/+}$ *gnrh2*$^{+/+}$ and *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ fish (Figure 4.12A-D). The double knockout males had mature spermatozoa in the lumens of the spermatocysts (Figure 4.12D), while the double knockout females had mature, late vitellogenic oocytes that were near to ovulation (Figure 4.12C). Finally, there were also no differences in fecundity (Figure 4.12E) and fertilization rate (Figure 4.12F) between *gnrh3*$^{+/+}$ *gnrh2*$^{+/+}$ x *gnrh3*$^{+/+}$ *gnrh2*$^{+/+}$ crosses and *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ x *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ crosses.

DISCUSSION

The original goal of this chapter was to generate and validate *lpxrfa*$^{-/-}$ and *gnrh3*$^{-/-}$ knockout lines in zebrafish with the TALEN technology to determine how Gnrh3 and Lpxrfa respond, respectively, in the complete absence of these key hormones. However, technical difficulties did not allow the production of a functional *lpxrfa*$^{-/-}$ knockout line. Over 500 WT embryos were injected with the *lpxrfa*-targeting TALEN mRNA, and hundreds of F0 and F1 fish were screened for *lpxrfa* mutations, using methods similar to that of the *gnrh3*$^{-/-}$ knockout line. However, only three mutations were found in the *lpxrfa* gene. Other than the 7 bp deletion in the *lpxrfa* coding region that was mentioned previously, one mutation was a 3 bp deletion in the same vicinity as the 7 bp deletion and simply resulted in the absence of one amino acid in the signal peptide, which would not result in a loss of Lpxrfa peptide. The other mutation was an 11 bp insertion in the same vicinity, which produced the same results as the 7 bp deletion. Regarding the 7 bp deletion, while fish that were homozygous for this mutation were obtained,

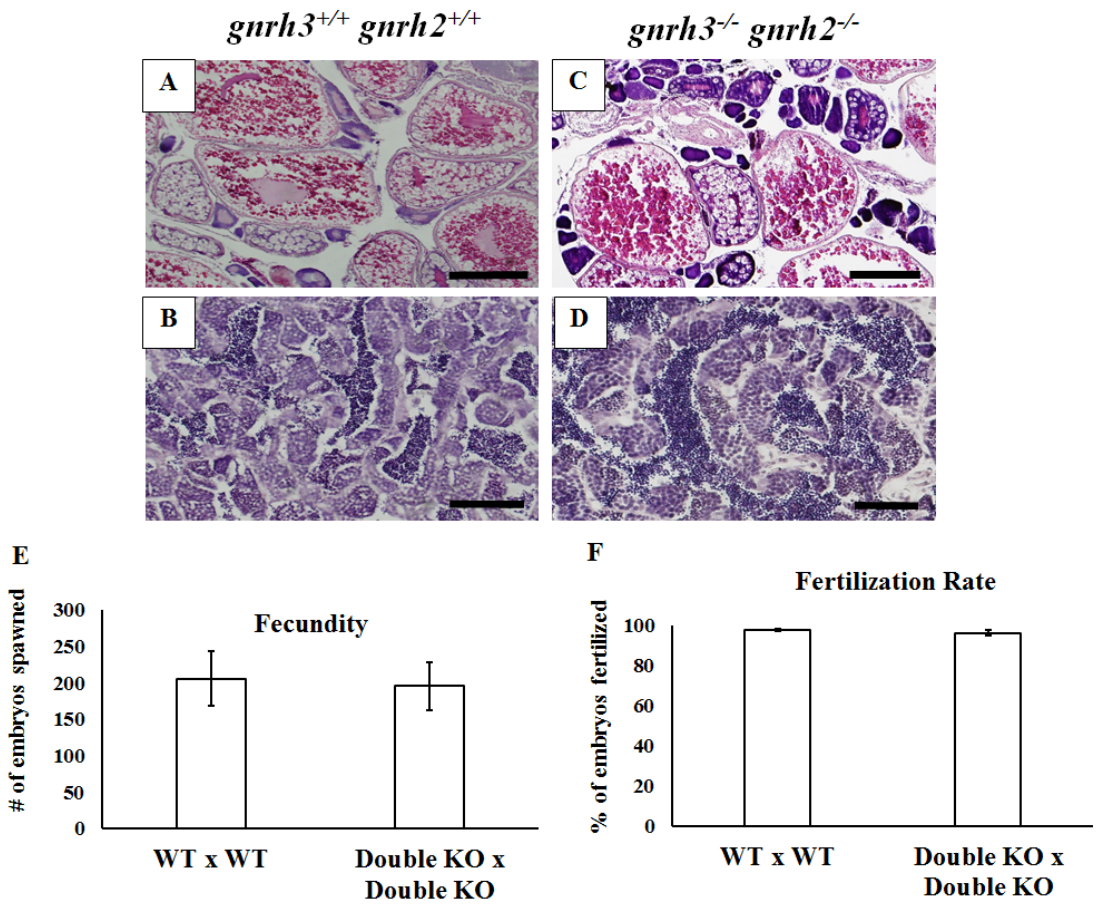

Figure 4.12. Gametogenesis and reproductive performance does not differ between *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ and *gnrh3*$^{+/+}$ *gnrh2*$^{+/+}$ adults for both sexes. (A-D) Female *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ ovaries contain all stages of vitellogenesis (C), including late-vitellogenic oocytes, similar to WT ovaries (A). Male *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ testes contain spermatozoa in the spermatocysts (D), similar to WT testes (B). (E-F) Fecundity (E) and fertilization rate (F) do not differ between *gnrh3*$^{+/+}$ *gnrh2*$^{+/+}$ and *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ adults. All values are presented as mean ± SEM with 3-6 replicates per group. Differences between spawning groups were determined by a one-tailed, homoscedastic Student *t*-test and are considered statistically significant when $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. Scale bars = 250 µm (ovaries) and 50 µm (testes).

immunohistochemistry results demonstrated that these homozygous fish still possessed the Lpxrfa protein. In other words, the mutant line was not a true knockout of *lpxrfa*.

In the *lpxrfa* gene, the 7 bp deletion (blue bracket in Figure 4.13) resulted in the production of a pre-mature stop codon near the deletion (black arrow in Figure 4.13). Because of this pre-mature stop codon and because of the presence of an in-frame ATG codon (methionine; red circle in Figure 4.13) after the 7 bp deletion and before the Lpxrfa-1 peptide in the zebrafish Lpxrfa precursor, we believe that an RNA polymerase was still able to produce a truncated, yet functional, *lpxrfa* transcript, which was subsequently translated into the Lpxrfa precursor peptide. Because this truncated peptide still included the three individual Lpxrfa peptides, the anti-zebrafish Lpxrfa would still be capable of recognizing it, leading to signal in the Lpxrfa immunohistochemistry. If transcription of *lpxrfa* began at this second methionine (red circle in Figure 4.13), the majority of the signal peptide of Lpxrfa would be missing. This could affect the ability of the Lpxrfa peptide to be transported throughout the cells. However, because the *lpxrfa*$^{-/-}$ fish still possessed Lpxrfa peptide, though truncated, we could not consider this line to be a true knockout line of *lpxrfa*. On the other hand, the production of a *gnrh3*$^{-/-}$ knockout line was successful, as validated by multiple immunohistochemistry experiments, and in the previous chapter, we analyzed how *lpxrfa* responds in the *gnrh3*$^{-/-}$ knockout line. This chapter, on the other hand, describes the generation, validation, and characterization of the *gnrh3*$^{-/-}$ knockout line in zebrafish in terms of factors downstream of Gnrh3 in the BPG axis and in terms of gametogenesis and

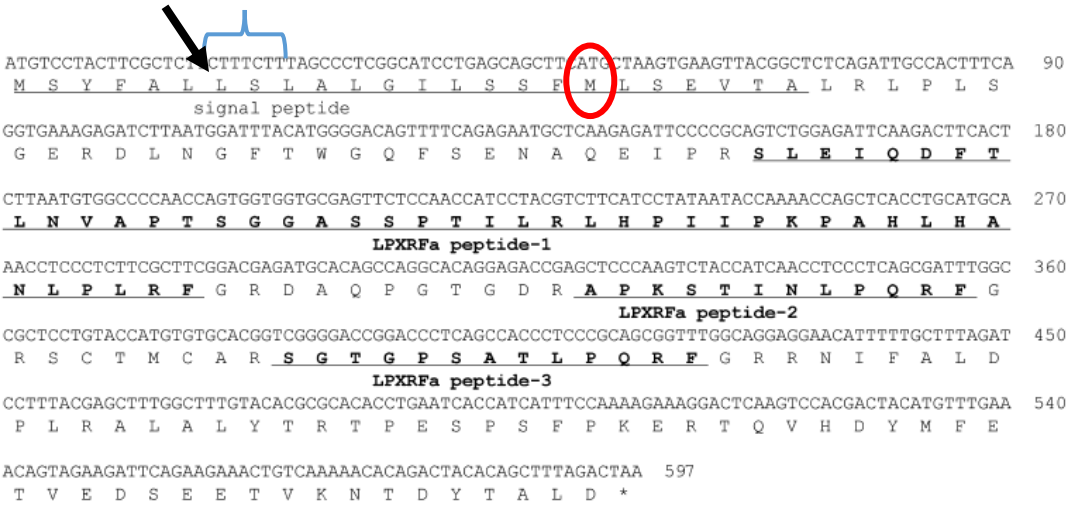

Figure 4.13. The *lpxrfa* mutation induced by the *lpxrfa*-targeting TALENs did not produce a true knockout line but most likely produced, instead, a truncated version of *lpxrfa*. The black arrow represents the location where the 7 bp deletion resulted in the production of a pre-mature stop codon. The red circle represents the ATG codon (methionine), after the 7 bp deletion (blue bracket) and before the Lpxrfa-1 peptide, where an RNA polymerase could begin transcription of a truncated *lpxrfa* transcript. Background image from Zhang et al. (2010).

reproductive performance.

In this study, we successfully developed a *gnrh3*$^{-/-}$ line with the TALEN technology and verified this line at the levels of the gene, transcript, and protein. The mutant line harbored a large deletion in the *gnrh3* gene, which resulted in a lack of the Gnrh3 peptide in Gnrh3 neurons and elsewhere in the brain, while the incomplete mRNA was still detectable in these neurons. Developmental mRNA levels of pituitary gonadotropin genes in the *gnrh3*$^{-/-}$ fish revealed higher levels for many time points; however, this effect was no longer visible by the time the fish reached

adulthood. Unexpectedly, the adult *gnrh3*$^{-/-}$ fish had normal reproductive function, as evidenced by normal gametogenesis and no changes in fecundity, fertility, and offspring survival. These findings strongly suggest that a compensatory mechanism is being activated in the *gnrh3*$^{-/-}$ fish to mitigate the effects of the complete lack of Gnrh3, since Gnrh3 cell ablation causes sterility in the zebrafish (Abraham et al. 2010). Therefore, we consider the possible explanations for the lack of the expected reproductive phenotype in the *gnrh3*$^{-/-}$ fish and discuss potential compensatory factors.

It is well-established that Gnrh is a major regulator of reproduction in many species of vertebrates (Marshall et al. 1990, Mylonas and Zohar 2000, Ottinger et al. 2002, Zohar et al. 2010), including zebrafish (Abraham et al. 2010). Gnrh3 is the hypophysiotropic form in zebrafish (Steven et al. 2003, Palevitch et al. 2007, Abraham et al. 2008), and the ablation of Gnrh3-expressing cells during early zebrafish development (4-6 dpf) results in sterility, at least in females (Abraham et al. 2010). Not only does the elimination of Gnrh3 negatively impact reproduction, it also impacts the ontogeny of Gnrh3 neurons. Transient *gnrh3* gene knockdown using anti-sense MO oligonucleotides resulted in misguided migration of Gnrh3 soma and fibers during neurogenesis (Abraham et al. 2008). The complete development and migration of the Gnrh3 neuronal system in zebrafish are essential to its proper function as is also seen in humans with Kallmann syndrome, in which a cessation of GNRH1 soma migration and subsequent hypogonadism are observed (Soussi-Yanicostas et al. 1998). Based on these results, it was reasonable to assume that *gnrh3* knockout in zebrafish would have profound effects on the ontogeny of the

Gnrh3 system and on reproductive performance. However, *gnrh3*$^{-/-}$ fish revealed no misguided migration of Gnrh3 neurons, as what was observed in *gnrh3* knockdown (Abraham et al. 2008). Or, if misguided migration was present, it was corrected by at least 34 dpf. This finding and the results that *gnrh3*$^{-/-}$ fish are reproductively normal demonstrate that the method used to remove functional gene products can have different resulting phenotypes.

Recently, much attention has been drawn to the fact that many gene knockout studies do not produce the expected phenotypes or those seen by knockdown studies using anti-sense MO oligonucleotides (Kok et al. 2015, Rossi et al. 2015) or by cell ablation studies. Assuming that gene knockout activates a compensatory mechanism, while it is not activated by gene knockdown, the discrepancy may be caused by differences in the timing or the duration of the elimination that dictates whether compensation commences. More specifically, the transient presence of the anti-sense MO oligonucleotide may be too short for the activation of compensation, or the morpholino may be introduced too late as it is not inherited, unlike what is observed in genetic knockouts. With regard to cell ablation, at least in the case of Gnrh3 soma (Abraham et al. 2010), the treatment leading to phenotypes (given at 4-6 dpf) most likely misses the window of time in which the compensatory mechanism is primed (probably very early in ontogeny or upon Gnrh3 differentiation). Mayer and Boehm (2011) illustrated the importance of the timing of treatment in a different system in mice. The kisspeptin/GPR54 system plays a major role in activating Gnrh neurons during murine puberty and reproduction (Gottsch et al. 2004, Han et al. 2005, Dungan et al. 2007). The ablation of *Kiss1*- and *Gpr54*-expressing cells early in development

resulted in fertile mice (Mayer and Boehm 2011); therefore, the ablation was probably conducted early enough to allow a potential compensatory mechanism to be activated. The same treatment applied to adults resulted in infertility (Mayer and Boehm 2011). These results may suggest that a critical time window for the activation of potential compensation exists very early in development. It would be worthwhile to explore the possibilities of establishing conditional *gnrh3* knockouts or conditional Gnrh3 cell ablations in zebrafish to see how a later loss of functional Gnrh3 affects puberty and reproduction. Altogether, it is reasonable to conclude that a compensatory mechanism is being activated in the *gnrh3*$^{-/-}$ fish at an early stage and that the inherited loss of Gnrh3 primes this mechanism. Because cell ablation of Gnrh3 neurons yielded sterile zebrafish (Abraham et al. 2010), we propose that the compensatory mechanism that can activate the reproductive BPG axis most likely exists, at least partially, within Gnrh3 neurons and is being activated before the differentiation of Gnrh3 neurons.

Interestingly, the activation of a compensatory mechanism that ensures reproductive success in zebrafish is not limited to one gene and is seen in other reproductive neuropeptide genes. The best example is of kisspeptin/GPR54 signaling. While *Kiss1*$^{-/-}$ mice (d'Anglemont de Tassigny et al. 2007) have been demonstrated to be infertile with hypogonadotropic hypogonadism, zebrafish *kiss1*$^{-/-}$, *kiss2*$^{-/-}$, and *kiss1*$^{-/-}$ *kiss2*$^{-/-}$ knockout lines exhibit no major differences in reproductive phenotypes (Tang et al. 2014). Even the targeted mutations of *kiss1r* and *kiss2r* individually or together (*kiss1r*$^{-/-}$ *kiss2r*$^{-/-}$) do not disrupt gonadal development and reproductive performance in zebrafish (Tang et al. 2014), also differing from the

results of *Gpr54*$^{-/-}$ mice (Funes et al. 2003). The inability of mice and humans to compensate for the loss of reproductively related factors in the central nervous system is puzzling, because at least mice are capable of activating compensatory mechanisms when other neuropeptides are mutated. For instance, serotonin and norepinephrine have been demonstrated to potentially compensate for a delayed growth phenotype in mice that do not produce functional neuropeptide Y (Gehlert et al. 2008). Another example was seen by Li et al. (2014), who found that phenotypes of embryonic fibroblasts from knockout mice are compensated by a functional homolog of the mutated gene. The combined results of the *gnrh3* and *kiss* genes may indicate that zebrafish activate a compensatory mechanism to ensure reproduction, whereas, although capable of for other genes, mice do not. It will be interesting to know if this also applies to other neuropeptides, such as neurokinin B—as mice and humans with mutated neurokinin B receptors are infertile (Young et al. 2010, Yang et al. 2011). In addition, the compensatory activation may be a common feature in basal vertebrates, as other teleost species also display such compensation (Takahashi et al. 2015).

After finding significant changes in the developmental expression of pituitary gonadotropins in the *gnrh3*$^{-/-}$ fish, we assessed the same genes in adulthood, studying males and females separately. For all genes in both sexes, there were no changes in mRNA levels between *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ adult fish. Consequently, some change occurred between development and adulthood to allow the *gnrh3*$^{-/-}$ pituitary gonadotropin mRNA levels to recover to WT levels. These results were also supported by the lack of changes in Gnrh3 fiber circuitry in juvenile *gnrh3*$^{-/-}$ fish and the normal pituitary Lh content, gametogenesis, and reproduction in adult *gnrh3*$^{-/-}$

fish. In general, gonadotropin mRNA levels are normally very low during development and gradually increase toward puberty to remain high throughout adulthood (Nozaki et al. 1990, Hassin et al. 1999, Hassin et al. 2000, Wong et al. 2004, Guzmán et al. 2009). The differences observed between larval and adult *gnrh3*$^{-/-}$ fish could be explained by an "adjustment period" for the compensatory factor, in which *gnrh3*$^{-/-}$ levels are different during development and are then adjusted to *gnrh3*$^{+/+}$ levels, after the compensatory mechanism is fully primed, sometime between development and adulthood.

Comparative analyses of gene silencing along multiple points of the BPG axis in zebrafish have revealed that the knockouts of reproductive genes upstream in the axis (*gnrh3*$^{-/-}$ and *kiss1*$^{-/-}$ *kiss2*$^{-/-}$; Tang et al. 2014) seem to have no major phenotypes compared to the knockouts of downstream reproductive genes (*fshβ*$^{-/-}$, *lhβ*$^{-/-}$, *fshβ*$^{-/-}$ *lhβ*$^{-/-}$, *fshr*$^{-/-}$, *lhr*$^{-/-}$, and *fshr*$^{-/-}$ *lhr*$^{-/-}$; Chu et al. 2014, Zhang et al. 2014, Zhang et al. 2015), which have sex-specific effects but mostly result in delayed gametogenesis and sometimes infertility. It is possible then that compensatory mechanisms are more abundant at the level of the brain in zebrafish to overcome the inherited loss of functional gene products than at the levels of the pituitary or gonads. This may be because many of the neuropeptides in the brain are pleiotropic, whereas the downstream elements are more functionally specialized. In addition, phenotypic differences between mice and zebrafish in knocking out the hypophysiotropic *Gnrh1* (Cattanach et al. 1977) and *gnrh3* (this study), respectively, and in knocking out *Kiss1*/*Gpr54* (d'Anglemont de Tassigny et al. 2007, Funes et al. 2003) and *kiss*/*kissr* (Tang et al. 2014), respectively, may indicate that basal vertebrates are more capable

of compensating for a lack of reproductive neuropeptides within the BPG axis than more recently evolved vertebrates. This may be explained by the concept that neuropeptides (and their cognate receptors) often appear in multiple isoforms in basal vertebrates.

When considering other reproductively relevant factors that could compensate for an inherited loss of Gnrh3, one of the most obvious candidates was Gnrh2, a midbrain tegmentum neuropeptide that is found in all vertebrates examined to date, except for rodents. The exact functions of Gnrh2 are not fully understood, but recent studies have implicated it in the regulation of feeding and reproductive behavior (Hoskins et al. 2008, Nishiguchi et al. 2012). Based on the available information about Gnrh2, there are three possible scenarios in which Gnrh2 may contribute as a compensation factor in the absence of Gnrh3 in *gnrh3*$^{-/-}$ fish: 1) Gnrh2 could be compensating from its soma in the midbrain tegmentum, since *gnrh2:eGFP* neurons project to the pituitary (Xia et al. 2014) and Gnrh2 peptide has been found in pooled zebrafish pituitaries, though at a much lower concentration than Gnrh3 (Steven et al. 2003). In addition, Gnrh2 displays a higher potency in activating the Gnrh receptor expressed in the pituitary than the hypophysiotropic Gnrh1 in the striped bass (Alok et al. 2000). 2) Gnrh2 could be expressed in Gnrh3 neurons in the forebrain, because, as mentioned previously (Abraham et al. 2010), some factor in the Gnrh3 soma is most likely regulating fertility (at least in females). 3) Alternatively, there could be a combination of these two scenarios that is contributing to the compensation in *gnrh3*$^{-/-}$ fish. In order to determine if Gnrh2 solely compensates for reproductive performance when Gnrh3 is completely absent, we generated *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ fish.

Surprisingly, the double knockout fish were also fertile and did not display any major observable phenotypes in reproduction. Therefore, Gnrh2 is most likely not the compensating factor for Gnrh3 in the *gnrh3*$^{-/-}$ fish, and a factor that is not an identified Gnrh isoform in the zebrafish is responsible for the compensation observed in our *gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$ line.

The identity of the compensation factor and the mechanism by which it takes over the functions of Gnrh3 are still unknown. In addition to isoforms that often cross-activate each other's receptors, other molecules that share some properties with the mutated factor could be responsible for the compensation. For instance, Rossi et al. (2015) demonstrated the upregulation of a particular family of proteins (Emilin) in *egfl7* mutants, which both share a similar functional domain. Therefore, it is possible that the compensatory factor for Gnrh3 could be a peptide, possibly yet to be identified, that has structural similarities to the Gnrh3 decapeptide, the Gnrh3 Gap, or the Gnrh3 precursor. So far, known compensatory factors in the central nervous system include neurotransmitters, such as serotonin and norepinephrine, which compensate for a loss of functional neuropeptide Y in mice (Gehlert et al. 2008). To this point, we have demonstrated that compensatory machinery still exists when Gnrh2 is eliminated in *gnrh3*$^{-/-}$ fish. It will be interesting to discover the identity of the compensating factor(s) utilized by the *gnrh3*$^{-/-}$ fish and whether the factor(s) compensating for Gnrh3 differ between development, puberty, and adulthood.

In conclusion, the current study has provided the development and validation of a *gnrh3*$^{-/-}$ line in zebrafish and characterized its endocrine components along the BPG axis, gametogenesis, and reproductive performance to reveal no major changes

in reproductive competence. These results represent the first targeted and heritable mutation of a Gnrh isoform in any organism. The *gnrh3*$^{-/-}$ zebrafish remains fertile, displaying normal reproductive performance, which is in stark contrast to phenotypes seen using other approaches (e.g., *gnrh3* knockdown, natural mutagenesis in mice and humans, ablation of Gnrh3-expressing cells) and/or other model systems (e.g., mice and humans). The results indicate that a compensatory mechanism is being activated, which is primed early on (probably upon Gnrh3 differentiation) and possibly within Gnrh3 neurons. It is possible, therefore, that basal vertebrates, like the zebrafish, possess the ability to better compensate for a complete lack of important reproductive neuropeptides compared to more evolutionary advanced vertebrates. This compensation in the zebrafish brain also appears to be more competent than that of reproductive genes in the zebrafish pituitary and gonads. Potential compensation factors and sensitive windows of time for compensation during development, puberty, and adulthood should be further explored to determine how fish remain reproductively competent in the complete absence of a key hormone, such as Gnrh3.

Chapter 5: Conclusions and Future Directions

CONCLUSIONS

Vertebrate reproduction is regulated by the BPG axis, in which internal and external stimuli are translated into key hormones that result in reproductive competence. GNRH has long been considered to be the major regulator of reproduction at the level of the brain, triggering the synthesis and release of gonadotropins in the adenohypophysis that, in turn, regulate gonadal functions, like steroidogenesis, gametogenesis, and final gamete maturation. Several neuropeptides upstream of GNRH that stimulate GNRH have been discovered in recent years, including kisspeptin, neurokinin B, and others. While many studies have shown that catecholamines (e.g., dopamine) in the brain can inhibit the production of gonadotropins, recent research has uncovered the first hypothalamic neuropeptide that is capable of exerting an inhibitory effect on gonadotropins: GNIH. Originally discovered in the quail brain (Tsutsui et al. 2000), GNIH and its orthologs are capable of reducing gonadotropin levels via direct contact with the pituitary and indirectly with GNRH neurons in the brain in birds and mammals (for review, see Bentley et al. 2006). While the reports on the inhibitory functions of GNIH are consistent for birds and mammals, Lpxrfa peptides in teleosts are capable of exhibiting stimulatory and/or inhibitory effects on gonadotropins (Moussavi et al. 2012, Zhang et al. 2010, Biran et al. 2014, Wang et al. 2015). This suggests that teleosts are an intermediate group that exhibit stimulatory and/or inhibitory effects of Lpxrfa, between jawless fishes that exhibit only stimulatory effects and birds/mammals that exhibit only inhibitory effects (Osugi et al. 2012, Tsutsui et al. 2012). However, information on Lpxrfa's

functions is limited in teleosts, and little research has attempted to understand the modes of action by which Lpxrfa affects reproduction in the brain and pituitary.

Therefore, the goal of this study was to understand the mechanisms by which Lpxrfa affects reproduction (independently and with Gnrh3) in terms of ontogeny of the reproductive control system and functions during adulthood in the zebrafish reproductive axis. Specifically, we aimed at studying Lpxrfa's anatomical and functional interactions in the brain (with Gnrh3 neurons) and in the pituitary with the gonadotropes. While studying the interactions of Lpxrfa and Gnrh3, our attention was diverted to also study how Gnrh3 exerts regulation on the reproductive BPG axis. Therefore, adding Gnrh3 to the scenario, we sought to understand how reproduction is coordinately controlled by these two neuropeptides at the level of the brain and pituitary via neuroanatomical, functional, and ligand-receptor interaction studies.

Through this research, Lpxrfa was demonstrated to be a hypophysiotropic neuropeptide located in the ventral zone of the periventricular hypothalamus that weakly innervates the neurohypophysis and, yet, has a strong inhibitory influence on gonadotropin expression. To exert these effects, Lpxrfa peptides activate Lpxrf-R2 and -R3 via the PKA/cAMP pathway. However, we demonstrate that Lpxrfa-2 and -3 may utilize the Kiss1ra receptor through inhibition more readily than the activation of Lpxrf-R2 and -R3 due to the high sensitivity of this inhibition, the high abundance of Kiss1ra, and the possible low abundance of Lpxrf receptors. In addition, Lpxrfa and Gnrh3 have a mutual regulation in adulthood, supported by neuroanatomical studies and functional assays, with Lpxrfa inhibiting *gnrh3* expression and Gnrh3 regulating the cyclicity of *lpxrfa*. However, during development, the relationship seems to be a

one-way regulation with Gnrh3 governing *lpxrfa* expression. Finally, although an *lpxrfa*$^{-/-}$ knockout line could not be established, a *gnrh3*$^{-/-}$ knockout line was generated and validated to understand how an inherited loss of Gnrh3 affects *lpxrfa*. Unexpectedly, the *gnrh3*$^{-/-}$ line also demonstrated no major changes in reproductive ontogeny and performance, offering potential compensation mechanisms that the organism utilizes when Gnrh3 is absent.

Lpxrfa/Lpxrf-R

This study has increased our understanding of the Lpxrfa/Lpxrf-R system in teleosts and how and where it exerts its functions on reproduction during both development and adulthood. In the adult zebrafish brain, Lpxrfa soma are located in a cluster in the ventral zone of the periventricular hypothalamus and interact with multiple brain regions, with extensive projections to Gnrh3 neurons in the pre-optic area and weak projections to the neurohypophysis, indicating the hypophysiotropic nature of Lpxrfa (Figure 5.1). Functionally, the weak Lpxrfa innervations of the pituitary result in zebrafish Lpxrfa-3 having negative effects on *lhβ* and *cgα* expression in adults at low physiological levels, while the extensive Lpxrfa projections to Gnrh3 soma in the pre-optic area result in reduced *gnrh3* expression in adults (Figure 5.2). These results offer two major pathways (i.e., direct contact with pituitary and Gnrh3 neurons in brain) by which Lpxrfa can exert its functions on the adult pituitary. During early development, however, Lpxrfa seems to have no effect on *gnrh3* expression (Figure 5.3), indicating the evolution of Lpxrfa functions from development (Figure 5.3) to adulthood (Figure 5.2). The inhibitory effects of Lpxrfa

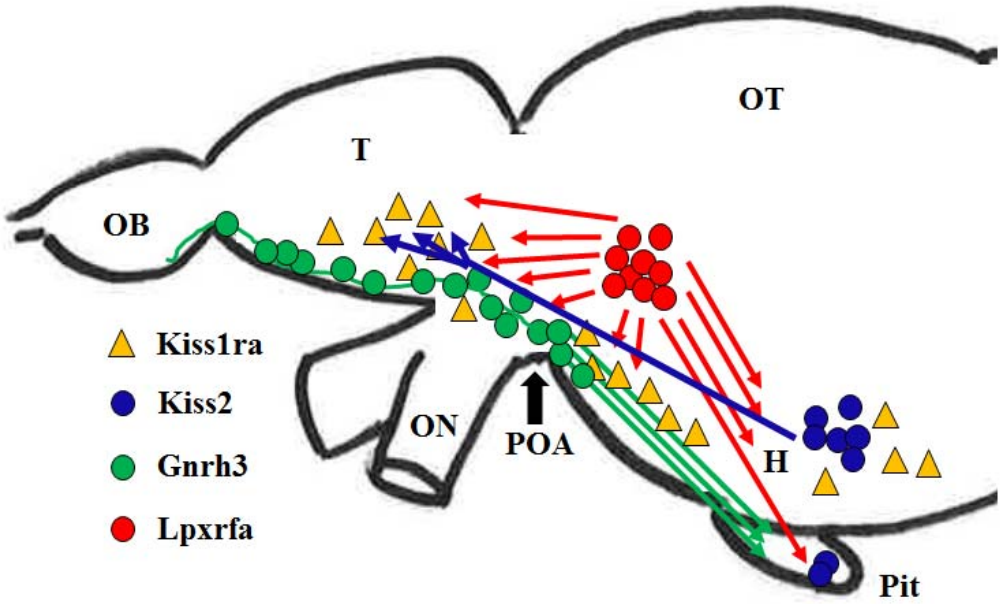

Figure 5.1. Neuroanatomical interactions between soma and fibers of important reproductive neuropeptides in the adult zebrafish brain-pituitary axis. Lpxrfa neurons (red circles) in the ventral zone of the periventricular hypothalamus project to: Gnrh3 soma (green circles) in the pre-optic area, *kiss1ra*-expressing cells (orange triangles) in the forebrain, and the neurohypophysis of the pituitary. Gnrh3 soma and fibers are located along the terminal nerve/ventral telencephalon and the pre-optic area/hypothalamus with fibers projections to the neurohypophysis (Abraham et al. 2008, Golan et al. 2015). Kiss2 neurons (blue circles) are located in the pituitary and ventral hypothalamus and project to *kiss1ra*-expressing cells in the forebrain (Servili et al. 2011). Arrows represent the fiber projections of the soma and are color-coded in the same manner as the soma. The number of arrows represents the intensity of fiber projections. OB = olfactory bulb. T = telencephalon. ON = optic nerves. POA = pre-optic area. H = hypothalamus. Pit = pituitary. OT = optic tectum.

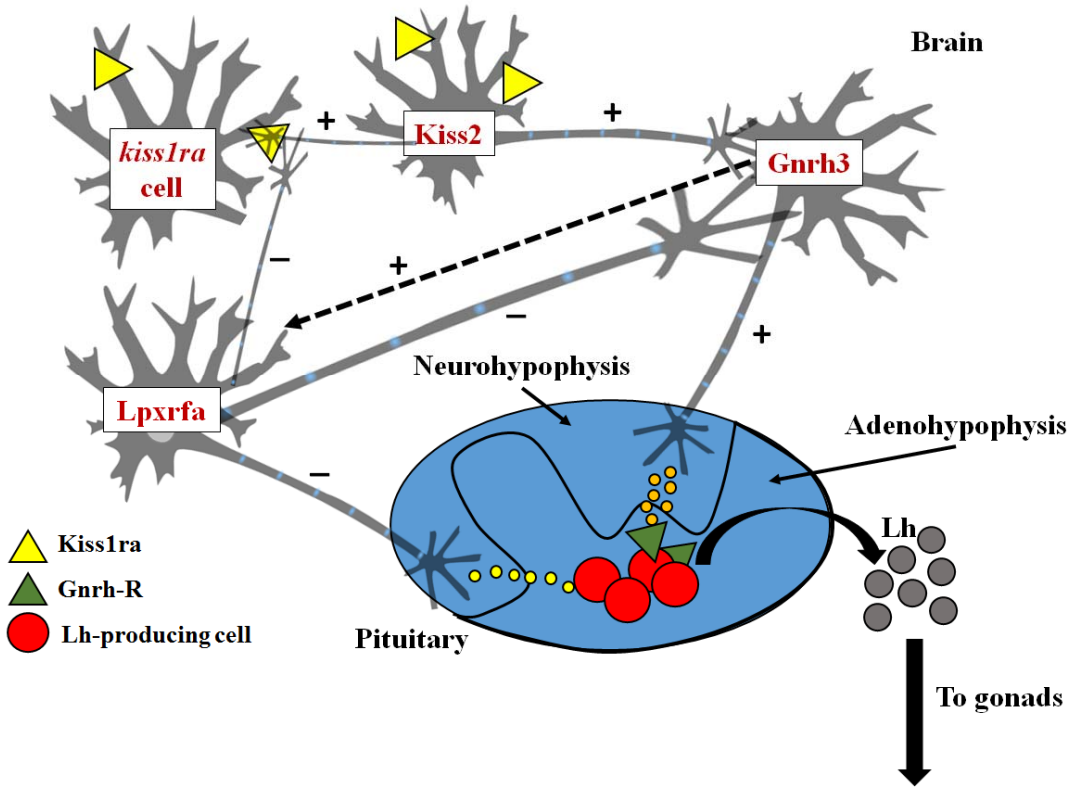

Figure 5.2. Neuroanatomical and functional pathways of important reproductive neuropeptides in the adult zebrafish brain that regulate gonadotropins (Lh) in the pituitary. Lpxrfa neurons project to the neurohypophysis, where they exhibit inhibitory (-) effects on *lhβ* and *cgα* levels, and to Gnrh3 soma, where they downregulate *gnrh3* levels. Lpxrfa neurons also project to *kiss1ra*-expressing cells, where they inhibit the activation of Kiss1ra (yellow triangles) by Kiss2. Gnrh3 neurons project to the neurohypophysis and interact with their cognate receptors (green triangles) to exert a stimulatory (+) effect on gonadotropes (red circle; Abraham et al. 2008, Golan et al. 2015). Gnrh3 neurons also regulate *lpxrfa* expression (dashed arrow), allowing mRNA levels to stay elevated in female brains in the evening. In addition, Kiss2 neurons express *kiss1ra*, project to Gnrh3 neurons, and project to other *kiss1ra*-expressing cells in the forebrain (Servili et al. 2011).

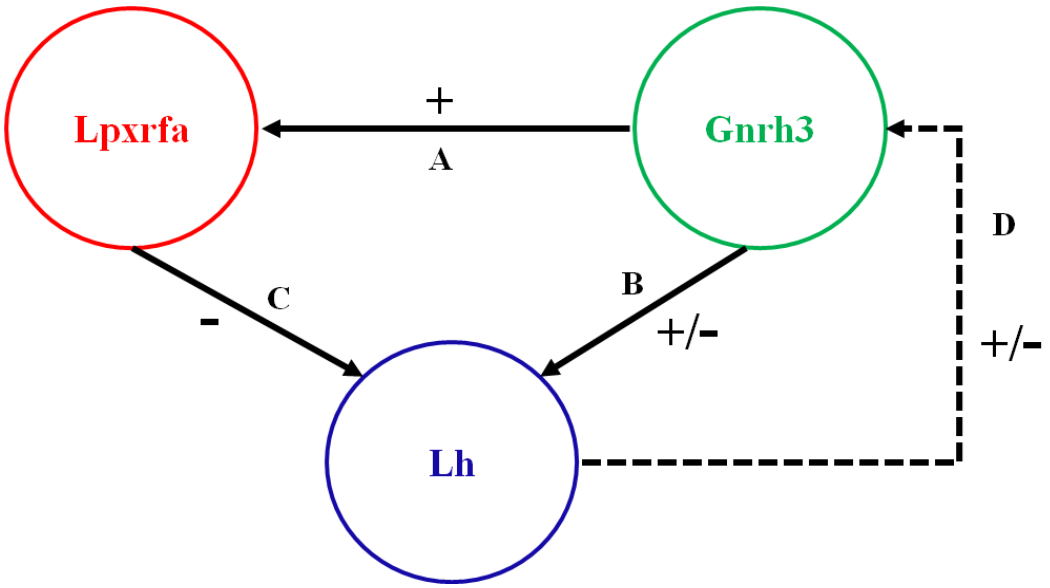

Figure 5.3. Functional roles of Lpxrfa and Gnrh3 in the brain and pituitary during development of the zebrafish. While Lpxrfa has a negative impact (-) on *gnrh3* expression during adulthood, we found no effect of Lpxrfa on *gnrh3* expression during development. Gnrh3, however, positively regulates (+) *lpxrfa* levels during both development (A) and adulthood. Although Gnrh3 is well-known for stimulating gonadotropin expression, the *gnrh3*$^{-/-}$ larvae exhibit higher levels of gonadotropin mRNAs than WT larvae, indicating that Gnrh3 may have stimulatory and inhibitory effects (+/-) on gonadotropins during development (B). On the other hand, Lpxrfa might be the direct source of the increased gonadotropin levels in the *gnrh3*$^{-/-}$ larvae (C): Since *gnrh3*$^{-/-}$ larvae exhibit low *lpxrfa* levels, the high gonadotropin levels present in these mutant fish might be explained by the depressed levels of the gonadotropin-inhibitor *lpxrfa*. Finally, the actions of neuropeptides are also affected by pituitary feedback (D).

on gonadotropins and Gnrh3 that is observed in adults is most likely to be consistently inhibitory, since zebrafish do not exhibit distinct, gradual stages of gonadal development, like the goldfish, during which Lpxrfa's effects (stimulatory vs inhibitory) can differ (Moussavi et al. 2012). Therefore, teleosts continue to represent an intermediate group (between jawless fishes and birds/mammals), in which Lpxrfa peptides of a particular species can exert stimulatory and/or inhibitory effects on reproduction (Osugi et al. 2012, Tsutsui et al. 2012).

To elicit these inhibitory effects in the adult zebrafish brain, Lpxrfa peptides utilize Lpxrf-R2 and -R3 through the PKA/cAMP pathway, similar to other cyprinids. In more advanced teleosts (e.g., perciforms), Lpxrfa utilizes both PKA/cAMP and PKC/Ca^{2+} pathways, possibly contributing to the stimulatory (Biran et al. 2014) and inhibitory (Wang et al. 2015) effects of Lpxrfa in this group. Lpxrfa neurons also project to multiple *kiss1ra*-expressing cells in the adult forebrain (Figure 5.1), supporting the inhibition of Kiss2-activated Kiss1ra by Lpxrfa-2 and -3 (Figure 5.2), which is more sensitive than the activation of Lpxrf-R2 and Lpxrf-R3 by Lpxrfa peptides and offers a third pathway that Lpxrfa can utilize to affect the pituitary. Thus, the Lpxrfa/Lpxrf-R (and Kiss2/Kiss1ra) system regulates Gnrh3 only during adulthood and serves as a gonadotropin-inhibitory system in the neuroendocrine control of reproduction.

Gnrh3

This study has also advanced our understanding of the hypophysiotropic Gnrh3 system in the zebrafish brain-pituitary axis, regarding its close relationship with Lpxrfa and its importance in reproductive ontogeny and performance. During

development, Gnrh3 positively regulates the expression of *lpxrfa*, allowing it to stay elevated at the appropriate levels, while *lpxrfa* does not appear to regulate *gnrh3* (Figure 5.3). The inherited loss of Gnrh3 during early development results in a reduction in *lpxrfa* expression and increases in gonadotropin expression, most likely due to the reductions in the gonadotropin-inhibitor *lpxrfa.* Therefore, Gnrh3 serves a role in early development to keep *lpxrfa* levels elevated, which most likely allow gonadotropin levels to remain suppressed at the appropriate levels that permit proper development of the gonadal system. However, although this reduction of *lpxrfa* expression is present during the development of the *gnrh3*$^{-/-}$ fish, it does not prevent the mutant fish from having full reproductive function as adults.

In the adult zebrafish, Gnrh3, in addition to the well-known function of stimulating gonadotropin synthesis/release from the pituitary, also positively regulates *lpxrfa* expression, particularly in the female brain (Figure 5.2). This regulation is manifested by Gnrh3's ability to keep *lpxrfa* mRNA levels elevated in the evening in the female brain, which, in turn, results in the reduction in Gnrh3 levels that is typically observed in the middle of the night for morning daily spawners. Because GNIH can be affected by melatonin (Chowdhury et al. 2010), Gnrh3 may also serve as a mediator between environmental cues, such as photoperiod, and Lpxrfa in the female adult brain. In addition, because Kiss2 neurons express *kiss1ra* (Servili et al. 2011) and stimulate Gnrh neurons in teleosts (Zmora et al. 2014; Figure 5.2), the brain levels of *lpxrfa* observed in WT female adults in the evening, which are likely regulated by Gnrh3, may downregulate the expression of *kiss2* in the medio-basal hypothalamus as seen in Zmora et al. (2015), thereby

offering another pathway for Lpxrfa to affect Gnrh3. Therefore, Gnrh3 serves as an important regulator of Lpxrfa during the majority of the zebrafish's life cycle, at least in the female brain.

Loss-of-Function

Finally, while we were unable to determine how Gnrh3 is affected when Lpxrfa is completely absent in the zebrafish, we did generate, validate, and characterize a *gnrh3*$^{-/-}$ knockout line in zebrafish to, first, understand how *lpxrfa* is affected by the inherited loss of Gnrh3 (as described above) and, second, understand how reproductive ontogeny and performance are affected by this loss. Because 1) null mutations in *Gnrh*/*GNRH* in mice/humans have resulted in hypogonadotropic hypogonadism and sterility (Cattanach et al. 1977, Chan et al. 2009), 2) ablation of Gnrh3-expressing cells in zebrafish resulted in a sterile, all-female population (Abraham et al. 2010), and 3) *gnrh3* knockdown in zebrafish resulted in misguided migration of the Gnrh3 system (Abraham et al. 2008), we hypothesized that a zebrafish *gnrh3*$^{-/-}$ knockout line would exhibit hypogonadotropic hypogonadism and sterility. Unexpectedly, *gnrh3*$^{-/-}$ fish exhibited no major changes in important factors downstream of Gnrh3 in the BPG axis. The mutant fish also demonstrated no differences in Gnrh3 neuronal migration during early development and no differences in gametogenesis and reproductive performance, as determined by fecundity, fertility, and offspring survival, in adults. In addition, zebrafish that lacked both Gnrh isoforms (*gnrh3*$^{-/-}$ *gnrh2*$^{-/-}$) also exhibited normal gametogenesis and reproductive performance.

A compensatory mechanism is, therefore, being activated in the *gnrh3*$^{-/-}$ fish (but not necessarily in *hpg* mice, humans with *GNRH* mutations, Gnrh3-cell ablated zebrafish, and zebrafish with *gnrh3* knockdown) to allow for normal reproductive function in the absence of a key reproductive neuropeptide. This compensation mechanism can most likely be activated only if the loss of the peptide is inherited or initiated very early in development. In addition, these results and others (Tang et al. 2014) indicate that basal vertebrates (e.g., teleosts) seem to be better capable of compensating for the loss of reproductive neuropeptides than more recently evolved vertebrates (e.g., mice and humans; Funes et al. 2003, d'Anglemont de Tassigny et al. 2007), which is probably due to the multiplicity of reproductive neuropeptides in basal vertebrates. Furthermore, because of the lack of a major reproductive phenotype in *gnrh3*$^{-/-}$ zebrafish and in zebrafish lacking kisspeptin (Tang et al. 2014), zebrafish appear to be better able to compensate for the loss of reproductive peptides at the level of the brain, more so than at the levels of the pituitary and gonads (Chu et al. 2014, Zhang et al. 2014, Zhang et al. 2015), most likely due to the pleiotropic nature of reproductive neuropeptides. Overall, zebrafish possess a valuable compensation mechanism that allows them to continue the important process of reproduction, even during the loss of a key reproductive neuropeptide.

In summary, this research has elucidated the mechanisms of action by which the Lpxrfa/Lpxrf-R system exerts its functions and by which Lpxrfa and Gnrh3 interact with each other in the reproductive axis of the zebrafish during both development and adulthood. Lpxrfa and Gnrh3 are important neuropeptides that negatively and positively regulate, respectively, the neuroendocrine control of

reproduction, and this regulation involves the complex interactions of multiple receptors and pathways within the reproductive axis. Because reproductive neuropeptides serve such important roles during development and adulthood, the teleost brain has evolved to allow compensatory mechanisms to be established, particularly for Gnrh3, to permit the continuation of the physiological processes of reproduction, when these neuropeptides are absent. Therefore, our understanding of the neuroendocrine control of teleost reproduction is now more comprehensive due to the clarification of the roles and functions exerted by Lpxrfa and Gnrh3 in the reproductive axis.

FUTURE DIRECTIONS

While the functions and relationships of Lpxrfa and Gnrh3 in the zebrafish reproductive axis have been characterized and described in this study, there are limitless possibilities for furthering this research. Particularly, the availability of the *gnrh3*$^{-/-}$ knockout line has permitted many more opportunities for understanding the physiology of the zebrafish when Gnrh3 is functionally absent. Some of our recommendations for continuing the zebrafish Lpxrfa-Gnrh3 research include, but are not limited to, the following:

1. Because *lpxrfa* is expressed in the gonads of zebrafish (Zhang et al. 2010), it is possible that Lpxrfa is participating in local gonadal functions, contributing to a potential "mini-reproductive axis" in the gonads. Therefore, we recommend studying the local, functional roles of Lpxrfa and Gnrh3 within the gonads and their effects on steroidogenesis, gametogenesis, and final gamete maturation.

2. Since Lpxrfa peptides demonstrate an ability to utilize Kiss1ra, we suggest incorporating kisspeptin receptor loss-of-function methods to understand how Lpxrfa elicits its functions when it cannot exert its inhibition of Kiss1ra.
3. Due to the inability to establish a true *lpxrfa*$^{-/-}$ knockout line in the zebrafish with the TALEN technology, we recommend utilizing the CRISPR-Cas9 knockout technology to develop a true *lpxrfa*$^{-/-}$ knockout line in the zebrafish to understand how reproduction and Gnrh3 are affected by the loss of this neuropeptide.
4. We propose the inclusion of RNA sequencing of *gnrh3*$^{+/+}$ and *gnrh3*$^{-/-}$ samples to determine any differentially expressed genes that may aid in identifying the compensation mechanism in the *gnrh3*$^{-/-}$ fish.
5. Because of the availability of an important tool like the *gnrh3*$^{-/-}$ knockout line, we advise using this line to understand how other important reproductive neuropeptides (e.g., kisspeptin, neurokinin B, etc.) are influenced by the inherited loss of Gnrh3.
6. Because we have demonstrated that a compensatory mechanism is most likely being activated very early in development (probably due to the inheritance of the loss of Gnrh3) in the *gnrh3*$^{-/-}$ fish, we recommend incorporating conditional knockout technology of the zebrafish Gnrh3 system to determine how a later loss of Gnrh3 function (e.g., late development, puberty, and/or adulthood) affects reproduction.

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