THE ROLES OF GNIH IN REPRODUCTIVE FUNCTION AND BEHAVIOR

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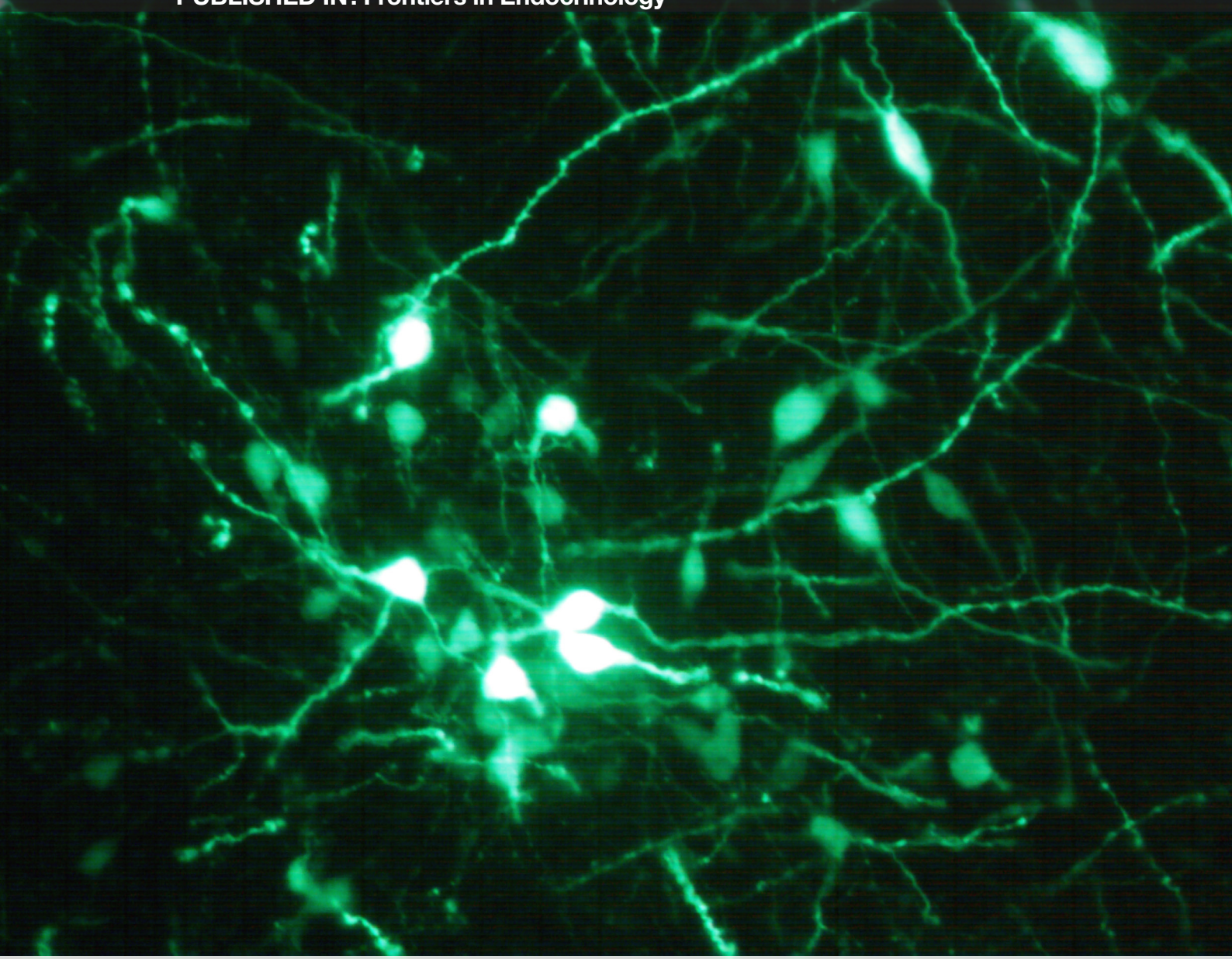

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THE ROLES OF GNIH IN REPRODUCTIVE FUNCTION AND BEHAVIOR

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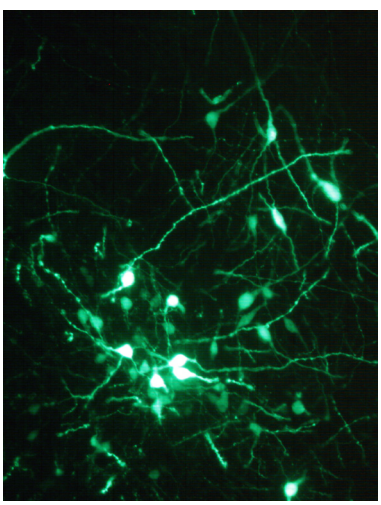

Enhanced green fluorescent protein (EGFP) tagged gonadotropin-inhibitory hormone (GnIH) neurons in the dorsomedial hypothalamic area of a male rat.

Image: Takayoshi Ubuka.

Since the discovery of gonadotropin-releasing hormone (GnRH) at the beginning of 1970s, it has been believed that GnRH is the only hypothalamic neuropeptide that regulates gonadotropin release in vertebrates. In 2000, however, a novel hypothalamic neuropeptide that actively inhibits gonadotropin release was discovered in Japanese quail and termed gonadotropin-inhibitory hormone (GnIH). Following seventeen years of research has revealed that GnIH is highly conserved across vertebrates including humans, and GnIH is involved in a number of physiological and behavioral functions related to reproduction. The aim of this e-book is to celebrate the discovery of GnIH and the progress of GnIH research by collecting review and original articles from leading scientists in this new research field.

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EDITORIAL
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Editorial: The Roles of GnIH in Reproductive Function and Behavior

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Editorial on the Research Topic

The Roles of GnIH in Reproductive Function and Behavior

Since the discovery of gonadotropin-releasing hormone (GnRH) at the beginning of 1970s, it has been believed that GnRH is the only hypothalamic neuropeptide that regulates gonadotropin release in vertebrates. In 2000, however, a novel hypothalamic neuropeptide that actively inhibits gonadotropin release was discovered in quail and termed gonadotropin-inhibitory hormone [GnIH, (1)]. GnIH is one of the RFamide peptides, which is also known as RFamide-related peptide (RFRP) in mammals. Following the discovery, the next 17 years of research revealed that GnIH is highly conserved across vertebrates including humans, and GnIH is involved in a number of physiological and behavioral functions related to reproduction (2–4). This research topic compiles original research and review articles describing the discovery and progress of GnIH research from leading scientists in the field.

The first review paper by Tsutsui et al. (2) describes how they discovered GnIH from the quail brain and showed its inhibitory effect on gonadotropin synthesis and release (Tsutsui et al.). They also describe studies over the past decade and a half that established the physiological function of GnIH as a key player regulating reproduction across vertebrates by acting on the brain and pituitary to modulate their reproductive physiology and behavior. They further introduce recent evidence indicating that GnIH regulates reproductive behavior through changes in neuroestrogen biosynthesis in the brain (5).

The review by Leon and Tena-Sempere describes their investigations on how GnIH (RFRP) acts centrally to suppress GnRH/gonadotropin secretion directly or indirectly cooperating with other stimulatory inputs such as kisspeptin in the dynamic regulation of the hypothalamic–pituitary–gonadal (HPG) axis. They focus on studies using pharmacological tools and functional genomics in rodent models. In 2006, RF9 was invented as a potent and selective antagonist of neuropeptide FF (NPFF) receptors including GnIH receptor (GPR147), which is also known as NPFF1 (6). Tena-Sempere's group was the first to show that central administration of RF9 evokes robust luteinizing hormone (LH) secretory responses in rats (7). They were also the first to create an NPFF1 null mouse (8). Although NPFF1 null mouse preserved pubertal progression and fertility, a rapid drop of LH was not observed in NPFF1 null mouse by food deprivation (8) suggesting the role of GnIH in the regulation of feeding (9) and stress (10).

Poling and Kauffman discuss the regulation of GnIH (RFRP-3) neurons by sex steroids and leptin during development and sexual maturation in rodents. They highlight significant changes in GnIH expression and neuronal activation during postnatal and pubertal development and discuss the role of GnIH receptors (GPR147; NPFF1) for normal pubertal timing and developmental LH

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secretion based on their studies (11). Geraghty et al. report their investigation on the role of GnIH (RFRP) and GnIH receptor (GPR147; NPFF1) in the regulation of age-related reproductive decline in female rats. Interestingly, females exhibited an increase in GnIH (RFRP) and GPR147 (NPFF1) mRNA expression in the hypothalamus before irregular estrous cycle in middle age rats. This transient increase was followed by subsequent decreases in kisspeptin and GnRH mRNA expression. Expression of GnIH (RFRP) and GPR147 (NPFF1) also increased in the ovaries with advancing age (Geraghty et al.). The results suggest a novel role of GnIH (RFRP) signaling system in the regulation of reproductive cessation.

Reproduction is regulated seasonally in species living in temperate zones and GnIHs (RFRP-1 and -3) are believed to play a critical role in the central control of seasonal reproduction. Henningsen et al. summarize the role and underlying mechanisms of GnIH (RFRP) in the seasonal control of reproduction, primarily focusing on mammalian species. In mammals, the GnIH (RFRP) system is persistently suppressed by short photoperiod and melatonin (12), which is opposite to quail (13). Central chronic administration of GnIH (RFRP-3) in short day-adapted male Syrian hamsters fully reactivates the reproductive axis (14), highlighting the importance of the seasonal changes in GnIH (RFRP) in proper regulation of the reproductive axis. Anjum et al. propose GnIH (RFRP) as a mediator between adiposity and impaired testicular function in their original research article. GnIH (RFRP) treatment increased food intake, upregulation of glucose transporter 4, and increased uptake of triglycerides in the adipose tissue of mice. On the other hand, treatment with GnIH (RFRP) decreased glucose uptake by downregulation of glucose transporter 8 expression and decreased testosterone synthesis. GnIH (RFRP) treatment also showed decreased expression of insulin receptor in the testis. Their findings suggest a new role of GnIH (RFRP) in fat accumulation, in addition to negative regulation of testosterone synthesis [Anjum et al.; (15)].

Various stressors suppress the HPG axis and consequently induce reproductive dysfunction. Iwasa et al. review the role of GnIH (RFRP) in stress-induced reproductive dysfunction. Psychological and immune stress increase GnIH (RFRP) expression and suppresses GnRH and gonadotropin secretion (10, 16). It was shown that glucocorticoid acts as a mediator of stress and GnIH (RFRP) (17). Soga et al. investigated the effect of early-life social isolation on serotonergic and GnIH neuronal system using enhanced green fluorescent protein (EGFP)-tagged GnIH transgenic rats (18) in their research article. They found that the total number of EGFP-GnIH neurons in socially isolated rats was the same as control rats, but c-Fos expression in GnIH neurons was significantly reduced in socially isolated rats. Serotonin fiber juxtapositions on EGFP-GnIH neurons were also reduced in socially isolated rats. Teo et al. found in their original research that socially isolated rats display greater CLOCK expression in the dark phase, while control rats display increased CLOCK expression in the light phase in EGFP-GnIH neurons in the dorsomedial hypothalamus. They also found that β-catenin expression pattern was disrupted in GnIH cells by social isolation (Teo et al.).

Fish represent more than the half of recognized living vertebrate species. Fish also include model species with scientific, clinical, and economic importance. Muñoz-Cueto et al. summarize all GnIH precursor and peptide sequences identified in fish, distribution of GnIH and GnIH receptor (GPR147; NPFF1) in central and peripheral tissues, physiological actions of GnIH on the HPG axis, as well as other reported effects of GnIH, and regulatory mechanisms of GnIH in fish. Finally, Ubuka and Parhar highlight the stimulatory effect of GnIH in the HPG axis, which was shown in mammals and in fish (2, 3, 12, 14) and investigate their pharmacological and physiological mechanisms as a perspective of future research.

AUTHOR CONTRIBUTIONS

TU wrote the manuscript. IP, LJK, and KT edited the manuscript.

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REVIEW
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Contribution of GnIH Research to the Progress of Reproductive Neuroendocrinology

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Since the discovery of gonadotropin-releasing hormone (GnRH) in mammals at the beginning of the 1970s, it was generally accepted that GnRH is the only hypothalamic neuropeptide regulating gonadotropin release in mammals and other vertebrates. In 2000, however, gonadotropin-inhibitory hormone (GnIH), a novel hypothalamic neuropeptide that actively inhibits gonadotropin release, was discovered in quail. Numerous studies over the past decade and a half have demonstrated that GnIH serves as a key player regulating reproduction across vertebrates, acting on the brain and pituitary to modulate reproductive physiology and behavior. In the latter case, recent evidence indicates that GnIH can regulate reproductive behavior through changes in neurosteroid, such as neuroestrogen, biosynthesis in the brain. This review summarizes the discovery of GnIH, and the contributions to GnIH research focused on its mode of action, regulation of biosynthesis, and how these findings advance our understanding of reproductive neuroendocrinology.

Keywords: gonadotropin-inhibitory hormone, gonadotropin-releasing hormone, gonadotropins, reproduction, reproductive behavior, melatonin, stress, social environment

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HISTORY OF NEUROENDOCRINOLOGY AS A FIELD

The discovery of "neurosecretion" in the first half of the twentieth century led to the creation of neuroendocrinology, a new research field of endocrinology. In the 1920s, Ernst and Berta Scharrer first proposed the new concept of neurosecretion, suggesting that hypothalamic neurons terminating in the neurohypophysis secrete neurohormones analogous to those of endocrine glands. This seminal idea was not accepted easily by the scientific community, leading to marked criticism, including the comments like: "We have just heard some very interesting things, … and also a great deal of nonsense." In 1949, however, Bargmann established the concept of neurosecretion proposed by Ernst and Berta Scharrer. Subsequently, hypothalamic neuropeptides, oxytocin (1) and vasopressin (2), were identified to be neurohormones that are secreted from the neurohypophysis.

The morphology of hypothalamic neurons that terminate at the median eminence (ME) made Harris (3) to hypothesize that these hypothalamic neurons may secrete neurohormones from the ME into the hypophysial portal system to regulate the secretion of anterior pituitary hormones. Harris further summarized the first map showing different areas in the hypothalamus responsible for various

pituitary hormone release, identified by contemporary lesions and electrical stimulation studies [(4), reviewed in Ref. (5)]. Halasz et al. (6) also contributed to the identification of the hypophysiotrophic area in the hypothalamus by pituitary transplantation method (5). McCann and Ramirez were the first to demonstrate the biological existence of luteinizing hormone (LH)-releasing factor (LHRF) in the basal middle hypothalamus [see Ref. (7) for a review]. Subsequently, the groups of Schally and Guillemin identified the structure of several neurohormones that regulate anterior pituitary hormone release, including thyrotropin-releasing hormone (TRH) (8, 9), gonadotropin-releasing hormone (GnRH) (10, 11), and growth hormone-inhibiting hormone (somatostatin) (12), in the brain of mammals. Schally and Guillemin were awarded a Nobel Prize in 1977 for the discoveries of these neurohormones. At the same time, Yalow also received this prize for her role in the development of radioimmunoassay that permitted the measurement of these neurohormones.

As described above, Schally's (10) and Guillemin's (11) groups discovered GnRH, a hypothalamic neuropeptide that stimulates the release of both LH and follicle-stimulating hormone (FSH) from gonadotropes in the anterior pituitary, in mammals. Subsequently, several GnRHs have been identified in other vertebrates (13–16). Although McCann et al. (17) suggested differential hypothalamic control mechanism of FSH secretion, it had been generally accepted that GnRH is the only hypothalamic neuropeptide regulating gonadotropin release in mammals and other vertebrates.

In 2000, however, Tsutsui and colleagues challenged this notion with the discovery of gonadotropin-inhibitory hormone (GnIH), a hypothalamic neuropeptide that actively inhibits gonadotropin release, in quail (18). Subsequent studies conducted by Tsutsui and colleagues over the past decade and a half demonstrated that GnIH is highly conserved among vertebrates, from agnathans to humans, acting as a key player regulating reproduction [for reviews, see Ref. (19–30)]. Recent studies by Tsutsui's group have demonstrated that GnIH has important functions beyond the control of reproduction (31, 32). Based on these findings, it now appears that GnIH not only acts on the pituitary but in the brain to affect a number of behaviors, including reproductive behavior through changes in neurosteroid, such as neuroestrogen, biosynthesis in the brain [(32), for a review, see Ref. (33)]. Thus, the following 15 years of GnIH research in collaboration with world's leading laboratories have permitted a more complete understanding of the neuroendocrine control of reproductive behavior and physiology [for reviews, see Ref. (19–22, 24–29, 34)].

Herein, this review summarizes the discovery of GnIH and the contribution of GnIH research over the past decade and a half, focusing on its mode of action, regulation of biosynthesis, and the ways that such contributions have contributed to the field of reproductive neuroendocrinology. This review also highlights the commonalities and diversity of GnIH structures and actions as well as the evolutionary origin of GnIH in chordates.

DISCOVERY OF GnIH AND ITS ROLE IN REPRODUCTION

Gonadotropin-inhibitory hormone was discovered in the brain of the Japanese quail while searching for a novel peptide possessing a C-terminal sequence Arg-Phe-NH_2 (RFamide peptide) in vertebrates (18). The first identification of an RFamide peptide dates back to the late 1970s, when Price and Greenberg purified a peptide Phe–Met–Arg–Phe–NH_2 (FMRFamide) from the ganglia of the venus clam (35). Since this initial discovery, various RFamide peptides that act as neurotransmitters, neuromodulators, and peripheral hormones had been identified in invertebrates. However, immunohistochemical studies in vertebrates suggested the presence of an unknown hypothalamic RFamide peptide(s) that may regulate the secretion of anterior pituitary hormones (36, 37). Based on this information, Tsutsui's laboratory searched for novel RFamide peptide(s) in the brain of the Japanese quail.

A breakthrough occurred in 2000 when Tsutsui and colleagues discovered a novel RFamide peptide Ser–Ile–Lys–Pro–Ser–Ala–Tyr–Leu–Pro–Leu–Arg–Phe–NH_2 (SIKPSAYLPLRFamide) that actively inhibited gonadotropin release in quail pituitary, providing the first demonstration of a hypothalamic neuropeptide inhibiting gonadotropin release in any vertebrate (18) (**Table 1**). Given its functional role, this neuropeptide was named GnIH (18) (**Figure 1**). In birds, cell bodies and terminals of GnIH neurons are located in the paraventricular nucleus (PVN) and ME, respectively (18). The C-terminal structure of GnIH is identical to chicken LPLRFamide which is the first reported RFamide peptide in vertebrates (38), but this peptide is likely to be a degraded fragment of chicken GnIH [(39), for reviews, see Ref. (20, 26, 27)] (**Table 1**). Subsequently, a cDNA encoding the precursor polypeptide for GnIH was identified in quail (40) and other avian species, such as chickens, sparrows, starlings, and zebra finches [for reviews, see Ref. (20, 26, 27)]. The GnIH precursor encompasses one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) possessing an LPXRFamide (X = L or Q) motif at their C-terminus in all avian species studied. GnIH was further identified as a mature peptide in starlings (41) and zebra finches (42), and GnIH-RP-2 was identified in quail (40) (**Table 1**).

Gonadotropin-inhibitory hormone is considered to be a key neuropeptide inhibiting avian reproduction because GnIH inhibits gonadotropin release in most avian species studied [for reviews, see Ref. (20, 26, 27)] (**Figure 1**). To demonstrate the biological mode of action of GnIH *in vivo*, Ubuka et al. (57) treated mature male quail with chronic GnIH. Chronic GnIH treatment decreases plasma LH concentration and the expressions of the common α, LHβ, and FSHβ subunit mRNAs. Additionally, Ubuka et al. (57) established that GnIH treatment induces testicular apoptosis and decreases the diameter of seminiferous tubules and testosterone concentration in mature birds. In immature birds, GnIH treatment suppresses normal testicular growth and testosterone concentration (57). Thus, GnIH appears to suppress gonadal development and maintenance by decreasing gonadotropin synthesis and release in birds (**Figure 1**).

To determine if these findings extend to mammals, including humans, Tsutsui and colleagues sought to identify GnIH in the hypothalamus of mammals and primates (43, 44, 47, 48, 58). All the identified and putative mammalian and primate GnIH peptides possess a common C-terminal LPXRFamide (X = L or Q) motif, like avian GnIH and GnIH-RPs [for reviews, see Ref. (20,

TABLE 1 | Molecular structure of mature GnIH peptides in chordates.

Chordates	Peptide name	Molecular structure	Reference
Mammals	*human RFRP-1*	MPHSFANLPLRFa	Ubuka et al. (43)
	human RFRP-3	VPNLPQRFa	Ubuka et al. (43)
	macaque RFRP-3	SGRNMEVSLVRQVLNLPQRFa	Ubuka et al. (44)
	bovine RFRP-1	SLTFEEVKDWAPKIKMNKPVVNKMPPSAANLPLRFa	Fukusumi et al. (45)
	bovine RFRP-3	AMAHLPLRLGKNREDSLSRWVPNLPQRFa	Yoshida et al. (46)
	rat RFRP-3	ANMEAGTMSHFPSLPQRFa	Ukena et al. (47)
	Siberian hamster RFRP-1	SPAPANKVPHSAANLPLRFa	Ubuka et al. (48)
	Siberian hamster RFRP-3	TLSRVPSLPQRFa	Ubuka et al. (48)
Birds	*quail GnIH*	SIKPSAYLPLRFa	Tsutsui et al. (18)
	quail GnIH-RP-2	SSIQSLLNLPQRFa	Satake et al. (40)
	chicken GnIH	SIRPSAYLPLRFa	McConn et al. (39)
	European starling GnIH	SIKPFANLPLRFa	Ubuka et al. (41)
	zebra finch GnIH	SIKPFSNLPLRFa	Tobari et al. (42)
Reptiles	*red-eared slider GnIH*	SIKPVANLPLRFa	Ukena et al. (49)
	red-eared slider GnIH-RP-1	STPTVNKMPNSLANLPLRFa	Ukena et al. (49)
	red-eared slider GnIH-RP-2	SSIQSLANLPQRFa	Ukena et al. (49)
Amphibians	*bullfrog GRP/R-RFa*	SLKPAANLPLRFa	Koda et al. (50) and Chartrel et al. (51)
	bullfrog GRP-RP-1	SIPNLPQRFa	Ukena et al. (52)
	bullfrog GRP-RP-2	YLSGKTKVQSMANLPQRFa	Ukena et al. (52)
	bullfrog GRP-RP-3	AQYTNHFVHSLDTLPLRFa	Ukena et al. (52)
	red-bellied newt LPXRFa-1	SVPNLPQRFa	Chowdhury et al. (53)
	red-bellied newt LPXRFa-2	MPHASANLPLRFa	Chowdhury et al. (53)
	red-bellied newt LPXRFa-3	SIQPLANLPQRFa	Chowdhury et al. (53)
	red-bellied newt LPXRFa-4	APSAGQFIQTLANLPQRFa	Chowdhury et al. (53)
Teleost fishes	*goldfish LPXRFa-3*	SGTGLSATLPQRFa	Sawada et al. (54)
Agnathans	*sea lamprey LPXRFa-1a*	SGVGQGRSSKTLFQPQRFa	Osugi et al. (55)
	sea lamprey LPXRFa-1b	AALRSGVGQGRSSKTLFQPQRFa	Osugi et al. (55)
	sea lamprey LPXRFa-2	SEPFWHRTRPQRFa	Osugi et al. (55)
Protochordates	*amphioxus PQRFa-1*	WDEAWRPQRFa	Osugi et al. (56)
	amphioxus PQRFa-2	GDHTKDGWRPQRFa	Osugi et al. (56)
	amphioxus PQRFa-3	GRDQGWRPQRFa	Osugi et al. (56)

Only structurally determined mature endogenous peptides are shown.

21, 26–29)] (**Table 1**). Therefore, these GnIH peptides were designated as LPXRFamide peptides on the basis of their structures. Mammalian and primate GnIH peptides are also called RFamide-related peptide 1 and 3 (RFRP-1 and -3) (**Table 1**). Kriegsfeld et al. (58) found that *in vivo* administration of avian GnIH centrally or peripherally to female Syrian hamsters inhibits LH release (58). Central administration of hamster GnIHs (RFRP-1 and -3) also inhibits LH release in Siberian hamsters (48). Central administration of rat GnIH (RFRP-3) to male rats also inhibits LH release (59) and GnRH-elicited gonadotropin release (60, 61). Reduction in LH pulse amplitude and inhibition of GnRH-elicited gonadotropin release and synthesis by mammalian GnIH (RFRP-3) have also been reported in ovine (62, 63) and bovine (64). Because the structure of human GnIH (RFRP-3) is the same as ovine GnIH (RFRP-3) (43), the hypohysiotropic action of human/ovine GnIH (RFRP-3) was assessed in ovine pituitary in collaboration with the Clarke laboratory. Human/ovine GnIH (RFRP-3) inhibits GnRH-stimulated secretion of both LH and FSH (62) demonstrating that, as with avian GnIH, mammalian and primate GnIH inhibit gonadotropin release and synthesis and GnRH-elicited gonadotropin secretion [for reviews, see Ref. (19–21, 26–29)] (**Figure 1**).

COMMONALITIES AND DIVERSITY OF GnIH STRUCTURES AND ACTIONS

To place these findings into a broader perspective, Tsutsui and colleagues further identified GnIH peptides in the brain of reptiles, amphibians, and fish. All of the identified and putative GnIHs in these vertebrates also possess a common C-terminal LPXRFamide (X = L or Q) motif, like avian, mammalian, and primate GnIHs (49–54, 65, 66) (**Table 1**). Thus, GnIH peptides exist in the brain of vertebrates from fish to humans [for reviews, see Ref. (20–30)]. Goldfish GnIH precursor cDNA encodes three GnIHs, gfLPXRFa-1, -2, and -3 (54). These goldfish GnIH peptides (gfLPXRFa-1, -2, and -3) have both inhibitory and stimulatory effects on gonadotropin synthesis and release, possibly depending on reproductive conditions (67–70). Zebrafish GnIH peptide, zfLPXRF-3, also has an inhibitory effect on gonadotropin release (71).

As mentioned above, GnIH peptides were identified in representative species of gnathostomes. However, GnIH peptide had not been identified in agnathans, the most ancient lineage of vertebrates (72). Therefore, Tsutsui and colleagues searched for agnathan GnIH in collaboration with the Sower and Nozaki

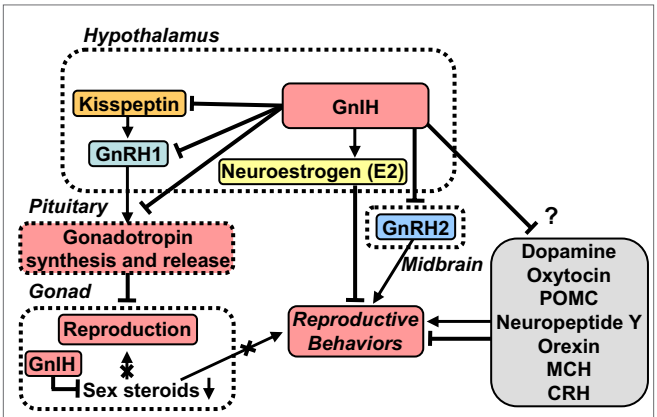

FIGURE 1 | A schematic model of GnIH actions on the regulation of reproduction and reproductive behaviors. GnIH is a newly discovered hypothalamic neuropeptide that inhibits gonadotropin release in the quail brain (18). GnIH is highly conserved among vertebrates. GnIH acts as a key player in the regulation of reproduction and reproductive behaviors across vertebrates. Cell bodies for GnIH neurons are localized in the paraventricular nucleus (PVN) in birds and in the dorsomedial hypothalamic area (DMH) in mammals. Terminals from GnIH neurons are localized to the median eminence (ME) and GnRH1 neurons in the preoptic area (POA) in birds and mammals. GnIH receptor is expressed in gonadotropes in the pituitary and GnRH1 neurons in the POA in birds and mammals. Thus, GnIH inhibits gonadotropin synthesis and release by directly acting on gonadotropes in the pituitary and by inhibiting the activity of GnRH1 neurons in the POA via GnIH receptor in birds and mammals. GnIH neurons project not only to GnRH neurons but also to kisspeptin neurons in the hypothalamus in mammals. Kisspeptin neurons express GnIH receptor in mammals. GnIH and GnIH receptor are also expressed in steroidogenic cells and germ cells in gonads, and GnIH possibly acts in an autocrine/paracrine manner to suppress gonadal steroid production and germ cell differentiation and maturation in birds and mammals. GnIH participates not only in neuroendocrine functions but also in behavioral control in birds and mammals. GnIH inhibits reproductive behaviors, such as sexual and aggressive behaviors, and stimulates feeding behavior by acting within the brains of birds and mammals. Furthermore, GnIH inhibits reproductive behaviors by changing the biosynthesis of neuroestrogen (E2) in the POA. GnIH neurons further project to many other neurons in the brain suggesting multiple actions of GnIH. See the text for details.

laboratories (55). Synteny analysis showed the existence of the gene for GnIH in sea lamprey, and Osugi et al. (55) cloned lamprey GnIH precursor cDNA that encodes GnIH peptides. Subsequently, three mature GnIH peptides were identified from the brain of sea lamprey by immunoaffinity purification and mass spectrometry (55) (**Table 1**). The identified lamprey GnIHs share a common C-terminal PQRFamide motif (55), unlike GnIHs identified of gnathostomes. However, phylogenetic analysis showed that the identified lamprey GnIH precursor groups with the LPXRFa peptide precursors of vertebrates, whereas the previously identified lamprey PQRFa peptide precursor (73) groups with the PQRFa peptide precursors of vertebrates. Accordingly, we concluded that the lamprey GnIH precursor gene is the ortholog of LPXRFa peptide gene (55).

Lamprey GnIH neurons are located in the hypothalamus (55) with immunoreactive fibers projecting to GnRH3 neurons (55). Few lamprey GnIH immunoreactive fibers were observed in the neurohypophysis compared to abundant lamprey GnRH3 immunoreactive fibers (55). Osugi et al. (55) then analyzed the effects of lamprey GnIH peptides on the expressions of lamprey GnRHs and the gonadotropin β subunit. It was found that one of the lamprey GnIH peptides increases the expressions of lamprey GnRH3 and gonadotropin β RNA (55). Thus, GnIH is present in the brain of lamprey, the oldest lineage of vertebrates, and may act on GnRH3 neurons to stimulate the expression of gonadotropin β in the pituitary (55). Based on these findings, it is speculated that GnIH emerged in agnathans as a stimulatory neuropeptide and changed into an inhibitory neuropeptide during vertebrate evolution.

EVOLUTIONARY ORIGIN OF GnIH

The C-terminal structure of most GnIH peptides is LPXRFamide (X = L or Q), making them a member of the RFamide peptide family [for reviews, see Ref. (20–30)]. Four more groups, i.e., the neuropeptide FF (NPFF; PQRFamide peptide) group, the kisspeptin group, the pyroglutamylated RFamide peptide (QRFP)/26RFamide group, and the prolactin-releasing peptide (PrRP) group, have been documented in vertebrates [for reviews, see Ref. (20, 21, 23, 30)]. Because the C-terminal structure of GnIH peptides is similar to NPFF peptides that have a C-terminal PQRFamide motif, further clarification of the NPFF peptide gene in agnathans was warranted. NPFF is a neuropeptide involved in pain modulation [for a review, see Ref. (74)]. Accordingly, Tsutsui and colleagues sought to identify the cDNAs of NPFF peptides in the brain of lamprey and hagfish (73, 75). Phylogenetic analysis established that agnathans possess both GnIH and NPFF precursor genes. Agnathan NPFF peptides were further identified in sea lamprey and hagfish. The identified agnathan NPFF peptides had the same C-ternimal PQRFamide motif as agnathan GnIH peptides (73, 75).

The findings that agnathans have both GnIH and NPFF genes and their mature peptides have the same C-terminal PQRFamide motif (55, 73, 75) suggest that the GnIH and NPFF genes were derived from a common ancestral gene in protochordates. To test this hypothesis, Tsutsui and colleagues identified an amphioxus PQRFamide peptide precursor cDNA that encodes three putative PQRFamide peptides (56). Subsequently, three endogenous amphioxus PQRFamide peptides were identified by immunoaffinity purification and mass spectrometry (56) (**Table 1**). Phylogenetic analysis showed that the amphioxus PQRFamide peptide precursor occurs before the divergence between the GnIH and NPFF groups in vertebrates (56). Synteny analysis showed that the conserved synteny region exists around the loci of the amphioxus PQRFamide peptide gene, GnIH gene, and NPFF gene in vertebrates (56). The amphioxus PQRFamide peptide gene is located near the HOX cluster, whereas the GnIH and NPFF genes in vertebrates are located near the HOXA and HOXC clusters, respectively, suggesting that the GnIH and NPFF genes may have duplicated through whole-genome duplications (56). Accordingly, the amphioxus PQRFamide peptide gene is considered to be close to the ancestor of the GnIH and NPFF genes (56, 76). Thus, the GnIH and NPFF genes may have diverged from a common ancestral gene in the protochordate through whole-genome duplication event during vertebrate evolution.

MODE OF GnIH ACTION ON GONADOTROPIN SECRETION

Identification of GnIH Receptor

To investigate the mode of action of GnIH on gonadotropin secretion in birds, Tsutsui and colleagues identified the receptor for GnIH in quail. They identified GnIH receptor as GPR147, a member of the G-protein-coupled receptor (GPCR) superfamily (77), which is also called NPFF receptor 1 (NPFF1). Membrane fraction of COS-7 cells that are transfected with GnIH receptor cDNA binds with high affinity to GnIH and GnIH-RPs (77). Since GnIH receptor is expressed in gonadotropes in the anterior pituitary, GnIH can act directly on gonadotropes to reduce gonadotropin release in birds [for reviews, see Ref. (19–21, 26–29, 34)] (**Figure 1**). In addition to acting on the anterior pituitary, GnIH neurons project to GnRH1 neurons (41, 78) that express GnIH receptor (41) (**Figure 1**). Thus, GnIH not only acts on gonadotropes but also acts on GnRH1 neurons to inhibit gonadotropin synthesis and release in birds [for reviews, see Ref. (19–21, 26–29, 34)] (**Figure 1**).

In mammals, Hinuma et al. (79) identified a specific receptor for mammalian GnIH, RFRP, which is identical to GPR147 and named it OT7T022 by the reverse pharmacological approach. In the human genome, there are approximately 700 GPCR genes, and the receptors whose ligands are still unknown are called orphan receptors. Hinuma et al. (79) searched for specific receptors for ligands by testing whether (1) increases in calcium ions, (2) increases in cAMP, or (3) decreases in cAMP happens in the cells transfected with the receptor by ligand stimulation [for a review, see Ref. (80)]. In the same year, Bonini et al. (81) reported two GPCRs for NPFF and designated them as NPFF1 (identical to GPR147) and NPFF2 (identical to GPR74). Bonini et al. (81) cloned these receptors by GPCR-targeted degenerate PCR using rat genomic DNA. As mentioned previously, it is thought that the GnIH (LPXRFamide peptide) and NPFF (PQRFamide peptide) genes have diverged from a common ancestral gene through gene duplication (55, 56, 76). It is also thought that GPR147 and GPR74 are paralogous from synteny analysis (82) and phylogenetic analysis (83). Analyses of binding affinities of GnIH and NPFF for GPR147 and GPR74 and their signal transduction pathways reveal that GnIH has a higher affinity for GPR147, whereas NPFF has potent agonistic activity for GPR74 (46, 81, 84), suggesting that GPR147 (NPFF1, OT7T022) is the primary receptor for GnIH.

GnIH Cell Signaling

To further understand the cellular cascade by which GnIH impacts gonadotropes in the anterior pituitary, Tsutsui and colleagues investigated GnIH signaling pathways in the mouse gonadotrope cell line, LβT2. First, the expression of GnIH receptor mRNA in LβT2 cells was shown by RT-PCR (85). Subsequently, the inhibitory effects of GnIH on GnRH-induced signaling pathways were demonstrated; mouse GnIHs effectively reduce GnRH-induced cAMP production and extracellular signal-regulated kinase (ERK) phosphorylation (85). Furthermore, mouse GnIHs reduce GnRH-induced LHβ expression and LH release (85). The stimulatory effect of GnRH on gonadotropin expression is suppressed by adenylate cyclase (AC) and protein kinase A (PKA) inhibitors but not by protein kinase C (PKC) inhibitor (85). Accordingly, mouse GnIH reduces GnRH-stimulated gonadotropin secretion by specifically interfering with GnRH actions via a AC/cAMP/PKA-dependent ERK pathway (85).

Following the discovery of GnIH, kisspeptin was discovered in mammals. Opposite to GnIH, kisspeptin has a stimulatory effect on GnRH neurons and the hypothalamic–pituitary–gonadal axis (HPG axis) in mammals (86–89). GnIH neurons project not only to GnRH1 neurons in the preoptic area (POA) but also to kisspeptin neurons in the hypothalamus and may regulate their activities [for reviews, see Ref. (19–21, 26–29, 90)] (**Figure 1**). GnIH neurons project to GnRH2 neurons and many other neurons suggesting multiple actions of GnIH [for reviews, see Ref. (19–21, 26–29)] (**Figure 1**).

REGULATION OF GnIH BIOSYNTHESIS IN THE BRAIN BY ENVIRONMENTAL AND INTERNAL FACTORS

Influence of Photoperiod Mediated by Melatonin

Investigating the regulatory mechanisms of GnIH expression in the brain has important implications for understanding the physiological role of GnIH. Photoperiodic mammals regulate reproductive activities according to the annual cycle of changes in the nocturnal secretion of melatonin (91). There is also evidence in birds that melatonin is involved in the regulation of several seasonal processes including gonadotropin secretion and gonadal activity (92–95), despite the accepted dogma that birds do not use seasonal changes in melatonin secretion to time their reproductive effort (96, 97).

To explore whether or not GnIH is part of the mechanism driving melatonin-induced seasonal changes in reproduction, Tsutsui and colleagues investigated the action of melatonin on the expression of GnIH in quail, a highly photoperiodic bird species. Initial findings demonstrated that melatonin induces GnIH expression in birds (**Figure 2**). More specifically, Ubuka et al. (98) first found that melatonin removal by pinealectomy, combined with orbital enucleation (Px plus Ex), decreases the expressions of GnIH mRNA and GnIH peptide labeling in the brain of quail (98). Ubuka et al. (98) further found that melatonin administration increases the expressions of GnIH mRNA and GnIH peptide in the brain of quail (98). Importantly, they found that Mel_{1c}, a melatonin receptor subtype, is expressed in GnIH neurons in quail (98). These findings established that melatonin acts directly on GnIH neurons to induce GnIH expression in this species (**Figure 2**). Chowdhury et al. (99) further demonstrated that melatonin not only increases GnIH expression but also increases GnIH release in quail (**Figure 2**). Interestingly, GnIH release is photoperiodically controlled in quail with diurnal changes that are negatively correlated with plasma LH concentration (99). As one would expect, GnIH release increases under short day (SD) photoperiods, when the duration of nocturnal secretion of melatonin increases (99). Together, these findings indicate that

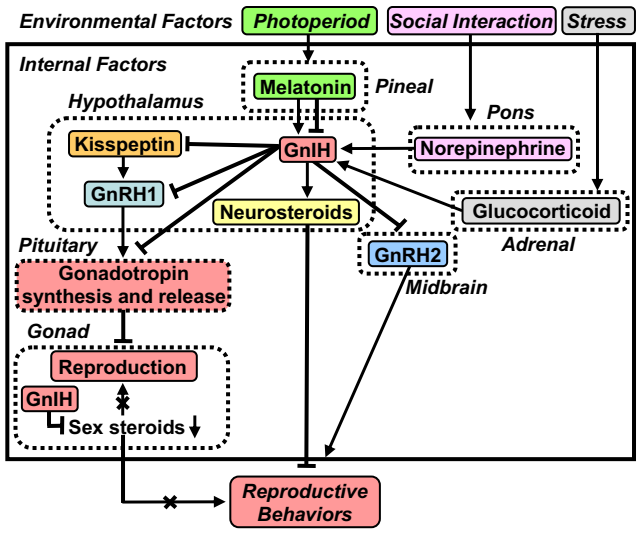

FIGURE 2 | A schematic model of the neuroendocrine integration of environmental factors and internal factors to control GnIH expression and release. The neuroendocrine integration of environmental factors, such as photoperiod, stress, and social interaction, and internal factors, such as GnIH, melatonin, glucocorticoid, and norepinephrine (NE), is important for the control of reproduction and reproductive behaviors. GnIH inhibits the expression and release of gonadotropins and the expression of reproductive behaviors in birds and mammals. GnIH expression and release are photoperiodically modulated via a melatonin-dependent process in birds and mammals. Melatonin increases GnIH expression in quail and rats, whereas melatonin decreases GnIH expression in hamsters and sheep. Stress increases GnIH expression mediated by the actions of glucocorticoids in birds and mammals. GnIH may be a mediator of stress-induced reproductive disruption. The social environment also changes GnIH expression and release mediated by the action of NE. See the text for details.

melatonin derived from the pineal gland and eyes acts directly on GnIH neurons via Mel_{1c} to induce GnIH expression and release in birds (29, 98–101) (**Figure 2**).

In contrast to the results seen in quail, melatonin reduces GnIH expression in Syrian and Siberian hamsters, both photoperiodic mammals (48, 102, 103) (**Figure 2**). Specifically, GnIH mRNA levels and the number of GnIH immunoreactive cell bodies are reduced in sexually quiescent Syrian and Siberian hamsters exposed to SD photoperiods, compared to sexually active animals maintained under long day (LD) photoperiods. These photoperiodic changes in GnIH expression are abolished in Px hamsters, and injections of LD hamsters with melatonin reduce the expression of GnIH to SD levels (48, 102). Analogous seasonal patterns of GnIH expression have been observed in European (104) and Turkish (105) hamsters as well as the semi-desert rodent, Jerboa (106). Although these results are suggestive of a role for GnIH in seasonal breeding, they are inconsistent with a straightforward model of seasonal reproductive control by this peptide. One possibility is that hamsters require enhanced GnIH expression to suppress GnRH during the initial period of regression, whereas this level of inhibition is not necessary in hamsters with a fully regressed reproductive axis and low testosterone (T) concentrations. Another possibility emerged from our findings in male Siberian hamsters. In this species, GnIH administration suppresses HPG axis function in LD, reproductive-competent animals but stimulates gonadotropin secretion in SD, reproductively quiescent animals (48), indicating that the impact of GnIH may depend on reproductive status or season. There are also reports showing that the expression of GnIH is regulated by melatonin and season in sheep (107, 108) and rats (109). Thus, as in quail, the expression of GnIH is photoperiodically modulated via a melatonin-dependent process in mammals (**Figure 2**).

We still do not have data as to why melatonin stimulates GnIH expression in quail (98) and inhibits GnIH expression in hamsters (48, 102) and other mammals. Although expression of melatonin receptor in GnIH neurons is still not demonstrated in mammals, one possibility is that melatonin triggers different intracellular signals in GnIH neurons in quail because mammals do not have Mel_{1c} melatonin receptor subtype (110). Another possibility is that melatonin indirectly regulates GnIH expression in mammals unlike quail. These possibilities should be tested in future studies.

Influence of Stress Mediated by Glucocorticoids

It is well known that stress can reduce reproduction across vertebrates (111). To explore whether stress changes GnIH expression in birds, Calisi et al. (112) investigated the effect of capture-handling stress on GnIH expression in male and female adult house sparrows. Calisi et al. (112) found that more GnIH-positive neurons are observed in fall birds versus those sampled in the spring, and GnIH-positive neurons are increased by capture-handling stress in spring birds. These observations indicate that stress influences GnIH during the breeding season (112). These findings suggested that stress may act through GnIH neurons to inhibit reproductive function in birds.

In mammals, Kirby et al. (113) showed that both acute and chronic immobilization stress lead to an upregulation of the expression of GnIH in the dorsomedial hypothalamic area (DMH) of male rats associated with inhibition of downstream hypothalamic–pituitary–testicular activity (**Figure 2**). Adrenalectomy blocks this stress-induced increase in GnIH expression. Immunohistochemistry revealed that GnIH neurons express glucocorticoid receptor (GR), suggesting that adrenal glucocorticoids act directly on GnIH neurons to increase GnIH expression (**Figure 2**). Together, these observations indicate that GnIH is an important integrator of stress-induced suppression of reproductive function in mammals (113).

Recently, Son et al. (114) demonstrated that GR mRNA is expressed in GnIH neurons in the PVN of quail, suggesting that glucocorticoids can directly regulate GnIH transcription (**Figure 2**). It was also found that treatment with corticosterone (CORT) increases GnIH mRNA expression in the quail diencephalon (114) (**Figure 2**). Subsequently, Son et al. (114) investigated the mechanism of activation of GnIH transcription by CORT using a GnIH-expressing neuronal cell line, rHypoE-23, derived from rat hypothalamus. Importantly, GR mRNA is expressed in rHypoE-23 cells, and CORT treatment increases GnIH mRNA expression (114). Son et al. (114) further found that CORT stimulates GR recruitment to the GC-response element (GRE)

present in the rat GnIH promoter region, providing further support that CORT induces GnIH expression via GR in GnIH neurons (**Figure 2**). Taken together, it appears that stress reduces gonadotropin release, at least in part, through an increase in GnIH expression. More recent evidence also indicates that GnIH might itself regulate the stress response in mice (115).

Influence of Social Interactions Mediated by Norepinephrine

In addition to the regulation of GnIH expression by environmental factors, photoperiod, and stress, the social environment may influence the GnIH system (**Figure 2**). To examine this possibility, Calisi et al. (116) investigated the impact of mating competition on GnIH. Nesting opportunities for pairs of European starlings were manipulated, and GnIH mRNA and GnIH content as well as GnRH content in the brain were examined. Birds with nest boxes have fewer numbers of GnIH-producing cells than those without nest boxes. However, GnRH content does not vary with nest box ownership. These observations suggest that GnIH may serve as a modulator of reproductive function in response to social environment (116).

Reproductive physiology and behavior are variable, both within and between individuals. It is known that the presence of a female bird as well as copulation rapidly decrease plasma T concentrations in male quail (117, 118). Based on these earlier observations, Tsutsui and colleagues investigated the neurochemical mechanism by which social stimuli alter reproductive physiology and behavior (**Figure 2**). Tobari et al. (31) first found that norepinephrine (NE) release increases rapidly in the PVN of quail when viewing a female conspecific (**Figure 2**). Likewise, GnIH mRNA expression increases in the PVN, with associated decreases in LH concentrations in plasma, when males view a female (**Figure 2**). Tobari et al. (31) then established a link between these two events by showing that NE application stimulates GnIH release from diencephalic tissue blocks *in vitro*. Double-label immunohistochemistry revealed that GnIH neurons are innervated by noradrenergic fibers and immunohistochemistry combined with *in situ* hybridization demonstrated that GnIH neurons expressed α2A-adrenergic receptor mRNA. Together, these observations indicate that female presence increases NE release in the PVN and stimulates GnIH release, resulting in the suppression of LH release in quail (31) (**Figure 2**).

MULTIPLE ACTIONS OF GnIH

Direct Regulation of Gonadal Activity

The aforementioned findings indicate that GnIH is a key player in the regulation of reproduction across vertebrates, reducing gonadotropin synthesis and release by decreasing the activity of GnRH1 neurons and decreasing the activity of pituitary gonadotropes, inevitably suppressing gonadal steroid secretion and spermatogenesis (**Figure 1**). In addition to these central actions of GnIH, several lines of evidence point to direct, local regulation of gonadal activity [for reviews, see Ref. (19, 20, 26–29, 32, 34)] (**Figure 1**). GnIH and GnIH receptor are expressed in steroidogenic cells and germ cells in the gonads of birds and mammals (119–125), with GnIH possibly acting in an autocrine/paracrine manner to suppress gonadal steroid production and germ cell differentiation and maturation (119–125) (**Figure 1**). There is also evidence in songbirds that gonadal GnIH responds directly to melatonin, metabolic challenge, and cues of stress in a seasonal manner (126–128)

Regulation of Feeding Behavior

It is becoming clear that GnIH participates not only in neuroendocrine functions but also in behavioral control. In environments where energy availability fluctuates, animals use photoperiod to phase breeding with anticipated times of maximal food availability (129). Should food become scarce during the breeding season, reproduction is temporarily inhibited (130, 131). Food deprivation and other metabolic challenges inhibit reproductive axis functioning and sexual motivation (132–136). GnIH may relay metabolic information to the HPG axis and regulate neural feeding circuits [for a review, see Ref. (19)].

Tachibana et al. (137) showed that intracerebroventricular (ICV) injections of GnIH, GnIH-RP-1, and GnIH-RP-2 stimulate food intake in chicks (137). In further support of a stimulatory role for GnIH in feeding, anti-GnIH antiserum suppresses appetite induced by fasting but does not modify feeding under *ad libitum* conditions (137). Similarly, Fraley et al. (138) reported that ICV injection of GnIH, but not of GnIH-RP1, suppresses plasma LH and stimulates feeding in adult Pekin ducks. To establish the neurochemical cascade underlying the actions of GnIH on feeding, Tachibana et al. (139) explored the possibility that the orexigenic effect of GnIH occurs via actions on the opioid and nitric oxide (NO) systems. It was found that the orexigenic effect of ICV injected GnIH is attenuated by coinjection of β-funaltrexamine (an opioid μ-receptor antagonist) but not ICI-174,864 (an opioid δ-receptor antagonist) and nor-binaltorphimine (an opioid κ-receptor antagonist) in chicks. It was also found that coinjection of a non-selective NO synthase inhibitor does not affect GnIH-induced feeding behavior (139). More recently, McConn et al. (39) investigated the central mechanism of the GnIH orexigenic response in chicks. It was found that neuropeptide Y (NPY) mRNA is increased, while pro-opiomelanocortin (POMC) mRNA is decreased in the hypothalamus following ICV injection of chicken GnIH. Additionally, ICV GnIH injections increase c-fos immunoreactive cells in the lateral hypothalamic area (LHA). McConn et al. (39) further found that in isolated LHA, melanin-concentrating hormone (MCH) mRNA is increased by ICV administration of GnIH. Together, these observations suggest that opioid μ-receptor-positive, NPY, POMC, and MCH neurons are likely involved in the GnIH orexigenic response.

In mammals, there are several reports indicating that ICV administration of GnIH increases food intake in rats (59) and sheep (140). Fu and van den Pol (141) showed that chicken GnIH and human GnIH inhibit POMC neurons and attenuate kisspeptin cell excitation by a mechanism based on opening potassium channels in mouse brain slices. Jacobi et al. (142) reported that GnIH inhibits the firing rate in POMC neurons and has a predominantly inhibitory effect on action potential activity in NPY neurons in mice. Jacobi et al. (142) also reported that NPY neurons have close contacts from GnIH fibers.

Together, these observations indicate that GnIH participates not only in reproduction but also in feeding behavior in birds and mammals.

Regulation of Reproductive Behaviors

Gonadotropin-inhibitory hormone also acts on the brain to regulate reproductive behaviors, such as sexual and aggressive behaviors (32, 143, 144) (**Figure 1**). For example, Bentley et al. (143) showed that a centrally administered physiological dose of GnIH inhibits copulation solicitation in estrogen-primed female white-crowned sparrows exposed to the song of males. It is known that GnRH2 enhances copulation solicitation in estrogen-primed female white-crowned sparrows exposed to the song of males (145). Because GnIH neurons terminate in close proximity of GnRH2 neurons and GnRH2 neurons express GnIH receptor in songbirds (41), GnIH may inhibit copulation solicitation by inhibiting GnRH2 neurons in female songbirds (143) (**Figure 1**). Ubuka et al. (144) directly investigated this possibility by applying RNA interference (RNAi) to the GnIH gene and examining the behavior of male and female white-crowned sparrows in collaboration with the Wingfield laboratory. GnIH RNAi reduces resting time, spontaneous production of complex vocalizations, and stimulates agonistic vocalizations. Additionally, GnIH RNAi enhances song production of short duration in male birds when they are challenged by playbacks of novel male songs. These observations indicate that GnIH gene silencing induces arousal. Ubuka et al. (144) further showed that the activities of male and female birds are negatively correlated with GnIH mRNA expression in the PVN. The density of GnIH neuronal fibers in the ventral tegmental area is decreased by GnIH RNAi in female birds, and the number of GnRH1 and GnRH2 neurons that receive close appositions of GnIH neuronal fiber terminals is negatively correlated with the activity of male birds (144) (**Figure 1**). Recently, Ubuka et al. (32) have demonstrated that GnIH also inhibits aggressive behavior in male quail. Thus, it is considered that GnIH decreases sexual and aggressive behaviors in birds [for reviews, see Ref. (19, 29, 34)] (**Figure 1**).

Similar results have been observed in mammals. For example, Johnson et al. (59) reported that ICV administration of GnIH suppresses male sex behavior in rats. Piekarski et al. (146) found that ICV administration of GnIH reduces sexual motivation and vaginal scent marking but not lordosis behavior in female hamsters. According to Piekarski et al. (146), GnIH administration alters fos expression in key neural loci implicated in female sexual behavior, including the medial POA, medial amygdala, and bed nucleus of the stria terminalis. These observations suggest that GnIH is an important modulator of female proceptive sexual behavior and motivation (**Figure 1**). Thus, as in birds, GnIH is not only in a position to regulate the HPG axis but may also act as a neuromodulator to drive the neural circuitry underlying socially motivated behavior.

Regulation of Neurosteroidogenesis

It is becoming increasingly clear that the interaction of neuropeptides and neurosteroids plays a role in the regulations of some brain functions [for a review, see Ref. (147)]. Recently, Ubuka et al. (32) found that GnIH activates cytochrome P450 aromatase (P450arom) and increases neuroestrogen synthesis in the avian brain (32) (**Figures 1** and **3**). Importantly, the actions of GnIH on neuroestrogen synthesis change the expression of aggressive behavior in birds (32) (**Figures 1** and **3**), providing a new concept that GnIH modifies the neurosteroid milieu in the brain to modulate aggressive behavior.

Further exploring the ability of GnIH to alter neurosteroid production has led to important insight into the control of aggressive behavior in quail. Sexually mature male quail frequently fight with intense aggressiveness, by using stereotyped actions (148,

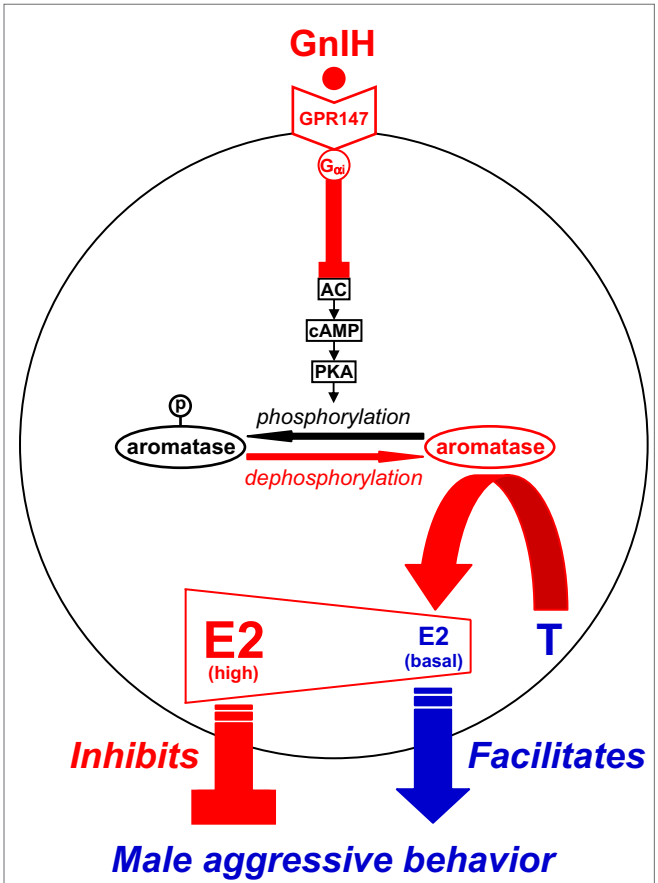

FIGURE 3 | Model of the intracellular mechanism of GnIH and its receptor (GPR147) that control male aggressive behavior by regulating the activity of aromatase and neuroestrogen synthesis in the brain. GPR147 is expressed in aromatase immunoreactive cells in the brain. GPR147 is coupled to $G_{\alpha i}$ protein that inhibits the activity of adenylate cyclase (AC) and decreases cAMP production and the activity of protein kinase A (PKA). Inhibition of AC/cAMP/PKA pathway may decrease phosphorylated aromatase and increase dephosphorylated aromatase. 17β-Estradiol (E2) synthesized from testosterone (T) by aromatase in the brain especially in the preoptic area (POA) regulates male aggression. The administration of GnIH activates aromatase by decreasing phosphorylated aromatase and stimulates neuroestrogen synthesis in the brain. GnIH may inhibit aggressive behavior by directly activating aromatase and increasing neuroestrogen synthesis in the brain beyond its optimum concentration for the expression of aggressive behavior. Partially adapted from Ubuka and Tsutsui (33).

149). Aggressive behavior of male quail is thought to be androgen dependent (148–150), but there is generally no correlation between aggressiveness and peripheral T concentration (150). It is also known that aggression in males is activated by aromatizable androgens, such as T and androstenedione (AD), but not by non-aromatizable androgens, such as dihydrotestosterone (DHT), and that administration of P450arom inhibitors blocks T-induced aggression (150, 151). Thus, the action of gonadal androgen on aggressive behavior requires its aromatization into estrogen (neuroestrogen) in the brain (152–154).

GnIH neurons project to the ME and other brain areas, such as the POA (18, 155, 156) and the periaqueductal central gray [PAG (41)] in birds. GnIH receptor is also expressed in the POA (41, 77) and PAG (41). These brain areas are known to regulate aggressive behavior (157, 158). The POA is also known to be the most critical site of aromatization of gonadal androgen by P450arom and neuroestrogen action for the activation of aggressive behavior in male quail (159, 160). Because GnIH decreases aggressive behavior in male birds (32, 144), GnIH may decrease this behavior by regulating P450arom activity and neuroestrogen synthesis in the brain (**Figures 1** and **3**). Ubuka et al. (32) therefore investigated whether GnIH neuronal fibers innervate P450arom cells and whether P450arom cells express GnIH receptor in the POA of male quail. It was found that abundant GnIH immunoreactive neuronal fibers are distributed in the vicinity of P450arom immunoreactive cells in the POA (32). It was also found that GnIH receptor is expressed in P450arom immunoreactive cells in the POA (32). Furthermore, GnIH stimulates P450arom activity and increases neuroestrogen synthesis in the POA through GnIH receptor (32) (**Figures 1** and **3**). These studies provided the first evidence that a hypothalamic neuropeptide can regulate neuroestrogen synthesis in the brain.

Importantly, the increase in neuroestrogen concentrations in the POA was associated with a decrease in aggressive behavior (32). Therefore, the effect of central administration of various doses of estradiol-17β (E2) on aggressive behavior was tested in male quail. Ubuka et al. (32) found that central administration of E2 at higher doses decreases aggressive behavior unlike E2 at lower doses (32). This observation indicates that the action of neuroestrogen is essential for the expression of aggressive behavior, but higher concentrations of neuroestrogen in the brain decrease this behavior. Taken together, GnIH decreases aggressive behavior by activating P450arom and increasing neuroestrogen synthesis in the brain beyond its optimum concentration for the expression of aggressive behavior of male birds (32) (**Figures 1** and **3**).

Ubuka et al. (32) further investigated the mode of action of GnIH on the stimulation of P450arom activity. There is important evidence that P450arom activity is not only controlled in the long term by transcription of the P450arom gene *Cyp19* by steroids, but also in the short term by phosphorylation by neurotransmitters, such as glutamate (161). Balthazart's group demonstrated that P450arom activity in the hypothalamus of male quail is rapidly downregulated by phosphorylation (161–165). Therefore, GnIH may activate P450arom by dephosphorylation of phosphorylated P450arom. Ubuka et al. (32) found that ICV administration of GnIH reduces phosphorylated P450arom in the POA in the short term compared with control birds (32). Ubuka et al. (32) also found that the action of GnIH on neuroestrogen synthesis in the POA is abolished by concomitant administration of RF9 (166, 167), a potent antagonist of GnIH receptor, or fadrozole (168, 169), an inhibitor of P450arom. Based on these findings, it is apparent that GnIH stimulates neuroestrogen synthesis in the POA by activating P450arom through dephosphorylation after binding to GnIH receptor in P450arom cells (**Figure 3**).

CONCLUSION AND FUTURE DIRECTIONS

The discovery of GnIH in 2000 and the contributions aimed at understanding its evolutionary history and functions have markedly advanced the progress of reproductive neuroendocrinology. Studies on GnIH over the past decade and a half have demonstrated that GnIH is a key player in the regulation of reproduction across vertebrates. It now appears that GnIH acts on the pituitary and the brain to modulate the reproductive axis and reproductive behaviors (**Figure 1**). In this review, the commonalities and diversity of GnIH structures and actions and the evolutionary origin of GnIH in chordates were also highlighted. The discovery of GnIH has changed our understanding of the regulation of reproductive physiology and behavior. As a result, more than 50 laboratories worldwide are now working on GnIH.

Following the discovery of GnIH, kisspeptin was also discovered in mammals. GnIH and kisspeptin are both comparatively new members of the RFamide peptide family and act on the HPG axis to downregulate and upregulate the reproductive system, respectively. Thus, we now know that GnRH is not the only hypothalamic neuropeptide regulating reproduction. Importantly, GnIH neurons project not only to GnRH neurons but also to kisspeptin neurons in the hypothalamus (**Figure 1**). GnRH neurons and kisspeptin neurons express GnIH receptor. We expect that future studies will reveal previously unknown interactions among GnIH, GnRH, and kisspeptin (**Figure 1**). The fact that GnIH neurons project to many other neurons in the brain suggests multiple actions of GnIH that have not yet been discovered (**Figure 1**).

GnIH activates P450arom activity in the brain (**Figure 1**) and may change the formation of other neurosteroids by activating or inactivating their steroidogenic enzymes. Furthermore, steroidogenic enzymes are expressed not only in the brain but also in the pineal gland (170–173). Future studies are needed to further develop the emerging concept that hypothalamic neuropeptides may modify the neurosteroid milieu in the brain and pineal gland to impact their function.

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Dissecting the Roles of Gonadotropin-Inhibitory Hormone in Mammals: Studies Using Pharmacological Tools and Genetically Modified Mouse Models

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Reproduction is essential for perpetuation of the species and, hence, is controlled by a sophisticated network of regulatory factors of central and peripheral origin that integrate at the hypothalamic–pituitary–gonadal (HPG) axis. Among the central regulators of reproduction, kisspeptins, as major stimulatory drivers of gonadotropin-releasing hormone (GnRH) neurosecretion, have drawn considerable interest in the last decade. However, the dynamic, if not cyclic (in the female), nature of reproductive function and the potency of kisspeptins and other stimulatory signals of the HPG axis make tenable the existence of counterbalance inhibitory mechanisms, which may keep stimulation at check and would allow adaptation of reproductive maturation and function to different endogenous and environmental conditions. In this context, discovery of the gonadotropin-inhibitory hormone (GnIH) in birds, and its mammalian homolog, RFRP, opened up the exciting possibility that this inhibitory signal might operate centrally to suppress, directly or indirectly, GnRH/gonadotropin secretion, thus reciprocally cooperating with other stimulatory inputs in the dynamic regulation of the reproductive hypothalamic–pituitary unit. After more than 15 years of active research, the role of GnIH/RFRP in the control of the HPG axis has been documented in different species. Yet, important aspects of the physiology of this system, especially regarding its relative importance and actual roles in the control of key facets of reproductive function, remain controversial. In the present work, we aim to provide a critical review of recent developments in this area, with special attention to studies in rodent models, using pharmacological tools and functional genomics. In doing so, we intend to endow the reader with an updated view of what is known (and what is not known) about the physiological role of GnIH/RFRP signaling in the control of mammalian reproduction.

Keywords: GnIH, RFRP, NPFF receptors, RF9, kisspeptin, GnRH, gonadotropins

INTRODUCTION

Reproductive maturation (including puberty) and function are indispensable for perpetuation of the species and, thus, are controlled by a sophisticated network of regulatory signals, which impact at the so-called hypothalamic–pituitary–gonadal (HPG) axis (1). In this system, the group of neurons that synthesize and release the decapeptide gonadotropin-releasing hormone (GnRH) acts as the common output pathway through which the brain, and thereby numerous internal and external cues, controls gonadotropins secretion. Accordingly, GnRH neurons are the target of complex regulatory actions, conducted by excitatory and inhibitory signals, which drive – directly or indirectly – the activity of this key neuronal population (1). In recent years, numerous neuropeptides and transmitters with ability to modulate GnRH neurosecretion have been identified. In fact, significant advances have been made in the characterization of stimulatory signals of GnRH neurons. A paradigmatic example is the identification of the puberty/fertility-stimulating neuropeptide, kisspeptin, which has profoundly changed our understanding of how the reproductive brain is controlled and how it interplays with other key neuroendocrine axes (1, 2). However, less progress has been made in the identification of inhibitory signals, which may counterbalance the effects of kisspeptins and other potent elicitors of GnRH/gonadotropin secretion, thereby playing an equally essential role in the precise and dynamic control of the HPG axis.

In this context, identification in 2000 of a novel peptide of the RF-amide superfamily, named gonadotropin-inhibitory hormone (GnIH) on the basis of its action as *Gonadotropin-Inhibitory Hormone*, raised considerable interest (3). For many neuroendocrinologists, this turned into excitement when the putative ortholog of GnIH, encoding the RF-amide peptides, RFRP-1 and RFRP-3, was identified in mammals (4), and the capacity of RFRPs to inhibit gonadotropin release was initially documented in several mammalian species (4–8). These findings paved the way for the characterization of the reproductive (and non-reproductive) roles of GnIH peptides in mammals, including not only their major effects and mode of action in the control of gonadotropin secretion but also their roles on related functions, such as the regulation of food intake (9). In this review, we intend to provide a succinct overview of recent data obtained in preclinical (mostly rodent) models, using pharmacological tools and functional genomics, which help to unveil the physiological relevance of GnIH/RFRP signaling in the regulation of the HPG axis. In addition, the putative role of this system as connecting factor between reproductive function and body energy homeostasis will be briefly discussed.

DISCOVERY AND MAJOR STRUCTURAL FEATURES OF GnIH/RFRP IN MAMMALS

While extensive description of the identification and major features of GnIH and their mammalian counterparts, RFRP, can be found elsewhere in this Special Issue, in this section we will provide, as a means of brief introduction, a succinct recapitulation of key aspects of this class of peptides in mammals, and how they were discovered. Of note, RF-amide peptide superfamily in mammals comprises a number of central regulators of different neuroendocrine axes, including the reproductive (i.e., RFRP and kisspeptins) and lactotropic (i.e., PRL-releasing peptides) axes (9, 10). These peptides share a common RF-amide (Arg-Phe-NH_2) signature at their carboxyl-terminal region (9). Notably, the first isolation of a RF-amide peptide occurred in an invertebrate species (11), and led to the discovery of a large series of peptides with a similar carboxyl-terminal RF-amide motif in different invertebrate and vertebrate species (9, 10). In this context, in 2000, Tsutsui and colleagues discovered in birds a 12 amino acid hypothalamic neuropeptide, with a Ser-Ile-Lys-Pro-Ser-AlaTyr-Leu-Pro-Leu-Arg-Phe-NH2 sequence, with the distinct capacity to inhibit gonadotropin release by cultured quail pituitaries (3). This was quite a remarkable finding, as no peptide with similar inhibitory activity on gonadotropin secretion had been previously identified in vertebrates; hence, this peptide was named GnIH by its activity as *Gonadotropin-Inhibitory Hormone* (3).

The avian GnIH is produced by a gene encoding a precursor protein of 173 amino acids that gives rise to three peptides after proteolytic cleavage: one is termed GnIH, and the other two are named GnIH-1 and GnIH-2 (12, 13). These peptides have a common carboxyl-terminal LPXRF-amide sequence, where X could be L or Q. In the pro-hormone, these sequences are flanked by glycine residues on the C-terminus, as well by an amidation signal and a basic amino acid on either end, as proteolytic cleavage site (14). Using these features as reference, similar sequences have been investigated in mammals by searching in gene databases. This has allowed the identification of orthologous genes and peptides in a number of mammalian species; some of these sequences/peptides are shown in a **Table 1** (*see next page*). Notably, these analyses allowed identification of two perfectly conserved canonical RF-amide peptides, with sequence homology to avian GnIH, in rats, mice, and hamsters, namely RFRP-1 and RFRP-3. Of note, while RFRP-1 displays higher structural homology with avian GnIH than RFRP-3 (4), pharmacological studies strongly suggest that RFRP-3 is likely the functional ortholog of GnIH in mammals (9, 15).

Indeed, phylogenetic analyses revealed that in different mammalian species, the RFRP gene encodes two major structurally related peptides: RFRP-1 and RFRP-3 (see **Figure 1**). These peptides have been identified, among others, in the bovine, rat, mouse, Syrian and Siberian hamster, monkey, and human species (4, 16–21). Additionally, human, macaque, and bovine genes, encoding an LPXRF-amide-like peptide, have been identified and named RFRP-2 (22). Yet, the homolog precursor cDNA in rodents does not encode an equivalent RFRP-2 peptide (10, 23, 24). Moreover, this peptide does not activate the same receptors as RFRP-1 and RFRP-3. Hence, its function as GnIH peptide has been questioned.

The biological actions of RFRP-1 and RFRP-3 peptides are exerted mainly through the G-protein-coupled receptor, NPFF1R (also termed Gpr147). However, RFRP-1 and RFRP-3 can also bind with lower affinity to the related receptor, NPFF2R (also termed Gpr74) (25). In rats, NPFF1R mRNA is expressed in central nervous system, specifically in the hypothalamus, spinal cord, amygdala, hippocampus, and *substantia nigra*, and in peripheral

TABLE 1 | RFRP peptides identified in mammals, with bird (quail) sequence provided for comparison.

Species	Peptide	Sequence	Reference
Mouse	RFRP-1[a] RFRP-3[a]	VPHSAAN**LPLRFa** NMEAGTRSHFPS**LPQRFa**	(22)
Rat	RFRP-1[a] RFRP-3	VPHSAAN**LPLRFa** ANMEAGTMSHFPS**LPQRFa**	(22) (16)
Human	RFRP-1 RFRP-3	MPHSFAN**LPLRFa** VPN**LPQRFa**	(22)
Bovine	RFRP-1 RFRP-3	SLTFEEVKDWAPKIKMNKPVVNKMPPSAAN**LPLRFa** AMAHLPLRLGKNREDSLSRWVPN**LPQRFa**	(22) (17)
Ovine	RFRP-1[a] RFRP-3[a]	SLTFEEVKDWGPKIKMNTPAVNKMPPSAAN**LPLRFa** VPN**LPQRFa**	(6)
Hamster (Siberian)	RFRP-1 RFRP-3	SPAPANKVPHSAAN**LPLRFa** TLSRVPS**LPQRFa**	(18)
Hamster (Syrian)	RFRP-1[a] RFRP-3[a]	SPAPANKVPHSAAN**LPLRFa** ILSRVPS**LPQRFa**	(4)
Quail	GnIH GnIH-RP-1[a] GnIH-RP-2	SIKPSAY**LPLRFa** SLNFEEMKDWGSKNFMKVNTPTVNKVPNSVAN**LPLRFa** SSIQSLLN**LPQRFa**	(3) (12) (12)

[a]*Deduced from cDNA sequence.*
a, *terminal amide group (characteristic of RF-amide motif).*

tissues, such as the pituitary gland, gonads, and eyes (22, 26). Furthermore, expression of NPFF1R has been found in the sheep pituitary and human gonadotrophs (20, 27, 28). In the hamster, NPFF1R expression has been observed in all types of germ cells, including sperm, and NPFF2R expression has been detected only in more differentiated germinal cells. In the monkey, RFRPs receptors are found in testicular Leydig cells, spermatogonia, and spermatocytes, as well as in granulosa cells, pre-antral follicles and corpora lutea in monkeys and humans (29).

Regarding neuronal populations expressing RFRPs, these have been detected mainly in the hypothalamic dorsomedial nucleus (DMN) or adjacent areas, and they have been found to project to several hypothalamic regions, including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamus, and the ventromedial nucleus (VMN); all these hypothalamic areas are known to play key roles in the control of reproduction and energy balance (30).

PHARMACOLOGICAL ANALYSIS OF GnIH/RFRP EFFECTS ON THE HPG AXIS IN MAMMALS

On the basis of the original work proposing RFRPs as functional orthologs of GnIH in mammals (4), in recent years a number of studies have aimed to characterize the profiles of (mostly brain) expression, as well as the effects and mechanisms of action of RFRPs on the HPG axis (mainly on gonadotropin secretion) in different mammalian species. For the sake of concision, in this section we will focus on the description of the effects RFRPs agonists, and especially of RFRP-3 as major functional analog of GnIH, on gonadotropin secretion in rodents (mainly) and other mammalian species (see **Table 2**). In addition, effects of RFRP-3 on related neuroendocrine axes will be also briefly mentioned.

Effects of the Administration of RFRP-3 in Rats

The limited (indirect) data available to date seem to indicate that in the rat the GnIH/RFRP system does not play an essential role in the regulation of the HPG axis during the developmental period that takes place before puberty. This is based on the fact that central administration of the agonist, RFRP-3, or antisense oligonucleotides (ODNs) against RFRP-3, did not alter the timing of puberty in male rats (31). However, RFRP-3 infusion elicited a significant decrease in luteinizing hormone (LH) levels, while it significantly increased plasma growth hormone (GH) levels compared to control rats, suggesting that RFRP-3 may be associated with peri-pubertal rise in GH secretion (31).

In turn, the GnIH/RFRP system seems to participate in both sexes in the maintenance of basal levels of gonadotropins in adulthood, exerting an inhibitory effect on the HPG axis, which is evidenced by the fact that central administration of RFRP-3 or its analog, RFRP3–8, induces a rapid decrease in serum LH levels (15). Similarly, central injection of RFRP-3 decreased the magnitude of the pre-ovulatory surge of LH, independently of the prolactin surge (32). The inhibitory effects of RFRP-3 were more clearly detected in gonadectomized (GNX) rats, used as putative model to ease identification of potential inhibitory effects against the prevailing elevated levels of gonadotropins caused by GNX. These analyses revealed consistent, albeit modest, inhibitory effects of RFRP-3, but not of RFRP-1, analogs on LH and follicle-stimulating hormone (FSH) levels, especially at high doses (15). As mentioned earlier, it is intriguing that RFRP-1 has been shown to display a higher degree of homology with the avian GnIH than RFRP-3 (25). Yet, the pharmacological analyses in rats strongly suggest that RFRP-3 is actually the functional homolog of GnIH in mammals (15). In fact, studies in ovine and bovine species have confirmed inhibitory effects of RFRP3–8 on

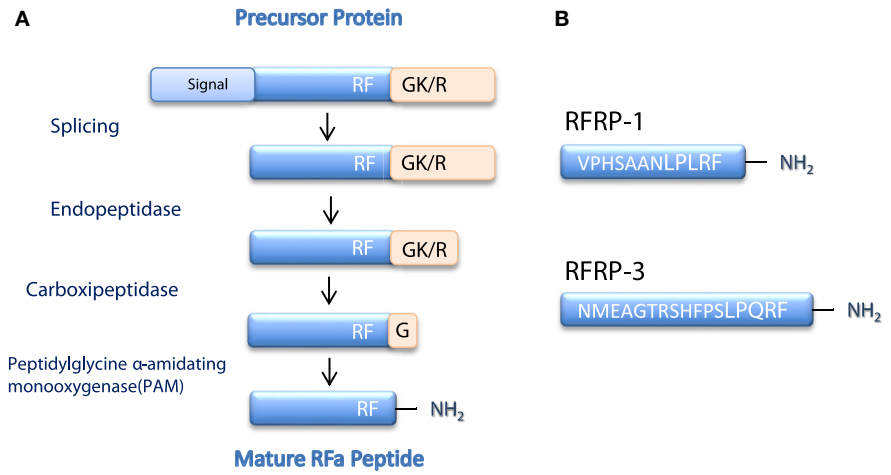

FIGURE 1 | In (A), a schematic representation of the cascade of proteolytic processing events necessary to generate a RF-amide mature peptide from a precursor protein is shown. Precursor proteins posses N-terminal regions that contain abundant hydrophobic residues, which form the signal peptide sequence. On the other hand, the precursor proteins contain a RFGR/K motif, in which R/K is a basic amino acid residue and G is an amide donor; cleavage of this area forms the terminal RF-amide signature. In **(B)**, the major structural features and sequences are shown of the two major peptides, RFRP-1 and RFRP-3, encoded by the RFRP gene. For RFRP-1, complete sequence homology is detected between hamster, mouse, and rat sequences. For RFRP-3, the mouse sequence is presented, which is similar to that of the rat peptide, except for one amino acid residue. Based on Ref. (4, 14) with modifications.

gonadotropin secretion by cultured pituitary cells (6, 33), with higher bio-potency than the larger 17-amino acid RFRP-3 fragment. Moreover, the capacity of central injection of RFRP3–8 to inhibit LH secretion has been documented not only in GNX female rats but also in intact and GNX male rats (15). Inhibitory actions of centrally injected RFRP-3 have been also reported on FSH secretion in male rats *in vivo*. Although unambiguous, the magnitude of such responses was somewhat modest and detected only at the high dose range (≥1 nmol/rat) (15).

In rats, the ability of RFRP to inhibit gonadotropin secretion directly at the pituitary gland remains controversial. In fact, some studies failed to detect GnIH/RFRP-immunoreactive fibers in the median eminence (34). Yet, expression of NPFF1R has been detected in the pituitary gland. In line with the latter, our pharmacological experiments supported a direct action of GnIH/RFRP at the pituitary in rats, as the octapeptide fragment of RFRP-3 was able to decrease basal and GnRH-stimulated secretion of LH by pituitaries from GNX males *ex vivo* (15). Interestingly, comparison of the effective dose-window revealed a dominant inhibitory action of RFRP-3 on GnRH-stimulated LH secretion at the low physiologic range (10^{-10} M), which is lost at higher concentrations (10^{-6} M) of the neuropeptide (15).

Additional studies have addressed the predominant (hypothalamic vs. pituitary) site of action of RFRP-3 in the inhibitory control of the HPG axis in rats by a combination of *in vivo* and *in vitro* approaches. Thus, comparison of the effects on LH secretion of central [intra-cerebroventricular (icv)] vs. systemic [intravenous (iv)] administration of RFRP-3 in adult ovariectomized (OVX) female rats revealed that, while iv administration of RFRP-3 significantly reduced plasma LH levels, icv RFRP-3 injections failed to alter neither the mean LH levels nor the frequency of the pulsatile LH secretion (8). The latter is at odds with other studies addressing the effects of central administration of RFRP-3, as described above, and points to a predominant pituitary action of RFRP-3. In the same vein, studies using cultured pituitary cells from female rats demonstrated a suppressive effect of RFRP-3 on LH secretion, selectively in the presence of GnRH (8). Alike, another study showed that RFRP-3 administration to OVX rats had no effects on basal secretion, but it inhibited GnRH-stimulated LH secretion by about 25% (34).

As final comment to this section, it has been documented that in adult males, icv injection of RFRP-3 has been shown to significantly increase GH secretion, independently of the time of day, while it did not alter plasma levels of thyroid hormone, or cortisol (5). In addition, RFRP-3 also has been related to the control of neuroendocrine and behavioral stress responses in rats. In this context, icv injection of RFRP-3 increased the expression of Fos protein in oxytocin neurons in the hypothalamus and plasma levels of adrenocorticotropic hormone and oxytocin (35).

Effects of the Administration of RFRP-3 in Hamsters

In hamsters, the role of the GnIH/RFRP system in the maintenance of basal levels of gonadotropins was initially suggested by the observation that in OVX Syrian hamsters, icv injection of GnIH induced a rapid reduction of LH levels; similar results were obtained after peripheral injections of the peptide (4). By contrast, however, a somewhat paradoxical stimulatory role of GnIH/RFRP on the gonadotropic axis has been demonstrated in male Syrian hamsters. Thus, acute central injection of RFRP-3 has been shown to induce c-Fos expression in GnRH neurons, and to increase LH, FSH, and testosterone secretion in male (but not female) Syrian hamsters (36). This result suggests that the effects

TABLE 2 | Summary of the reported effects of the administration of RFRP-3 in mammals.

Species	RFRP-3	Reference
Rat	Anti-RFRP ODN treatment in peri-pubertal rats caused a significant increase in plasma LH, but not FSH, levels	(31)
	RFRP-3 administration to peri-pubertal rats caused a significant decrease in plasma LH levels and significant increase in plasma GH levels	
	Central (icv) administration of RFRP-3 significantly reduced plasma LH levels and increased circulating GH, but did not alter plasma levels of FSH, thyroid hormone or cortisol	(5)
	RFRP-3, but not of RFRP-1, analogs had inhibitory effects on LH and/or FSH levels	(15)
	RFRP-3 suppressed the pre-ovulatory GnRH/LH surge independently of the prolactin surge	(32)
	In adult OVX rats, systemic (iv) administration of RFRP-3 lowered plasma LH levels, while icv injections	
	RFRP-3 failed to alter neither the mean levels of LH nor the frequency of the pulsatile LH secretion	(5)
	In cultured pituitary cells, the suppressive effect of RFRP-3 on LH secretion was not clearly detectable in the absence of GnRH, but in the presence of GnRH, RFRP-3 significantly suppressed LH secretion *in vitro*	
	RFRP-3 administration to OVX rats had no detectable effects on basal LH secretion, but inhibited GnRH-stimulated LH secretion	(34)
	Icv injection of RFRPs increased the plasma levels of ACTH and oxytocin	(35)
	In adult males, icv injection of RFRP-3 significantly reduced plasma levels of LH and increased GH independently of the time of day; however, it did not alter plasma FSH, thyroid hormone, or cortisol levels	(8)
Hamster	In female (OVX) Syrian hamsters, GnIH administration (central or peripheral) rapidly inhibits LH secretion	(7)
	In male Syrian hamsters, acute central injection of RFRP-3 induces c-Fos expression in GnRH neurons and increases LH, FSH, and testosterone secretion	(36)
	Central (icv) administration of RFRP-1 or RFRP-3 to male Siberian hamsters decreased LH and FSH levels in long-day conditions, but stimulated LH secretion in short-day conditions	(18)
Mouse	In electrophysiological studies, an inhibitory effect of RFRP-3 on the excitability of GnRH neurons has been shown in male and female mice	(37)
	RFRP-3 has been shown to lower plasma LH levels *in vivo* (after icv injection) and LH secretion *in vitro* (pituitary explants)	(38)
	RFRP-3 has been suggested to inhibit testicular steroidogenesis and spermatogenesis, either indirectly through GnRH or by directly influencing germ cell proliferation, survival, and apoptosis	(39)
	GnIH inhibited follicular development and steroidogenesis in the mouse ovary	(40)
Sheep	Peripheral administration of GnIH decreased the amplitude of LH pulses, while GnIH decreased the secretion of LH and FSH *in vitro*	(6)
Human	RFRP-3 reduced FSH, LH, and forskolin-stimulated progesterone secretion by human granulosa cells	(41)

of RFRP-3 administration on the gonadotrophic axis may be sex dependent, at least in the Syrian hamster (36). Of note, icv administration of RFRP-1 or RFRP-3 to male Siberian hamsters induced a significant decrease in LH and FSH levels under long-day photoperiodic conditions, while, in sharp contrast, both peptides stimulated LH secretion after administration to hamsters under a short-day regimen (18). This would suggest that, in addition to sex, external light cues and photoperiod would play a dominant role in defining inhibitory vs. stimulatory responses to GnIH/RFRP in hamsters.

Effects of the Administration of RFRP-3 in Mice

Electrophysiological studies have shown an inhibitory effect of RFRP-3 on the excitability of GnRH neurons in male and female mice (37). The effects were found to be of rapid onset, dose dependent, and repeatable, suggesting a typical neurotransmitter mode of action of RFRP-3 on GnRH neurons. However, it is noted that RFRP-3 displayed mixed actions on the firing rate of GnRH neurons, with >40% being inhibited but 12% being activated by RFRP-3 (37). In any event, these observations as a whole support a role for RFRP-3 in the modulation of GnRH neuron activity, as major mechanisms for its action in the regulation of gonadotropin secretion (37). To date, the effects of RFRP-3 on gonadotropin secretion *in vivo* have been scarcely studied in the mouse. In fact, to our knowledge, only one study, coming from our group, has shown an inhibitory effect of RFRP-3 on LH secretion *in vivo* in the mouse, using a standard OVX model. Thus, central (icv) administration of 5 nmol RFRP-3 to OVX mice evoked a 25% suppression of LH levels (38). Likewise, pituitary explants from male mice, exposed to RFRP-3 *in vitro*, displayed a suppression of LH secretion (38). In addition, a recent study showed that RFRP-3 treatment induces significant changes in body mass, circulating steroid level and testicular activity in mice (39). Notably, RFRP-3 treatment also caused dose-dependent histological changes in spermatogenesis, such as a decline in germ cell proliferation and survival markers and an increase in apoptotic markers in testis. This study also suggested that the inhibitory effect of RFRP-3 in the testis might be mediated through local production of GnRH. Thus, RFRP-3 could inhibit testicular steroidogenesis and spermatogenesis either indirectly through GnRH or by directly influencing germ cell proliferation, survival, and apoptosis (39). Additionally, GnIH has been shown to inhibit follicular development and steroidogenesis in the mouse ovary (40).

Effects of the Administration of RFRP-3 in Ovine and Primate Species

While, for sake of concision, we have focused our review in rodent studies, it is interesting to note that *in vivo* and *in vitro* studies have demonstrated an inhibitory effect of GnIH on reproduction in ewes, where the peripheral administration of GnIH decreased the amplitude of LH pulses, while *in vitro* GnIH decreased the secretion of LH and FSH (6).

In addition, it is notable that the RFRP/NPFF1R system has been shown to be expressed in the gonads of primates; thus, indirectly suggesting that GnIH/RFRP might exert a direct role in the control of gonadal physiology. In this sense, treatment of human granulosa-lutein cells with RFRP-3 reduced FSH, LH,

and forskolin-stimulated progesterone secretion (41). To our knowledge, the effects of GnIH/RFRP on the secretory profiles of gonadotropins in primates (including humans) have not been reported to date.

ANALYSIS OF GnIH/RFRP ROLES ON THE HPG AXIS USING ANTAGONISTS: STUDIES WITH RF9

As summarized in previous sections, the available pharmacological data strongly suggest that RFRPs are involved in the regulation of the HPG axis in mammals, by delivering (predominantly) an inhibitory signal to the central elements of the gonadotropic axis. However, as also reviewed above, the integral analysis of the pharmacological data surfaces some inconsistencies and differences, which have fueled the debate on the actual physiological relevance, relative importance (vs. other regulatory systems) and major sites of action of this neuropeptide system in the control of gonadotropin secretion in mammals (25). To some extent, this is due to the fact that the study of the physiological actions of GnIH/RFRP has been mostly based on the use of indirect experimental approaches, involving expression analyses or the testing of pharmacological doses of exogenous agonists. By contrast, direct assessment of the roles of endogenous RFRPs in mammals has been hampered by the lack of potent and selective antagonists of RFRP signaling.

In this context, in 2006, the compound RF9 was reported as a potent and selective antagonist of NPFF receptors, with binding affinity and antagonistic activity at the level of both NPFF1R and NPFF2R (42). Notably, initial pharmacological characterization of this compound focused on the analyses of NPFF-mediated events; it was shown that RF9 effectively blocks the effects of NPFF on heart rate and blood pressure, and it was capable to prevent opioid-induced hyperalgesia and tolerance in rats, phenomena that are presumably mediated via NPFF2R (42). However, it was not until 2010 when the first study addressing the impact of RF9-mediated RFRPR blockade, as putative receptor pathway for mediating GnIH/RFRP actions, on gonadotropin secretion in rodents was published by our group (43).

The effects of RF9 on gonadotropin secretion were initially explored in cycling female rats. Intracerebral injection of RF9 evoked robust LH secretory responses in cycling rats at the two stages of the cycle tested, estrus and diestrus-1 (D-1), with peak values at 30 min and persistently elevated LH levels during the 120-min period after RF9 injection. The testing of a wide range of doses of RF9 (10 and 100 pmol, and 1, 5, and 20 nmol; icv injection) demonstrated consistent stimulation for doses of RF9 from 5 nmol onwards (43). Similar dose–response curves were generated in adult male rats after icv injection of different doses of RF9. Consistent stimulation of LH secretion was detected in male rats from doses of 1 nmol/icv onward. In addition, RF9 elicited significant elevations of circulating FSH levels in cycling females at estrus, but not at D-1; moreover, only the dose of 20 nmol RF9 icv was capable of eliciting unambiguous FSH responses in male rats (43).

In mice, RF9 elicited robust LH secretory peaks at 15 min after its icv injection, which represented >20-fold increase over basal levels; LH responses to RF9 in mice were similar in amplitude between males and females (43, 44). However, central injection of RF9 failed to evoke significant FSH responses in female mice, whereas it elicited a modest 35% increase in serum FSH levels in adult males. In addition, RF9 blocked the inhibitory effects of NPFF on GnRH neuron pacemaker activity (45).

The potential interplay of GnIH/RFRP with sex steroid levels and the functional status of the HPG axis in the control of gonadotropin secretion have been pharmacologically explored also in various species using RF9. Thus, iv administration of RF9 increased plasma LH levels in orchidectomized (ORX) male rats, but this stimulatory effect was completely blunted after blockade of GnRH actions (46). On the other hand, the stimulatory effects of RF9 on gonadotropin secretion were detected despite the prevailing suppression of gonadotropin levels by testosterone or estradiol. In fact, central administration of RF9-induced extraordinarily potent *in vivo* LH responses in ORX mice receiving a fixed dose of testosterone, while RF9 reversed the inhibitory effects of testosterone on GnRH release frequency from brain slices in vitro (47). In turn, blockade of estrogen receptor-α partially attenuated gonadotropin responses to RF9 in rats (43). In other species, like the ewe, icv injection of RF9 during anestrus or the breeding season caused a clear elevation of plasma LH levels, with a more pronounced effect during the anestrous season (48). Furthermore, peripheral administration of RF9 as a bolus or as a constant iv infusion to anestrous ewes induced a sustained increase in plasma LH levels (48). A summary of the reported effects of RF9 in different mammalian species is presented in **Table 3**.

Despite the fact that the above pharmacological evidence is suggestive of a tonic suppression of gonadotropin tone by GnIH/RFRP, as blockade of this signaling pathway by RF9 causes a robust increase in LH (and to a lesser extent, FSH) levels, recent doubts have been raised about the interpretation of hormonal studies using this GnIH/RFRP antagonist. A call of caution for interpretation of the gonadotropic effects of RF9 was made already in initial publications, given the capacity of RF9 to block both NPFF1R and NPFF2R. Yet, the function of RF9, as putative selective antagonist of NPFFRs, together with the conspicuous lack of other NPFF1R antagonist, made RF9 an appealing candidate for neuroendocrine studies.

These doubts have recently substantiated in the context of analyses of the potential interplay of GnIH/RFRP and kisspeptins using RF9 as pharmacological probe. Initial studies demonstrated that co-injection of RF9 and kisspeptin-10 (Kp-10) resulted in elevated LH levels that were similar to those observed after icv administration of RF9 alone, except for a longer duration of those evoked by the combined administration of RF9 and Kp-10 (43). In fact, co-administration of RF9 and Kp-10 elicited FSH secretory responses that were not statistically different from those of RF9. To further explore the putative interplay between GnIH/RFRP and kisspeptins, the effects of RF9 on gonadotropin secretion were evaluated in Gpr54 KO mice, which are genetically engineered to lack kisspeptin signaling. While persistent LH responses to RF9 were observed in the absence of functional kisspeptin receptors, the absolute magnitude of such responses was severely blunted, as it was about one-tenth of that observed in WT animals (44).

TABLE 3 | Summary of the reported effects of RF9 administration on the HPG axis in mammals.

Species	RF9	Reference
Rat	Intravenous treatment with RF9 increased circulating LH levels in ORX male rats, but failed to evoke LH secretion after blockade of GnRH actions	(42)
	Central administration of RF9 evoked a dose-dependent increase of LH and FSH levels in adult male and female rats	
	Administration of RF9 further augmented the gonadotropin-releasing effects of kisspeptin (duration of responses), and its stimulatory effects were detected despite prevailing suppression of gonadotropin secretion by testosterone or estradiol	(43)
	In males, systemic administration of RF9 modestly stimulated LH secretion *in vivo* and had no direct effects in terms of gonadotropin secretion by the pituitary *in vitro*	
	Co-administration of the kisspeptin antagonist, p234, blunted LH responses to RF9	(44)
Mouse	Central (icv) injection of RF9 elicited with robust LH secretory responses in mice	(43)
	LH responses to RF9 were severely blunted in Gpr54 KO mice, with absolute magnitudes that were only one-tenth of WT mice	(44)
	Central (icv) administration of RF9 induced potent LH responses in ORX mice getting a fixed dose of testosterone, but these were absent in ORX Gpr54 KO mice with similar testosterone replacement	
	RF9 blocked the inhibitory effects of NPFF on GnRH neuron pacemaker activity and reversed the inhibitory effects of testosterone on GnRH secretory frequency	(45)
	Central (icv) administration of RF9 evoked very potent LH secretory responses in mice genetically devoid of NPFF1R	(51)
Sheep	Central (icv) and peripheral administration of RF9 induced significant increases in LH plasma concentrations in the ewe, especially in the anestrous season	(48)

Moreover, LH responses to RF9 were totally suppressed in ORX Gpr54 KO mice receiving testosterone implants (44). Altogether, these data strongly suggest that a preserved kisspeptin signaling is essential for the manifestation of the potent gonadotropin-releasing effects of RF9. Along with this view, it was recently demonstrated that the excitatory effects of RF9 on GnRH neuronal firing do not occur in Gpr54 KO mice (49). Furthermore, it has been very recently shown that pharmacological blockade of kisspeptin receptors, by the use of the antagonist p234, blunted RF9-induced LH secretion in female rats (50). Admittedly, however, these observations do not necessarily exclude the possibility that the lowering of inhibitory actions of GnIH/RFRP, eventually caused by RF9, might need preserved kisspeptin inputs to translate into detectable LH secretory responses.

Yet, very recent studies, combining *in vitro* and *in vivo* analyses, further support a primary action of RF9 directly via Gpr54 (51). Thus, analyses using CHO cells stably expressing Gpr54 have demonstrated the capacity of RF9 to bind the kisspeptin receptor and to activate its canonical intracellular signaling cascade, including increases in intracellular calcium and inositol phosphate, as well as ERK phosphorylation (51). In good agreement, our studies *in vivo* have demonstrated that RF9 evokes very potent LH-releasing responses in a mouse line genetically devoid of NPFF1R, the Npff1r$^{-/-}$ mouse (see Analysis of the GnIH/RFRP Roles in the HPG Axis Using Functional Genomics: *the Npff1r$^{-/-}$ Mouse*). Moreover, while LH responses to RF9 were severely blunted in Gpr54 null mice, the stimulatory effects of RF9 were rescued by selective re-expression of Gpr54 in GnRH neurons (51). Altogether, these observations strongly suggest that at least a substantial component of the secretory effects of RF9 on gonadotropin secretion stems from its capacity to activate Gpr54, rather than its blocking effects on NPFF1R.

As final note to this section, a very recent study has reported the identification and pharmacological characterization of a novel NPFFR antagonist, termed GJ14, with improved specificity in terms of receptor interaction and blockade (52). Notably, this study has documented that infusion of GJ14 effectively blocked the anxiogenic and corticosterone-stimulatory effects of RFRP-3 in mice (52). To our knowledge, the impact of this novel antagonist on the function of the HPG axis has not been reported so far. Similarly, additional *in vivo* studies are warranted to further evaluate the specificity of GJ14 and its eventual (lack of) interaction with other central regulators of GnRH/gonadotropin secretion.

ANALYSIS OF THE GnIH/RFRP ROLES IN THE HPG AXIS USING FUNCTIONAL GENOMICS: *THE Npff1r$^{-/-}$ MOUSE*

As summarized in previous sections, compelling pharmacological evidence suggests that GnIH/RFRP plays a role in the inhibitory control of the HPG axis in mammals. However, the actual physiological relevance of this system remained incompletely defined, mainly because of conflictive results concerning the nature, magnitude, and major sites of action of RFRP-3 in the control of gonadotropin secretion. To some extent, these uncertainties were due to the lack of appropriate experimental models and investigative tools to address the physiological functions of GnIH/RFRP *in vivo*. A major advancement in this area, however, took place recently, when the reproductive characterization of the first mouse line with genetic inactivation of NPFF1R was published by our group (38). This piece of work, which analyzed the impact of congenital ablation of NPFF1R on fecundity, litter size, puberty, adult gonadotropic function, gonadal feedback, and NPFF/Kiss1 interactions, is considered relevant to ascertain the relative importance of this system in the control of reproductive function in mammals (38).

As expected, mice deficient for NPFF1R did not respond to icv administration of RFRP-3 (38), in contrast to the preserved inhibitory responses observed in WT mice, where RFRP-3 suppressed LH secretion, in keeping with previous literature (15). In addition, while pituitary explants from WT male mice responded to RFRP-3 with a significant suppression of LH secretion *in vitro*, this inhibitory response was not detected in the pituitaries from Npff1r$^{-/-}$ animals (38). The latter data evidence that RFRP-3 can partially act directly at the pituitary level to suppress LH secretion

in mice. Interestingly, whereas pituitaries from both WT and NPFF1R KO mice responded to GnRH with robust LH secretory responses *in vitro*, the magnitude of such responses was (modestly) higher in NPFF1R KO mice, suggesting that null animals are devoid of inhibitory mechanisms that restrain stimulated LH secretion (38).

In keeping with the presumable role of GnIH/RFRP as inhibitor of gonadotropin secretion, NPFF1R-deficient male and female mice had preserved fertility. In fact, the mean size of litters from NPFF1R KO pairs was significantly higher than that of WT breeders (38). Pubertal analyses of null animals evidenced that KO males, but not females, displayed constitutively elevated LH levels before and during puberty, whereas FSH levels were similar between genotypes during the pubertal transition. Pubertal progression was not apparently altered by the congenital lack of NPFF1R, as evidenced by similar mean ages of occurrence of external signs of puberty: balano-preputial separation in males and vaginal opening in females. In addition, testicular and ovarian maturation, as well as ovulatory dynamics, were similar in WT and KO mice, and NPFF1R KO female mice showed preserved estrous cyclicity and pre-ovulatory LH surges of similar magnitude as in WT mice (38).

In addition, the functionality of the gonadotropic axis in the absence of GnIH/RFRP signaling was further explored by studying LH responses to three major regulators of gonadotropin secretion, namely GnRH, Kp-10, and senktide, in NPFF1R null male mice. Of note, senktide is an agonist of neurokinin B (NKB), which has been shown to be co-expressed in a subset of Kiss1 neurons, located in the ARC, where NKB would operate as activator of kisspeptin release onto GnRH neurons (2). Systemic [intra-peritoneal (ip)] injection of GnRH elicited enhanced LH responses in KO mice, in good agreement with the responses observed in the incubations of pituitary explants. In turn, central administration of Kp-10 induced an increase in LH levels similar in both genotypes, while activation of NKB signaling induced lower LH responses in NPFF1R KO mice compared to WT animals. Such differences in the patterns of response to these factors (GnRH, Kp-10, NKB) are probably due to the differences in their sites of action as GnRH acts at the pituitary whereas NKB operates mainly at the level of ARC Kiss1 neurons, and how these sites are differentially affected by the lack of GnIH/RFRP actions (38). In addition, the analysis by *in situ* hybridization of the levels of Kiss1 mRNA in the two major hypothalamic areas of expression (the ARC and the anteroventral periventricular nucleus, i.e., AVPV) in NPFF1R KO mice showed that the overall expression level of Kiss1 and the number of Kiss1 neurons were significantly higher in the ARC, but not in the AVPV, of NPFF1R KO animals, suggesting that RFRP signaling carries out a tonic repression of Kiss1 gene expression at this site (38). Of note, it has been reported that approximately one-forth of ARC Kiss1 neurons actually co-express NPFF1R, while only a very small fraction of AVPV Kiss1 neurons expressed NPFF1R or NPFF2R (53).

The putative interplay between GnIH/RFRP and kisspeptin was further explored by the generation of a double KO mouse, where both inhibitory (NPFF1R) and stimulatory (Gpr54) receptors were genetically ablated. With this approach, we aimed to explore whether elimination of the inhibitory signal driven by RFRP might (at least partially) compensate the profound hypogonadotropic hypogonadism induced by the lack of kisspeptin signaling. However, analysis of this double NPFF1R/Gpr54 KO mouse line revealed that the absence of RFRP signaling was not sufficient to rescue nor did it improve the severe gonadal failure of Gpr54 KO mice, as this double mutant line showed remarkable phenotypic and hormonal similarities with single Gpr54 null mice (38). These findings strongly suggest that, despite some interplay between kisspeptin and GnIH/RFRP signaling, kisspeptin function clearly predominates in the central control of the HPG axis.

Finally, the roles of GnIH/RFRP in the negative feedback control of gonadotropin secretion were also explored using Npff1r$^{-/-}$ mice. To this end, protocols of GNX were applied to male and female WT and KO mice. Removal of sex steroids revealed a delay in LH responses to the removal of gonadal steroids in the mutant mice of both sexes. However, in longer term, the absence of the NPFF1R caused sexually dimorphic alterations in post-GNX responses: while in female mutant mice, circulating levels of LH were significantly higher than those in WT from 7-days post OVX onward; in male mice, LH responses were normalized 7-days after ORX, and remained similar to those of WT from that period onward. These data suggest that in females, but apparently not in males, the GnIH/RFRP system operates as (moderate) brake to modulate the increase in circulating LH levels following the removal of negative feedback signals from the gonads (38).

In sum, functional genomic approaches have allowed to characterize the neuroendocrine (gonadotropic) impact of the constitutive lack of GnIH/RFRP signaling, thereby providing a direct assessment of the relative importance of this system in the control of the HPG axis. These analyses revealed alterations, such as increased litter size, increased gonadotropin levels at certain developmental stages, increased Kiss1 expression in the ARC, and altered post-OXV responses in the Npff1r$^{-/-}$ mouse, which are in general compatible with an inhibitory role of GnIH/RFRP signaling in the control of reproductive function. It must be stressed, however, that most of these changes were moderate in nature and subordinated to preserved kisspeptin signaling (38). Yet, the possibility exists that developmental compensation (e.g., via NPFF2R, whose expression is preserved in this KO model) might have masked to some extent the phenotypic impact of the lack of NPFF1R signaling.

GnIH/RFRP AS PUTATIVE LINK FOR THE INTEGRAL CONTROL OF REPRODUCTION AND METABOLISM

The existence of a close relationship between energy balance and reproduction is well established and there is compelling evidence that forms of metabolic stress, such as conditions of negative energy balance, result in the inhibition of reproductive function. Similarly, alterations of the HPG axis may be linked to metabolic perturbations (54). Despite this evidence, the basic mechanism by which this reciprocal control occurs remains partially unknown. This has prompted the analysis of the metabolic effects of different factors primarily involved in the control of reproductive function and vice versa.

In this context, evidence has been recently presented supporting a potential role of GnIH/RFRP as a potential link between reproductive and metabolic homeostasis. Thus, central (icv) administration of RFRP-3 has been shown to stimulate food intake in adult male rats (55). In turn, it has been reported that RF9 decreased food intake without affecting body weight in female rats (50); yet, the possibility exists that RF9 might evoke such anorectic responses acting via Gpr54, as kisspeptin has been shown to suppress feeding as well (56). However, microinjections of RFRP-3 in the central part of amygdala resulted in a significant decrease of food intake in rats; an effect that was eliminated after RF9 pre-treatment (57). All in all, the pharmacological data available suggest a predominant orexigenic role of RFRP-3, supported also by data obtained in sheep (55); yet, at some brain areas, GnIH/RFRP signaling might evoke food-suppressing responses. Assuming that orexigenic factors are activated in conditions of negative energy balance (as to promote food seeking), it is tenable that GnIH/RFRP might contribute to the suppression of gonadotropic function observed in condition of energy deficit (55).

This possibility has been recently addressed using the Npff1r$^{-/-}$ mouse and protocols of short-term fasting as form of metabolic stress (38). While in WT animals, food deprivation caused a significant lowering of LH levels already at 12-h after beginning of fasting, such a rapid drop of LH was not detected in NPFF1R KO mice subjected to a similar fasting regimen, despite the lowering of body weight was proportionally higher than that in WT animals (38). This observation suggests that activation of GnIH/RFRP signaling might contribute to the suppression of gonadotropin levels observed in conditions of negative energy balance. Indeed, previous studies have shown an increase in the activity of RFRP-positive neurons during periods of undernutrition in hamsters (58). Interestingly, NPFF1R null mice displayed also partially preserved gonadotropic function in response to other forms of metabolic stress linked to reproductive alterations, such as diet-induced obesity. Thus, feeding with high-fat diet (HFD) for 9 weeks evoked >40% weight gain in WT mice, which was associated with a significant decrease in circulating LH levels. By contrast, in NPFF1R KO mice, HFD feeding did not induce significant changes in circulating LH levels, in spite of a similar weight gain as in WT mice (38). Overall, these observations suggest that the mechanisms by which extreme metabolic conditions inhibit gonadotropin secretion probably involve changes in signaling by GnIH/RFRP, such as an increase of the inhibitory tone by RFRP.

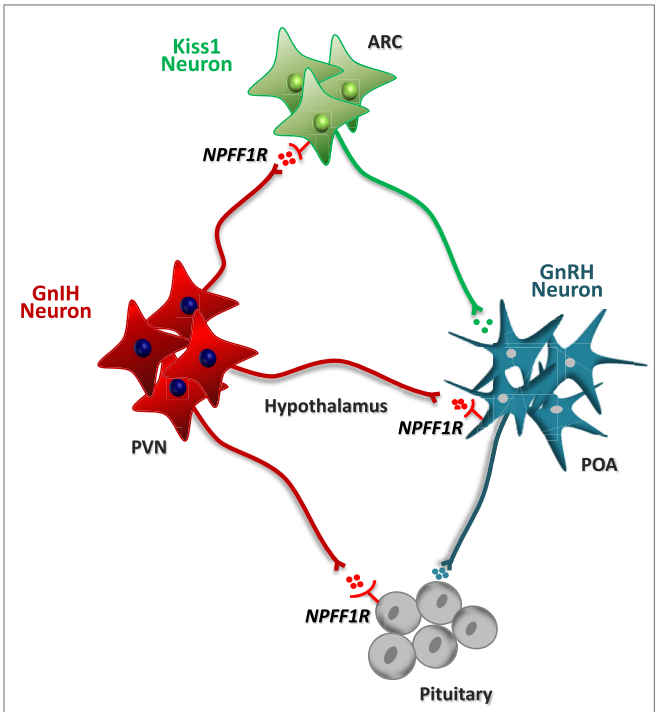

FIGURE 2 | Schematic diagram of the putative interplay of neurons expressing GnIH/RFRP with key elements of the HPG axis, including pituitary gonadotrophs and hypothalamic GnRH and Kiss1 neurons. A prominent population of GnIH neurons has been identified in the paraventricular nucleus (PVN). On the basis of neuroanatomical studies (e.g., on neuronal projections) and expression analyses (e.g., of receptors), different sites of action of the GnIH/RFRP system have been proposed, as depicted in the scheme. Admittedly, however, the relative importance of the different sites of action might vary between mammalian species. Alike, direct gonadal effects of GnIH/RFRP (not depicted in this scheme) have been proposed also on the basis of experimental data.

CONCLUDING REMARKS

Identification of GnIH in birds, and of its mammalian orthologs, RFRPs, has opened up new avenues for our understanding of the central mechanisms for the control of the HPG axis. As illustrated in **Figure 2**, expression and functional studies strongly suggest that GnIH/RFRP signaling may play a role in the dynamic control of key elements of the HPG axis, including prominently pituitary gonadotrophs and hypothalamic GnRH and (eventually) Kiss1 neurons. However, while the pharmacological data gathered to date point to a predominant inhibitory effect of GnIH/RFRP on gonadotropin secretion across mammals, some controversy persists regarding the nature (stimulatory in some instances), major sites of action (hypothalamic vs. pituitary) and relative importance (as compared with other neuropeptides) of the GnIH/RFRP system in the control of the gonadotropic axis in different species. Alike, the interplay of RFRP with other central transmitters with key roles in the reproductive brain, such as kisspeptins, has been suggested, but further investigation is needed to fully expose the interactive partners and interdependence of GnIH/RFRP with key central and peripheral regulators of the HPG axis. In this review, we intended to provide a succinct view of the state-of-the-art of the field, by summarizing recent pharmacological data and studies using genetically modified models. By doing so, we aimed to define what we know, and we do not know, about the physiology of GnIH in mammals, as a means to set the scene for further research in this exciting and rapidly evolving area of Neuroendocrinology.

AUTHOR CONTRIBUTIONS

In collaboration with the other co-author, this author reviewed the literature, wrote the review, and prepared/edited the Tables and Figures.

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Regulation and function of RFRP-3 (GnIH) neurons during postnatal development

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RFamide-related peptide-3 (RFRP-3) [mammalian ortholog to gonadotropin-inhibiting hormone (GnIH)] potently inhibits gonadotropin secretion in mammals. Studies of RFRP-3 immunoreactivity and *Rfrp* expression (the gene encoding RFRP-3) in mammalian brains have uncovered several possible pathways regulating RFRP-3 neurons, shedding light on their potential role in reproduction and other processes, and pharmacological studies have probed the target sites of RFRP-3 action. Despite this, there is currently no major consensus on RFRP-3's specific endogenous role(s) in reproductive physiology. Here, we discuss the latest evidence relating to RFRP-3 neuron regulation and function during development and sexual maturation, focusing on rodents. We highlight significant changes in RFRP-3 and *Rfrp* expression, as well as RFRP-3 neuronal activation, during key stages of postnatal and pubertal development and also discuss recent evidence testing the requisite role of RFRP-3 receptors for normal pubertal timing and developmental LH secretion. Interestingly, some findings suggest that endogenous RFRP-3 signaling may not be necessary for the puberty timing, at least in some species, forcing new hypotheses to be generated regarding this peptide's functional significance to sexual maturation and development.

Keywords: RFRP-3, GnIH, development, Gpr147, Rfrp, reproduction, puberty

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Introduction

Since their identification 15 years ago, both gonadotropin-inhibiting hormone (GnIH) and its mammalian ortholog, RFamide-related peptide (RFRP-3), have become important research focuses of neuroendocrinologists and reproductive biologists. When given exogenously, both peptides potently inhibit the hypothalamic–pituitary–gonadal axis, and numerous studies have studied the *in vivo* and *in vitro* effects of GnIH and RFRP-3 on luteinizing hormone (LH) secretion (1–6). In rodents, as with most mammals, the neurons that produce RFRP-3 are a scattered population localized exclusively within and immediately adjacent to the hypothalamic dorsal-medial nucleus (DMN). This was first characterized by *in situ* hybridization (ISH) for *Rfrp* mRNA (7) and confirmed by immunohistochemistry with RFRP-3 or GnIH antibodies (2, 8).

In rodents, the primary focus of this review, RFRP-3 is thought to regulate LH secretion through inhibition of gonadotropin-releasing hormone (GnRH) neurons rather than by direct action on the pituitary. This model is supported by data, collected primarily in adult animals, showing that RFRP-3 neural fibers appose GnRH neurons (2, 9, 10), RFRP-3's high affinity receptor, Gpr147, is expressed in some GnRH neurons (11), GnRH neuron electrical firing changes when RFRP-3 is applied to hypothalamic explants (4, 5), and RFRP-3 treatment suppresses LH secretion in a

GnRH-dependent manner (10). RFRP-3 may also regulate LH secretion through additional indirect circuits in the brain, as only a subset of GnRH neurons in rodents actually express *Gpr147* mRNA (11). For example, RFRP-3 signaling may modulate the activity of arcuate kisspeptin neurons, which also express the Gpr147 receptor (12), or other upstream neuronal populations. Interestingly, there are two reports of RFRP-3 stimulating LH secretion in hamster species (13, 14), suggesting that in seasonal rodents, RFRP-3 may have stimulatory and inhibitory roles in reproduction. Similar stimulatory effects of RFRP-3 have not been reported in mice or rats in adulthood or development.

How do RFRP-3 Immunoreactivity and *Rfrp* mRNA Levels Change in the Brain During Development?

RFamide-related peptide immunoreactivity and *Rfrp* mRNA are first detectable in the rat hypothalamus on embryonic day 16 (E16) or E17 (15). Using BrdU labeling to mark neurogenesis, we know that RFRP-3 immunoreactive neurons are born as early as E12, with most RFRP-3 neurons born on E13-E14 (16), consistent with the neurogenesis of the DMN region itself (17). There are minimal detectable RFRP-3 projections during the embryonic stage, as RFRP-3 fibers are nearly absent before or at birth (15). However, by the second and third postnatal weeks, RFRP-3 immunoreactive fibers are clearly visible in some proximal hypothalamic nuclei, such as the arcuate, lateral hypothalamic area, and paraventricular nuclei, as well as non-hypothalamic sites, such as the thalamus and midbrain (15). RFRP-3 fibers do not reach some of their more distal targets, such as the spinal cord, until after puberty is complete (15).

The first two studies to examine postnatal developmental changes in neural *Rfrp* mRNA in rodents used qPCR on either hypothalamic dissections or micropunches. Substantial increases in *Rfrp* mRNA between neonatal/juvenile and peripubertal rats were observed in both sexes (18, 19). However, Iwasa's study showed a significant decrease in *Rfrp* expression after puberty in male, but not female, rats, whereas Quennell and colleagues reported no significant decrease for either sex between peripubertal ages and adults.

Our lab also examined *Rfrp* expression over development by measuring *Rfrp* levels in newborn and adult mice by ISH. We found that not only did *Rfrp* cell number change markedly over postnatal development, but the levels of *Rfrp* mRNA in each cell was dramatically different between the two ages. At birth, there were numerous *Rfrp* neurons detected, but in adulthood, the number of detectable neurons expressing *Rfrp* was notably less (**Figure 1**). However, the relative amount of *Rfrp* mRNA per neuron, indicated by the number of silver grains per individual

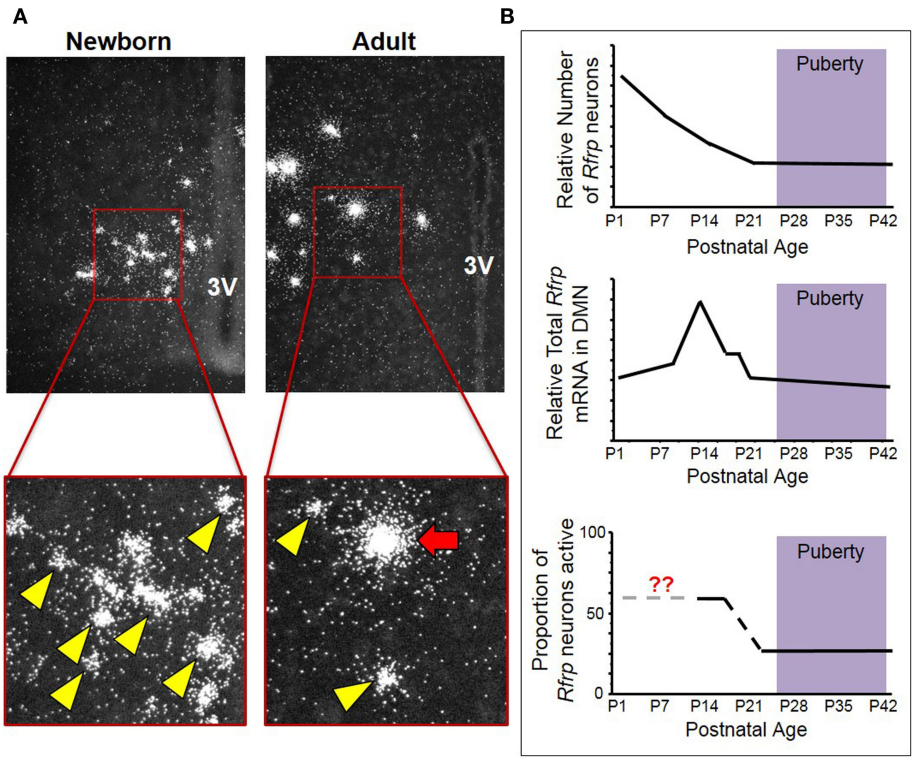

FIGURE 1 | Changes in neural *Rfrp* expression during postnatal development in mice. **(A)** Between the day of birth and adulthood, there are significant changes in the expression of certain subtypes of *Rfrp* neurons (categorized based on their *Rfrp* expression levels). Low-expressing (LE) *Rfrp* cells (yellow arrowheads) are the predominate subtype at birth whereas high-expressing (HE) *Rfrp* cells (red arrows) are first detectable during the 2nd week of postnatal life and remain present through adulthood. **(B)** Three graphic representations of *Rfrp* cell number, total *Rfrp* expression levels in the brain, and *Rfrp* neuronal activation (measured by c-Fos co-expression) during rodent postnatal development. P = postnatal day. The purple shading represents the approximate ages when puberty occurs.

cell cluster, was higher in adults. This dramatic change in *Rfrp* mRNA levels per cell demonstrated that while *Rfrp* cell number is *decreasing* during postnatal development, each *Rfrp* cell is a producing, on average, *more Rfrp* mRNA as the animal ages.

To further investigate this intriguing developmental change, ISH was performed for *Rfrp* mRNA at multiple postnatal ages in mice, and each individual *Rfrp* cell was categorized as either low expressing (LE) or high expressing (HE) based on the level of mRNA being expressed by that cell (11, 20). We found that the total number of detectable *Rfrp* cells was highest at birth and significantly dropped through all postnatal ages, with the fewest *Rfrp* neurons present in adulthood. This developmental pattern of *Rfrp* neurons was the same for both sexes (20). Interestingly, the total amount of *Rfrp* mRNA present in the DMN increased around PND 6 and peaked at PND 12 before dropping again at subsequent older ages. This robust juvenile increase in total *Rfrp* mRNA reflects a large developmental increase in the number of identifiable HE *Rfrp* cells, which substantially increase in prevalence and peak around PND 12 before dropping significantly in number at older pubertal and adult ages (20). Conversely, the number of LE *Rfrp* cells decreases slowly and steadily from birth to adulthood, being lowest in adulthood (20). Currently, the phenotypic and functional difference between HE and LE *Rfrp* cells is unknown. However, the HE cell population appears to be the more responsive of the two subtypes to physiological challenges, such as sex steroid treatments and leptin deficiency, as HE cells change more severely than the LE subpopulation (11, 20).

We also examined later postnatal ages in female mice around puberty, using a detailed day-to-day time course. The total number of *Rfrp* cells was found to decrease around PND20-21, around weaning and ~1 week before the onset of female puberty (21). However, this outcome differs from the previously published qRT-PCR rat experiments (18, 19), as neither of those studies showed a consistent decrease in *Rfrp* expression between prepubertal and adult animals. Additionally, the observed juvenile increase in total *Rfrp* expression in mice appears to occur later in development in rats, even when accounting for different developmental timelines between rats and mice. In mice, using ISH, *Rfrp* expression peaks in the second week of life and drops to adult-like levels by the end of third week of postnatal life, about a week before external markers of puberty are noticeable. In rats, *Rfrp* expression increases steadily during postnatal and pubertal development, and only drops to adult-like levels at ages after puberty is completed [note: a significant post-pubertal decrease was only observed in male rats (19)]. However, a newer experiment using qRT-PCR to measure *Rfrp* expression in brains of female mice produced a similar pattern as in our findings: total *Rfrp* expression increases in prepubertal mice then drops at the time of vaginal opening (22). We note that all values in that study were normalized to *Rfrp* expression on PND 24, which is after the age when most *Rfrp* gene expression changes are typically observed in mice in our ISH experiments. Furthermore, those data are from animals that were pooled based on the day they completed vaginal opening, rather than a specific postnatal day. While the implications of the discrepancies in these developmental profiles are currently unclear, the consistent finding is that there is a marked upregulation of RFRP-3 between birth and puberty in rodents. This conclusion is supported by both mRNA and protein data in several species, but the functional significance of this developmental change remains to be determined.

What Regulates Developmental Changes in Neural *Rfrp* Expression?

Few studies have moved beyond routine descriptive characterization of changes in RFRP-3 or *Rfrp* expression during postnatal life. One study questioned whether the majority of changes observed in *Rfrp* expression and cell number during postnatal development reflected neuronal apoptosis as a possible underlying mechanism (11). Using knockout mice deficient in BCL2-associated X protein (BAX, a major factor causing neuronal apoptosis), we found, in adult male mice, a small but significant increase in the total number of *Rfrp* cells present in the brain compared to WT mice. This higher number of overall *Rfrp* cells was primarily due to increased numbers of detectable LE *Rfrp* cells. However, while the adult BAX KO mice had more total *Rfrp* cells than WTs, the observed difference in cell number was not nearly as great as the difference seen between the day of birth and adulthood in normal WT mice. Thus, because adult BAX KO males did not have a newborn-like number of *Rfrp* neurons, it appears that BAX-mediated apoptosis is not solely responsible for the large overall decrease in cell number seen during development. Therefore, in addition to a minor effect of apoptosis via BAX, other regulatory factors or developmental processes must also be involved in modifying the temporal expression of *Rfrp* neurons throughout development.

Since neuronal apoptosis (via BAX) cannot completely explain the decrease in *Rfrp* neuron number during postnatal development, these neurons are likely undergoing regulatory changes in *Rfrp* expression that (1) decrease the *Rfrp* mRNA expression in some cells to a degree that makes them undetectable by ISH while (2) simultaneously increasing *Rfrp* expression in a subset of neurons, thereby generating the HE *Rfrp* cell population that is virtually absent at birth but which emerges in juvenile life. The mechanisms causing this maturation of the *Rfrp* system may be intrinsic to this neuropeptide cell population, but more likely, an extrinsic factor is acting on the *Rfrp* neuron population to dictate specific changes in gene expression in the various subtypes of *Rfrp* neurons.

A factor that was hypothesized to regulate this developmental change was the adipocyte hormone, leptin. Leptin is well known for its regulation of body weight by regulating feeding behavior and energy expenditure. Additionally, there are demonstrated developmental effects of leptin on hypothalamic neurite outgrowth during postnatal juvenile life. In rodents, during the second week of postnatal life, serum leptin levels transiently increase several fold for several days and then return to normal low levels by postnatal day 16 (23). This juvenile "leptin surge" has functional significance in the hypothalamus, regulating neurite outgrowth and projections from the arcuate nucleus to the DMN, where RFRP-3 neurons reside (24, 25). In regards to RFRP-3, we found that a small subset of *Rfrp* neurons express the long form leptin receptor mRNA, suggesting that leptin could potentially act directly in those neurons. Supporting this possibility, we detected impaired *Rfrp* expression in adult *Obese* (Ob) mice (20), which produce a non-functional leptin peptide, rendering them morbidly obese in adulthood and reproductively incompetent

(26, 27). Because there was a significant alteration of *Rfrp* mRNA expression in adult Ob mice, we tested whether *Rfrp* neuron maturation during juvenile life was dependent on proper leptin actions via the leptin surge. First, we measured serum leptin during postnatal development in normal female mice to determine if changes in leptin levels correlate with changes in *Rfrp* expression. We found that both leptin and *Rfrp* expression are similarly low at birth and the late neonatal period, and that both measures then start to rise in juvenile life. Serum leptin levels were highest just before and during the peak of *Rfrp* expression observed around postnatal day 12 (20), showing nice symmetry in the timing of developmental changes in these two measures. This experiment was followed up with an examination of *Rfrp* expression in postnatal Ob mice and their WT littermates. We hypothesized that leptin signaling is required, directly or indirectly, for the normal pattern of *Rfrp* neuron development in juveniles. However, despite the strong correlation between developmental *Rfrp* and leptin changes observed in normal mice, we found no differences between Ob and WT mice in any measure of *Rfrp* expression during postnatal development (20). Since leptin is not required for the developmental changes in *Rfrp* expression, other metabolic hormones, such as ghrelin or insulin (28–30), or reproductive hormones from maturing gonads (31–34), may be involved in driving the *Rfrp* developmental pattern during these postnatal ages.

What Role Does RFRP-3 Have in Peripubertal Life?

Given the dramatic developmental changes in *Rfrp* expression and neuronal activation during prepubertal and peripubertal life, it is possible that RFRP-3 regulates pubertal timing. Several investigators have used different methods to block RFRP-3's production or action to test this hypothesis. First, a small interfering RNA was designed to knockdown RFRP-3 in prepubertal male rats (35). After 2 weeks of treatment, the knockdown successfully decreased RFRP-3 immunoreactivity and increased serum LH. However, this treatment was surprisingly unable to alter the timing of pubertal development (35). Second, a Gpr147 KO mouse line was developed in order to test the necessity of RFRP-3 signaling for mouse reproductive function (36). Gpr147 is the primary G-protein coupled receptor for RFRP-3, as determined by receptor–ligand binding and functional assays (7, 37). Gpr147 KO mice were confirmed to be unresponsive to exogenous RFRP-3, showing no decrease in LH after central RFRP-3 injections. Yet, surprisingly, Gpr174 KO females had no significant advancement or delay in pubertal timing, as measured by vaginal opening. Collectively, these two independent findings suggest that endogenous RFRP-3, acting on Gpr147, does not have a major role in timing puberty onset, at least in rodents. However, estrous cyclicity was slightly impaired in adult Gpr147 KO mice, which had a small but significant decrease in the time spent in diestrous, suggesting RFRP-3 may still have meaningful roles in reproduction in adulthood.

Despite the findings above, it remains possible that RFRP-3 may still have a developmental role in controlling LH secretion in juvenile or prepubertal mice, but not robustly altering puberty timing. Indeed, the same study that demonstrated normal puberty in Gpr147 KOs reported that prepubertal Gpr147 KO males have higher LH levels than their WT counterparts, suggesting that endogenous RFRP-3 is important in modulating prepubertal LH secretion. Interestingly, this genotype difference was normalized after puberty, with both KOs and WTs having similar adult LH levels (36). Therefore, RFRP-3 may not have a critical role in the timing of puberty onset or duration, but may still act to suppress LH secretion prior to the pubertal period, which would match the developmental stage when neural *Rfrp* levels are highest. Most recently, our lab examined the neuronal activation of multiple reproductive neuropeptides during the prepubertal and peripebuteral periods in C57BL6 female mice (21). Of the three reproductive neuropeptide systems examined, kisspeptin, neurokinin B and RFRP-3, *Rfrp* neurons were the only population to demonstrate a significant change in neuronal activation during the peripubertal period. More specifically, *c-Fos* co-expression, which marks recently activated neurons, was significantly higher in *Rfrp* neurons on PND 15 than on PND 21, reflecting lower neuronal activation at the latter age. Afterward, throughout subsequent pubertal ages (~PND 22-30), *c-Fos* induction in *Rfrp* neurons remained consistent around 40% versus the higher levels of neuronal activation ~60% at the earlier prepubertal age (PND 15). From these data, we infer that RFRP-3 secretion and actions on the reproductive axis are decreased at PND 21 and

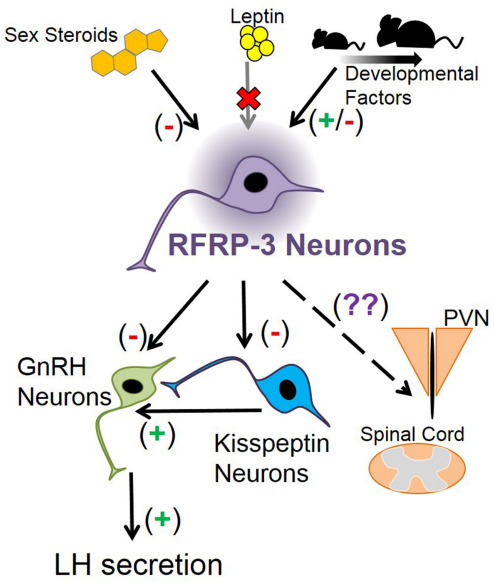

FIGURE 2 | Schematic depicting how *Rfrp* neurons may fit into mouse neuroanatomy and physiology. Sex steroids are known to inhibit *Rfrp* expression, primarily through estrogen pathways, while leptin appears to have no major direct effect on *Rfrp* neurons, at least in development. There appear to be other developmental factors that regulate the development of *Rfrp* expression which are both stimulatory and inhibitory that remain to be determined. RFRP-3 neurons have efferents that may regulate LH secretion by acting directly on GnRH neurons, or indirectly on GnRH neurons through arcuate kisspeptin neurons or other yet-to-be identified neuronal populations. Other RFRP-3 neuronal target areas include the paraventricular nucleus (PVN) and the spinal cord, and RFRP-3 has unknown effects on these non-reproductive regions.

throughout puberty relative to a stronger inhibitory tone that potentially exists on PND 15. Additionally, we speculate that at some point between PND 15 and PND 21, there are either changes in the upstream stimulatory input onto RFRP-3 neurons or that the RFRP-3 neurons are intrinsically changing their activity and secretion by an undetermined mechanism.

Collectively, the above experiments suggest that RFRP-3 is unlikely to be a major player in substantially impacting the timing of pubertal onset or progression because (1) knocking down RFRP-3 production during puberty has no effect on pubertal time, (2) Gpr147 KO mice have normal timing of vaginal opening, and (3) any notable decrease in *Rfrp* gene expression or neuronal activation occurs multiple days, if not longer, before external markers of puberty are evidenced. Nonetheless, RFRP-3 may still prove to have role in restraining the reproductive axis before puberty can start or proceed. A recently published report demonstrated that RFRP-3 is able to suppress LH secretion in prepubertal female mice, but only in the presence of estradiol (22). It currently remains unclear why RFRP-3 had no stimulatory effect in the absence of estrogen. Regardless, this is the first report to examine the effect of RFRP-3 on LH secretion in prepubertal mice, and confirms the assumption that RFRP-3 can have bioactivity at these younger non-adult ages. Within the context of dramatic developmental changes in *Rfrp* expression and RFRP-3 neuronal firing discussed above, these new data suggest that RFRP-3 may have a role in maintaining the reproductive axis in a prepubertal quiescent state until other pubertal regulators initiate puberty.

Conclusion

RFamide-related peptide-3 suppresses LH secretion when given exogenously. However, additional exploration into RFRP-3's functional position in the mammalian reproductive axis has been limited. RFRP-3 neurons have distinct and quantifiable changes throughout development, hinting at a role in regulating puberty, but this was not corroborated by knockout and knockdown studies. Thus, RFRP-3's developmental function is not immediately clear (**Figure 2**). Knockout mouse studies have shown that RFRP-3 neuron development is not strongly regulated by BAX-mediated apoptosis or by leptin, opening the possibility of other novel mechanisms that may influence the maturation of RFRP-3 neurons (**Figure 2**). Thus, RFRP-3's specific developmental role is still being elucidated, leaving multiple avenues to explore. How does RFRP-3 regulate LH secretion in prepubertal animals, and why is it influenced by estrogen? Why does RFRP-3 neuron activation decrease after weaning? Why do some *Rfrp* neurons express high levels of *Rfrp* mRNA while others do not? Is there a functional significance to the robust *Rfrp* changes between birth and juvenile life and then again before adulthood? Answering these questions will further identify the role of RFRP-3 in development and reproduction in general.

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The Role of RFamide-Related Peptide-3 in Age-Related Reproductive Decline in Female Rats

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Reproductive senescence, the point in time when females cease to show estrous cyclicity, is associated with endocrine changes in the hypothalamus, pituitary, and gonads. However, the mechanisms triggering this transition are not well understood. To gain a better understanding of the top-down control of the transition from reproductive competence to a state of reproductive senescence, we investigated middle-aged female rats exhibiting varying degrees of reproductive decline, including individuals with normal cycles, irregular cycles, and complete cessation of cycles. We identified hormonal changes in the brain that manifest before ovarian cycles exhibit any deterioration. We found that females exhibit an increase in RFamide-related peptide-3 (RFRP3) mRNA expression in the hypothalamus in middle age prior to changes in estrous cycle length. This increase is transient and followed by subsequent decreases in kisspeptin (*KiSS1*) and gonadotropin-releasing hormone (*GnRH*) mRNA expression. Expression of *RFRP3* and its receptor also increased locally in the ovaries with advancing age. While it is well known that aging is associated with decreased GnRH release and downstream disruption of the hypothalamic–pituitary–gonadal (HPG) axis, herein, we provide evidence that reproductive senescence is likely triggered by alterations in a network of regulatory neuropeptides upstream of the GnRH system.

Keywords: GnIH, RFRP3, GnIH/RFRP3, aging, reproductive senescence

INTRODUCTION

Female reproduction is a complex process that requires precise neurochemical timing. From puberty through senescence, a complex interplay among central neuroendocrine circuits, peripheral hormonal systems, and internal and external cues is required for successful procreation. The hormonal regulation that is involved in successful reproductive functioning is well studied. However, less is known about the neural and hormonal mechanisms initiating the degradation of the reproductive axis as females approach reproductive senescence.

Reproductive senescence in females is defined as the point in an organism's life when it ceases to show regular cyclic ovarian activity, exiting from an active reproductive stage to an inactive reproductive life, akin to menopause in humans. In humans, menopause is characterized as three stages – premenopausal, when females still maintain regular menstrual cycles; perimenopausal,

when cycles become increasingly irregular; and postmenopausal, defined as 1 year post amenorrhea (1) – and is associated with specific endocrine changes in both the brain and the ovaries (2–4). Rats represent a useful, non-human model for the study of menopause as they experience reproductive decline analogous to that of humans. With advancing age, middle-aged rats transition from regular 4- to 5-day estrous cycles to irregular cycle lengths of over 6 days, followed by a complete cessation of cyclicity, characterized as either a state of persistent estrus or diestrus. This final transition occurs in female rats between 12 and 15 months of age (5), appropriately corresponding to about 45–50 years old in women (4). The hypothalamic–pituitary–gonadal (HPG) axis is highly conserved across mammals, and the hypothalamic and ovarian changes in the rodent resemble menopausal changes in humans (6).

In female rodents, ovulation requires the precisely timed release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. In rats, the transition to reproductive senescence is associated with reductions in GnRH neuron activity (7, 8) and secretion (9, 10) on the day of the preovulatory luteinizing hormone (LH) surge, resulting in an attenuation of LH and ovulation (10, 11). These changes are likely due to a modification in the network of upstream hypothalamic mediators of GnRH function. In particular, two neuropeptides identified in the past 15 years have been shown to regulate GnRH in opposing ways. Kisspeptin (the product of the *KiSS1* gene) found in the anteroventral periventricular (AVPV) and arcuate nuclei stimulates GnRH release, whereas RFamide-related peptide-3 (RFRP3) found in the dorsal medial hypothalamus of rodents acts to inhibit GnRH release (12–14). Kisspeptin mRNA levels decrease with aging (15), along with a decrease in the number of immunoreactive kisspeptin neurons in the AVPV in middle age (16). In contrast, the role of RFRP3 in female reproductive aging is yet to be discovered.

In this study, we examined *RFRP3* expression in regularly cycling young (3 months old), regularly cycling middle aged (8 months old), irregularly cycling (10 months old), and acyclic (12 months old) Long–Evans female rats. To explore the possibility that alterations in neurochemical systems upstream of GnRH neurons initiate the transition to reproductive senescence, we measured *RFRP3*; its receptors *GPR147*, *KiSS1*, and *GnRH*; and pituitary gonadotropin mRNA expression throughout aging. We found that *RFRP3* expression increased in middle-aged animals along with reductions in *KiSS1* and *GnRH* expression with advancing age. Expression of *RFRP3* and *GPR147* also increased with age in the ovaries. These data reveal an association between alterations in the RFRP3 and kisspeptin systems with age-related reproductive decline both centrally and peripherally.

MATERIALS AND METHODS

Experimental Subjects

Adult female Long–Evans rats were housed in trios and exposed to a 12/12-h light–dark cycle. Lights came on at 0700 hours and *ad libitum* food and water were available. For all studies, rats were acclimated to their housing conditions for a week, and then vaginal smears were obtained daily to determine cyclicity for 30 days before the studies commenced. All tissue was collected between the hours of 0900 and 1200 hours. In the irregularly cycling group, only females that showed irregular cycles for at least 15 of the 30 days (cycles above 6 days in length) were used in the 10-month-old group ($n = 21$ of a total of 30 animals measured). For the acyclic group, only 12-month-old animals that exhibited persistent estrus or diestrus for at least 15 of the 30 days were used ($n = 12$ of a total of 18 animals measured). "Young" control animals were measured in the diestrus phase of the estrous cycle. All animal care and procedures were approved by the University of California Berkeley Animal Care and Use Committee.

Real-time Reverse Transcriptase PCR

Rats were lightly anesthetized with isoflurane and rapidly decapitated before bilateral hypothalami (dissected from bregma −2.5 to bregma −3.5), pituitary, and whole ovaries were dissected on ice in RNA-later (Thermo Fisher Scientific, Santa Clara, CA, USA) and flash-frozen in liquid nitrogen. For all studies, total RNA was extracted using Trizol extraction methods and purified with DNase (DNA-free, Ambion). cDNA was synthesized following the manufacturer's instructions for iScript cDNA synthesis kit (BioRad), and then RT-PCR was run using the manufacturer's instructions for SsoAdvanced SYBR supermix (BioRad). Samples were run in a BioRad CFX96 real-time PCR system. After the PCR was complete, specificity of each primer pair was confirmed using melt curve analysis, and samples run on a 2% ethidium bromide agarose gel with a 50-bp DNA ladder (Invitrogen) to verify the generation of a single product of correct size. Rat primers were designed using the NCBI Primer BLAST software, which verifies specificity. The primer efficiencies were determined by standard curve, and C_t values were determined using PCR miner (17) and normalized to the ribosomal reference gene, ribosomal protein L16P (RPLP). There were no significant differences in RPLP values across any groups. Fold change was evaluated using the delta delta C_T method as outlined in Pfaffl 2001 (18).

Primer sequences are given below.

Primer	Forward	Reverse	Temp	Product size
RPLP	ATCTACTCCG CCCTCATCCT	GCAGATGAGG CTTCCAATGT	55	159
RFRP3	CCAAAGGTTT GGGAGAACAA	GGGTCATGGC ATAGAGCAAT	55	110
GPR147	GGTCAGAACG GGAGTGATGT	AGGAAGATG AGCACGTAGGC	55	119
LHβ	GCAAAAGCCA GGTCAGGGATAG	AGGCCCAC ACCACACTTGG	55	92
FSHβ	TTCAGCTTTCCC CAGGAGAGATAG	ATCTTATGGTCT CGTACACCAGCT	55	305
Cga	CTATCAGTGTATG GGCTGTTG	CTTGTGGTAG TAACAAGTGC	55	199
KISS1	TGGCACCTGTG GTGAACCCTG	ATCAGGCGAC TGCGGGTGGCA	61.4	202
GnRH	GCAGATCCCTA AGAGGTGAA	CCGCTGTTGT TCTGTTGACT	55	201

Statistical Analysis

Differences in gene expression examined *via* RT-PCR were analyzed by a one-way ANOVA followed by Tukey's multiple comparison test for *post hoc* analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Statistics were performed using the Prism software.

RESULTS

Hypothalamic Neuropeptide and Receptor Expression Level in the Hypothalamus across the Reproductive Span

To characterize changes in the reproductive axis during the transition from reproductive competence to reproductive senescence in female rats, naturally aging rats that exhibited different stages of reproductive decline were utilized. Expression profiles in four groups were examined: a 3-month regularly cycling group labeled "young," an 8-month regularly cycling group referred to as "middle-aged cycling," a 10-month group of irregularly cycling rats referred to as "irregularly cycling," and a 12-month group of acyclic animals referred to as "acyclic."

Hypothalamic *RFRP3* mRNA levels were significantly higher in middle-aged and acyclic females compared with young cycling females (relative fold change, 8 months old, 2.15 ± 0.23, 12 months old, 1.97 ± 0.25, **Figure 1A**). *RFRP3* was significantly lower in irregularly cycling females (10 months old, 0.24 ± 0.04, **Figure 1A**) compared with all other groups. Expression of the RFRP3 receptor, *GPR147*, was significantly higher in middle-aged animals regularly cycling females, relative to young controls (relative fold change, 1.5 ± 0.09, **Figure 1B**).

Kisspeptin is a potent activator of GnRH, and others have found that *KiSS1* decreases with aging (16). In our study, we also found a significant decrease in expression of *KiSS1* in all groups compared with young controls (relative fold change, 8 months old, $0.002 \pm 1.9 \times 10^{-3}$, 10 months old, $0.001 \pm 1.8 \times 10^{-4}$, and 12 months old, $0.002 \pm 4.7 \times 10^{-4}$, **Figure 1C**). We also see a downstream decrease in *GnRH* mRNA levels, as the acyclic animals exhibit a significant downregulation of GnRH compared with both the young controls and regularly cycling middle-aged animals (0.45 ± 0.05, **Figure 1D**).

Pituitary Expression Level in the Pituitary across the Reproductive Span

Next, we examined gonadotropin changes in the pituitary and found no differences in expression of the RFRP3 receptor, *GPR147* across the four groups (**Figure 2A**). However, the expression level of the LH subunit β (LHβ) in the pituitary was decreased in the middle-aged regularly cycling group (relative fold-change, 0.57 ± 0.22, **Figure 2B**) and irregularly cycling animals (0.73 ± 0.31, **Figure 2B**) relative to young controls. In acyclic rats, LHβ mRNA expression was not significantly different

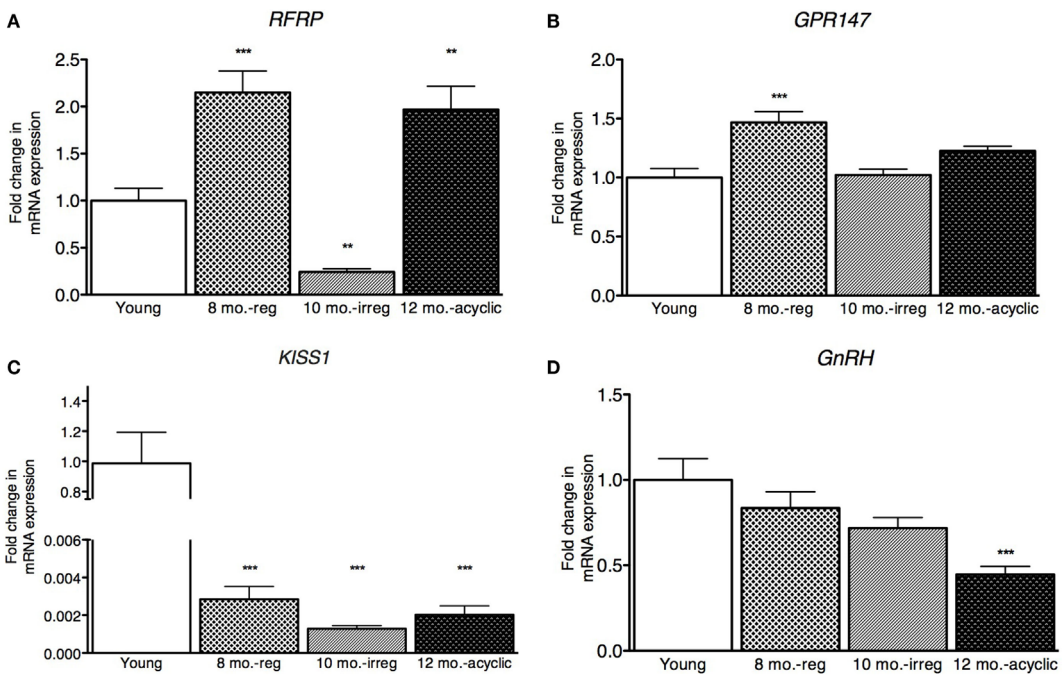

FIGURE 1 | Hypothalamic peptide mRNA expression in female rats. (A–D) Gene expression changes in the hypothalamus at different age ranges through middle age. Young = 3-month regularly cycling females, 8-month-old reg. = 8-month-old females with regular estrous cycles, 10-month-old irreg. = 10-month-old females exhibiting irregular cycles, i.e., over 6–7 days rather than 4–5, and 12-month-old acyclic = 12-month-old rats that exhibit persistent estrous or diestrous over a period of more than 14 days. mRNA levels of all (mean ± SEM, $N = 18$/group for young and 8-month-old reg., $N = 21$ for 10-month-old irreg., and $N = 12$ for 12-month-old acyclic) were determined using qRT-PCR relative to the ribosomal reference gene RPLP. Estrous cycle staging was determined by inspection of daily vaginal smears (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

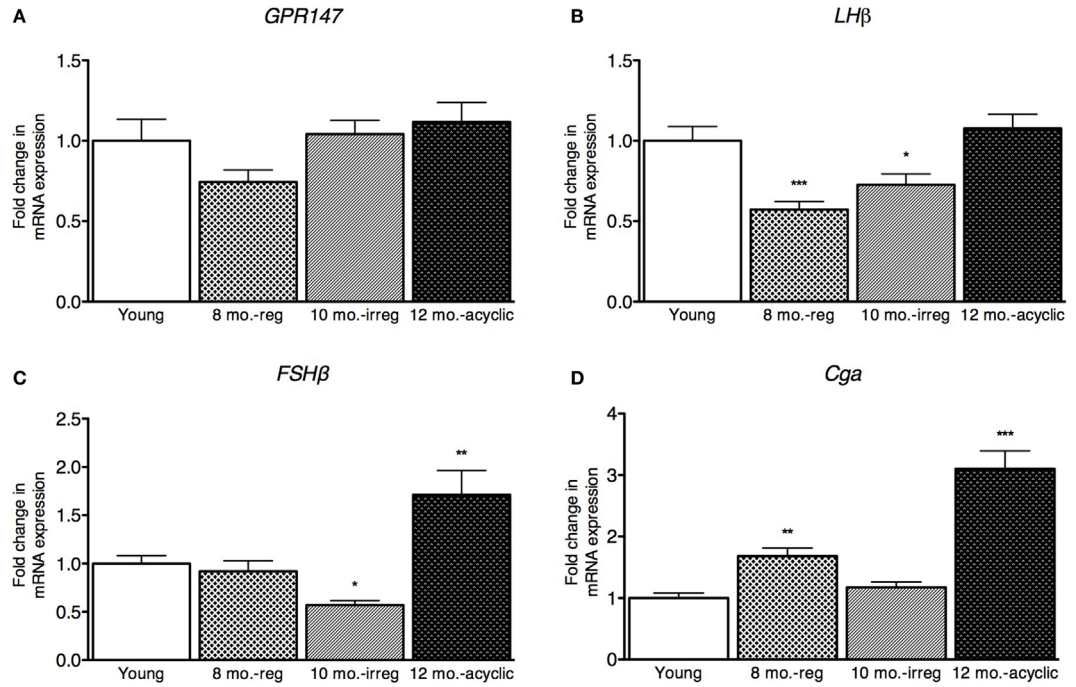

FIGURE 2 | Pituitary mRNA expression in female rats. (A–D) Gene expression changes in the pituitary at different age ranges through middle age. Young = 3-month regularly cycling females, 8-month-old reg. = 8-month-old females with regular estrous cycles, 10-month-old irreg. = 10-month-old females exhibiting irregular cycles, i.e., over 6–7 days rather than 4–5, and 12-month-old acyclic = 12-month-old rats that exhibit persistent estrous or diestrous over a period of more than 14 days. mRNA levels of all (mean ± SEM, $N = 18$/group for young and 8-month-old reg., $N = 21$ for 10-month-old irreg., and $N = 12$ for 12-month-old acyclic) were determined using qRT-PCR relative to the ribosomal reference gene RPLP. Estrous cycle staging was determined by inspection of daily vaginal smears (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

from young controls (1.1 ± 0.31). FSHβ expression was decreased in irregularly cycling females (0.57 ± 0.21, **Figure 2C**) relative to young controls, but increased in acyclic females, compared with the young controls (1.71 ± 0.88). Synthesis of LH and FSH requires a common glycoprotein alpha subunit (Cga), in addition to a specific beta subunit. Cga expression was increased in the middle-aged group (1.68 ± 0.55, **Figure 2D**) relative to young and 10-month-old irregularly cycling females and the acyclic group (3.00 ± 1.00) relative to all other groups.

RFRP3 and GPR147 Expression Increase Dramatically in the Aging Ovary

Next, we explored how *RFRP3* and *GPR147* changed in the ovaries during reproductive decline. We found that *RFRP3* was higher in the ovaries in the acyclic group (2.5 ± 0.29, **Figure 3A**) compared with young and middle-aged cycling females. *GPR147* was upregulated in both middle-aged and irregularly cycling females compared with young animals (8 months old, 2.9 ± 0.34 and 10 months old, 2.44 ± 0.21, **Figure 3B**).

DISCUSSION

The present findings suggest that the inhibitory neuropeptide, RFRP3, may play a role in the initiation of reproductive decline in female rats, prior to change in cyclicity. Specifically, we found that hypothalamic RFRP3 expression increases significantly in middle age, concomitant with an increase in the RFRP3 receptor, *GPR147*, in the hypothalamus and gonads. These findings imply a transient increase in RFRP3 signaling around 8 months of age, prior to the onset of cycle irregularity. This transient increase in inhibitory signaling possibly acts as an initiating signal to inhibit regular cycling, leading to subsequent reproductive decline. This initial inhibitory signaling at 8 months is followed by a decrease in *KiSS1* in the hypothalamus. RFRP3 receptor mRNA has been shown to colocalize with KiSS1 neurons (19), suggesting that increased RFRP3 expression in middle age prior to reproductive cessation might act to signal *KiSS1*-expressing cells to further reproductive decline. Though transient, this effect may be sufficient to trigger a downstream decrease in HPG axis activity, as we see decreases in *LH*β and *FSH*β subunits in early aging. Since RFRP3 mRNA levels were measured in RNA extracted from whole hypothalamic in this study, it cannot provide nucleus-specific localization. However, RFRP3 expression was only documented in the DMH (12, 20). The data presented in this study provide a timeline of changes in expression of the major regulators, providing correlative evidence for the role played by RFRP3. Future studies using RFRP shRNA or overexpression viral vector or pharmacological inhibition of the RFRP3 receptor GPR147 would be required in order to prove the mechanistic role

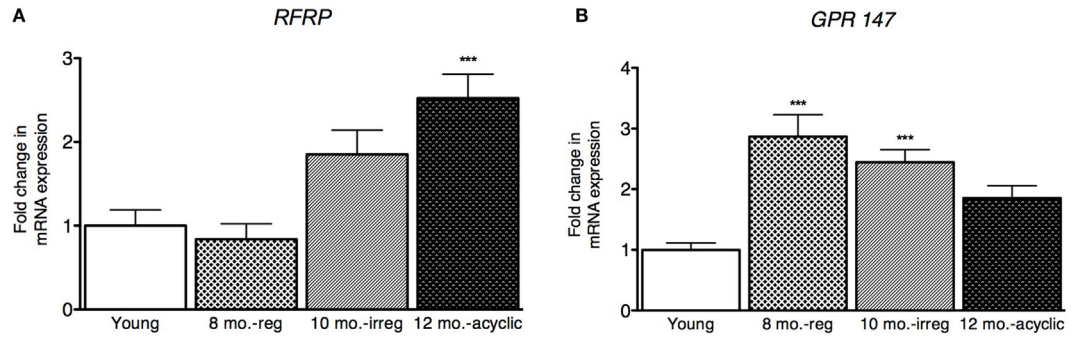

FIGURE 3 | Ovarian hormone mRNA levels in female rats. **(A,B)** Gene expression changes in the ovary at different age ranges through middle age. Young = 3-month regularly cycling females, 8-month-old reg. = 8-month-old females with regular estrous cycles, 10-month-old irreg. = 10-month-old females exhibiting irregular cycles, i.e., over 6–7 days rather than 4–5, and 12-month-old acyclic = 12-month-old rats that exhibit persistent estrous or diestrous over a period of more than 14 days. mRNA levels of all (mean ± SEM, $N = 18$/group for young and 8-month-old reg., $N = 21$ for 10-month-old irreg., and $N = 12$ for 12-month-old acyclic) were determined using qRT-PCR relative to the ribosomal reference gene RPLP. Estrous cycle staging was determined by inspection of daily vaginal smears (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

of RFRP3 in the timing of senescence. For example, a recently published inducible RFRP3 shRNA lentiviral vector (21) could be used to test the effect of blocking the transient increase in RFRP3 on onset of senescence. Alternatively, use of a GPR147 antagonist would provide information as to the role of RFRP3 in reproductive senescence, and the compound GJ14 might be a useful tool in this respect (22).

Previous studies have shown that LH serum concentrations decrease with aging, whereas FSH serum concentrations increase (6). This decrease in LH is likely triggered by a decrease in GnRH stimulation from the hypothalamus. It is hypothesized that an increase in FSH is seen in rodents, in contrast to what is observed in women, because follicles continue to develop throughout aging. However, these follicles are acyclic as they do not produce corpus lutea. The increase in *LH*β, *FSH*β, and *Cga* mRNA levels in acyclic, aged females has been seen in previous studies (23, 24) and is hypothesized to represent a decrease in the ability to transcribe gonadotropin proteins successfully, or potentially a decrease in the ability to release the gonadotropins. Here, we show that, similar to our hypothalamic findings, early decreases in LH and FSH transcription precede cycle cessation and increase thereafter, with this latter decrease likely representing the loss of negative feedback control.

Perhaps the most striking finding in this study is the increase in both *RFRP3* and *GPR147* in the ovaries as aging progresses. The increase in RFRP3 is maintained through at least 12 months of age female become acyclic. What is particularly interesting is the different kinetics of RFRP3 regulation in the hypothalamus and the gonads. RFRP3 increases transiently in the hypothalamus in middle-aged animals while still cycling normally, is suppressed in irregularly cycling females, and is upregulated again when cycling ceases. In contrast, RFRP3 expression in the ovaries increases after cycle irregularity begins and is maintained at least until cycles cease. This differential expression dynamics can potentially reflect hypothalamic RFRP signaling inducing subsequent downstream suppression of the axis. The correlative evidence described in this study would require further experimental manipulations to discover the mechanistic hierarchy. For instance, is the increase of ovarian RFRP3 induced by the hypothalamic RFRP3 increase, or occurs independently? There is evidence for local RFRP3 production in the ovary that might be independently regulated, separate from signals from the hypothalamus, providing support for this latter possibility (25–27). Decreased gonadal steroid production throughout the stages of reproductive decline (6) is thought to result from decreased preovulatory gonadotropin release. Our data suggest that this decrease in gonadal steroids may be due to local inhibition *via* RFRP3 in the ovaries.

Successful reproduction is dependent on the coordination of many endocrinological events in the brain and the periphery. In the aging rat, HPG axis functionality decreases, manifesting as decreases in GnRH synthesis and release, decreases in LH, and a decrease in gonadal steroids. However, the regulatory cues that initiate the transition from function to decline are largely yet unknown. We have described here the dynamic regulation of key neuropepetides in the HPG axis along this transition. Importantly, a transient increase in the expression of RFRP3 and its receptor precedes irregular cycling, and later cycling cessation, adding a new piece to the puzzle of reproductive decline.

AUTHOR CONTRIBUTIONS

AG, GB, LK, and DK designed experiments and wrote the manuscript. AG and SM performed experiments. AG analyzed data.

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REVIEW
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RFRP Neurons – The Doorway to Understanding Seasonal Reproduction in Mammals

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Seasonal control of reproduction is critical for the perpetuation of species living in temperate zones that display major changes in climatic environment and availability of food resources. In mammals, seasonal cues are mainly provided by the annual change in the 24-h light/dark ratio (i.e., photoperiod), which is translated into the nocturnal production of the pineal hormone melatonin. The annual rhythm in this melatonin signal acts as a synchronizer ensuring that breeding occurs when environmental conditions favor survival of the offspring. Although specific mechanisms might vary among seasonal species, the hypothalamic RF (Arg–Phe) amide-related peptides (RFRP-1 and -3) are believed to play a critical role in the central control of seasonal reproduction and in all seasonal species investigated, the RFRP system is persistently inhibited in short photoperiod. Central chronic administration of RFRP-3 in short day-adapted male Syrian hamsters fully reactivates the reproductive axis despite photoinhibitory conditions, which highlights the importance of the seasonal changes in RFRP expression for proper regulation of the reproductive axis. The acute effects of RFRP peptides, however, depend on species and photoperiod, and recent studies point toward a different role of RFRP in regulating female reproductive activity. In this review, we summarize the recent advances made to understand the role and underlying mechanisms of RFRP in the seasonal control of reproduction, primarily focusing on mammalian species.

Keywords: RFRP, mediobasal hypothalamus, melatonin, seasonal reproduction, photoperiod, TSH

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INTRODUCTION

Animals living in temperate and boreal latitudes experience marked seasonal changes in their environment. To overcome these environmental changes, thus increasing their chances of survival, they show seasonal changes in several aspects of their physiology, i.e., reproduction, metabolism, and behavior. Annual variations in day length are translated into an endocrine message, namely, the nocturnal secretion of the pineal hormone melatonin, which acts as a potent seasonal synchronizer of biological functions, especially reproductive activity (1–4).

The effects of photoperiod on reproductive function have long been established, and recent studies have made much progress in describing key components and pathways involved in this adaptive process. The RFamide peptide kisspeptin (kp), a very potent stimulator of gonadotropin-releasing hormone (GnRH) release, shows photoperiodic variations and was therefore thought to be a likely candidate for the photoperiodic control of reproduction in seasonal breeders, but increasing evidence now points toward another RFamide peptide, the RF (Arg-Phe) amide-related peptide

(RFRP) as the critical intermediate between the melatonin-dependent photoperiodic signal and central control of the reproductive axis (5–8).

Herein, we review our current understanding of the RFRP system in seasonal breeders, reporting the mechanisms through which melatonin impacts on RFRP synthesis and the effects of RFRPs in the seasonal control of reproduction including species-dependant and sex-specific variations in the RFRP system.

THE PINEAL HORMONE MELATONIN SYNCHRONIZES REPRODUCTION WITH SEASONS

In seasonal species, reproductive activity is restricted to a particular time of the year so that birth occurs when warmer temperatures and better accessibility to food increase the chances of survival of the offspring. Early studies have demonstrated that synchronization of reproductive activity with season is driven by the pineal hormone melatonin (2). Photic information reaches the pineal gland *via* the retino–hypothalamo–pineal pathway that during nighttime generates a release of norepinephrine, which acts as a potent and reliable regulator of the rhythmic release of melatonin from the pineal gland. As a consequence, melatonin is synthesized and secreted in a diurnal fashion with a dramatic increase during nighttime that returns to nearly undetectable levels at daytime with the duration of elevated melatonin depending on night length (1–4, 8, 9). In long photoperiod (LP) breeders, i.e., the Syrian hamster (*Mesocricetus auratus*) that is widely used as a rodent model to study seasonal reproduction, short day lengths represented by a long nocturnal duration of melatonin secretion inhibits the reproductive axis, and removal of the melatonin signal by pinealectomy prevents this short day inhibition of reproductive activity (2). In contrast to small rodents, larger mammals with a longer gestation time, such as the sheep, are sexually active in short photoperiod (SP) and becomes sexually quiescent after transfer to LP conditions (10). Although the reproductive timing is opposite in hamsters and sheep, in both cases, the photoperiodic changes in circulating levels of melatonin synchronize reproduction with seasons.

Melatonin Modes And Sites of Action on the Reproductive Axis

Three melatonin receptor subtypes have been characterized so far; MT1 (Mel1a), MT2 (Mel1b), and Mel1c with its mammalian ortholog GPR50 (11, 12). Using the highly specific 2-[^{125}I]-iodomelatonin, high affinity melatonin-binding sites have been found in the hypothalamus and the *pars tuberalis* (PT) of mammals, and among species, the highest concentration of melatonin receptors is found in the PT (8, 13–16). The MT1 subtype seems to be dominantly expressed throughout species and is known to be responsible for the neuroendocrine integration of season (17, 18). Indeed, Siberian hamsters (also known as the Djungarian hamster or *Phodopus sungorus*) show seasonal reproductive responses to melatonin despite lacking a functional MT2 receptor (19). Maywood et al. found that site specific lesions of iodomelatonin-binding sites in the mediobasal hypothalamus (MBH) prevent testicular regression in Syrian hamsters exposed to SP (20) and in Siberian hamsters, melatonin infusion into or lesions of the suprachiasmatic nucleus (SCN), alter the reproductive response to seasonal changes (21, 22). Finally, in sheep, the premammilary region of the hypothalamus contains melatonin-binding sites (23, 24) and melatonin implantations in the area of this structure, but not the PT, were shown to prevent synchronization of reproduction with photoperiod (24–27). Altogether, these data have pointed toward the potential importance of these hypothalamic regions for proper integration of the melatonin-dependent photoperiodic signal onto the reproductive axis. However, it has not been possible to determine whether and how melatonin would act directly on these hypothalamic sites. Although a direct hypothalamic effect of melatonin cannot be excluded, accumulating evidence now points toward the PT as the major site for the hypothalamic integration of the melatonin signal in seasonal breeders (8, 28–30).

TSH, THYROID HORMONES, AND THE MELATONIN-DRIVEN REPRODUCTIVE ACTIVITY

In 2003, Yoshimura and colleagues made a remarkable finding that unveiled a link between the thyroid-stimulating hormone (TSH) pathway and seasonal reproduction. They showed that light-induced type 2 thyroid hormone deiodinase (Dio2) expression in the MBH and subsequent hormone conversion of thyroxine (T4) into the bioactive triiodothyronine (T3) regulate the photoperiodic response of gonads in birds (31). Since this discovery, it has been shown that TSH expression in the PT is regulated by photoperiod in a melatonin dependent manner and that TSH stimulates Dio2 expression in seasonal mammals (29, 32, 33). MT1-expressing cells in the PT synthesize TSH, and its production in the PT is strongly inhibited by the SP pattern of melatonin (34–36). Recent work moreover disclosed that melatonin regulates the photoperiodic changes in TSH expression in the PT *via* differential effects on clock gene expression and on the transcription of the co-activator EYA3 (37–39). Another primary response to photoperiodic changes in melatonin is the opposite regulation of Dio2 and type 3 thyroid hormone deiodinase (Dio3) expression in the MBH (29, 31, 33, 40–43). While Dio2 catalyzes the conversion of T4 into T3, Dio3 catalyzes the conversion of T3 to the biological inactive T2. Thus, in concert, Dio2 and Dio3 regulate the hypothalamic T4/T3 balance according to photoperiod, with a higher production of T3 in LP as compared to SP (5, 43–45). Thyroid hormones have long been known to be important for the transitions between the breeding and non-breeding states, i.e., in 1940s, thyroidectomy in starlings was reported to result in persistent breeding (46). Similarly, thyroidectomized sheep remain in the breeding state when changing from spring to anestrous and T4 replacement can reverse these effects, but interestingly, thyroidectomy displays no effect in the transition from the anestrous state to the breeding state (47–50). Moreover, T3 administration in the LP-breeding Siberian hamster blocks the SP-induced gonadal regression (44, 51, 52).

Both deiodinases are highly expressed in a population of specialized glial cells in the ependymal cell layer lining the third ventricle named tanycytes (53). Interestingly, tanycytes co-express the TSH receptor (TSHR), and recent data clearly show that activation of these receptors increases *Dio2* expression, thereby increasing levels of T3 in the MBH, in a number of seasonal mammals (5, 29, 33, 43, 54–57). In line with these observations, a recent study shows that PT-derived TSH, in contrast to *pars distalis*-derived TSH, does not stimulate the thyroid gland, but rather acts *via* TSHR on the tanycytes (58). Altogether, these studies have unveiled a conserved photoperiodic transduction pathway explaining how the melatonin signal is integrated in the PT and transduced into a local thyroid message in the MBH (8) (**Figure 1**).

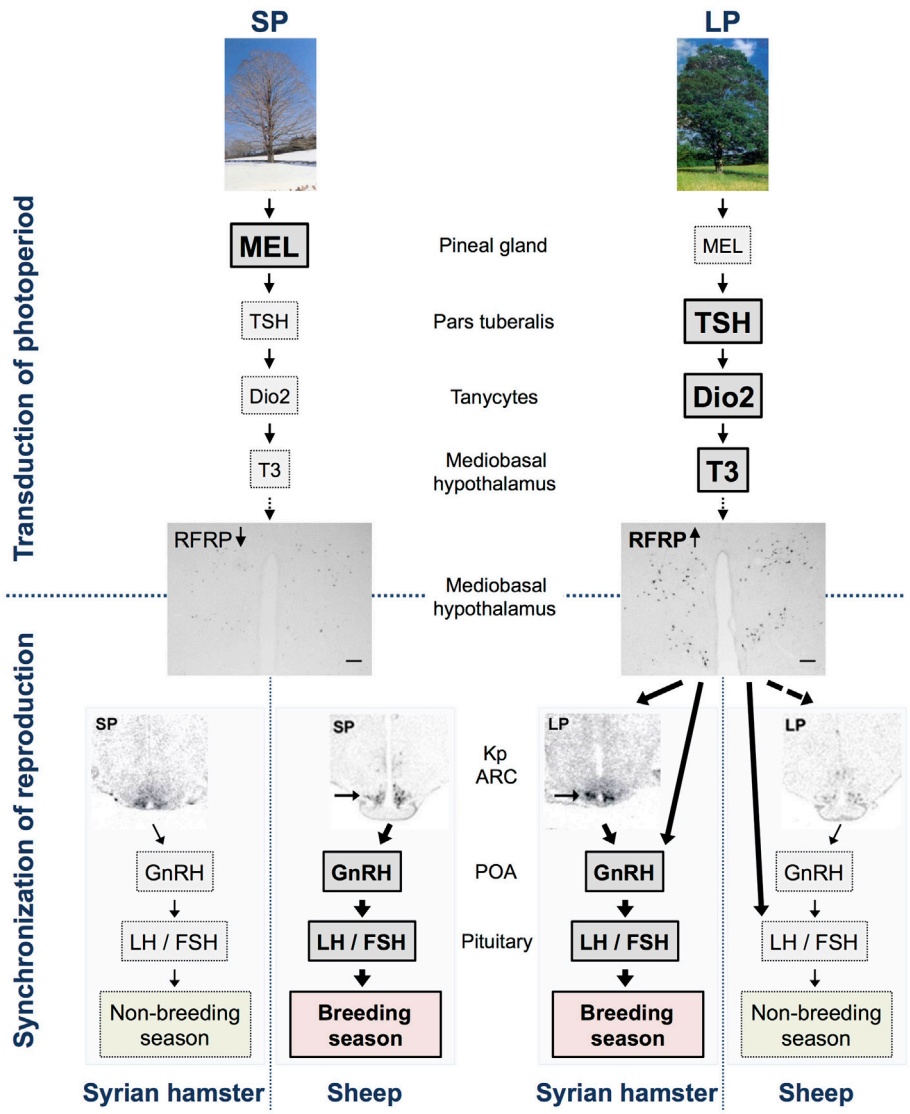

FIGURE 1 | Model of the transduction of photoperiod and seasonal regulation of the reproductive axis in long (Syrian hamsters) and short day (sheep) breeders. In short photoperiod (SP), the large production of melatonin from the pineal gland inhibits TSH synthesis in the *pars tuberalis*, whereas the lower production of melatonin in long photoperiod (LP) allows the synthesis and release of TSH. TSH is transmitted *via* TSH receptors expressed in tanycytes surrounding the third ventricle and activates the enzyme deiodinase 2 (Dio2). Dio2 ultimately controls and increases the local availability of the active form of the thyroid hormone, T3, in the mediobasal hypothalamus. Subsequently, T3 regulates the expression of RFRP also in the mediobasal hypothalamus so that there is a high expression in LP and a low expression in SP in both LP and SP breeders, as demonstrated with pictures of RFRP-ir neurons in brains from Syrian hamster kept in LP and SP [scale bar 100 μm, taken from Ref. (59)]. In Syrian hamsters, RFRP subsequently acts either directly on GnRH neurons or indirectly *via* kisspeptin (kp) neurons (indicated by arrows) or other interneurons in the arcuate nucleus (ARC) to synchronize reproduction with season. In sheep, RFRP regulates the reproductive axis directly at the level of the pituitary (indicated by arrow) and possibly also directly or indirectly *via* kp neurons (indicated by dotted arrow) and/or GnRH neurons. Expression of the gene encoding kp in the ARC displays an opposite photoperiodic regulation in the two species being elevated in both LP-adapted sexually active Syrian hamsters [see arrow in picture taken from Ref. (33, 60)] and SP-adapted sexually active sheep [see arrow in picture taken from Ref. (61)]. This model does not describe specific effects of RFRP-3 reported in each species, sex, and photoperiod, and readers are referred to **Table 1** for a detailed summary.

Thyroid hormones mediate their neuroendocrine effects through still undefined hypothalamic sites since no cellular phenotyping of their receptors have been reported in the hypothalamus. Interestingly, however, a recent study found that chronic TSH administration in SP-adapted Siberian and Syrian male hamsters reactivates the reproductive axis, while at the same time increasing the expression of two known hypothalamic regulators of reproductive output, RFRP and Kp, suggesting that the melatonin signal reaches the reproductive axis *via* the TSH/thyroid hormone pathway acting on these neurons (5) rather than *via* direct hypothalamic effect as previously suggested (20).

HYPOTHALAMIC REGULATION OF SEASONAL REPRODUCTION

Hypothalamic control of the reproductive axis is commonly regulated among species through the release of GnRH from GnRH fiber terminals projecting to the median eminence. GnRH is released into the portal blood system from which it regulates the synthesis and release of pituitary gonadotropins. Despite the marked decrease of GnRH release during sexual quiescence, most seasonal species display an unchanged number and level of GnRH neurons and GnRH-immunoreactivity (ir) in the different photoperiods (62, 63). GnRH synthesis and release is regulated upstream by various signals, especially two RF-amide peptides released from, respectively, RFRP neurons in and around the dorso/ventromedial hypothalamus (DMH/VMH) and kp neurons in the anteroventral periventricular nucleus (AVPV) and medial preoptic nucleus (MPN), and in the arcuate nucleus (ARC). Kp peptides are potent stimulators of the reproductive axis and acts directly on GnRH neurons through their cognate receptor GPR54 (64, 65). Interestingly, kp expression both in the MPN/AVPV and ARC was found to be significantly downregulated by melatonin in SP-adapted sexually inactive Syrian hamsters (60, 66). In the SP-breeding sheep, kp expression is oppositely upregulated in SP (61, 67, 68), suggesting that ARC kp expression reflects the breeding state rather than the seasonal state of the animal (**Figure 1**). In both hamsters and sheep, continuous infusion of kp during sexual quiescence fully restores reproductive activity (60, 69–71), and kp neurons are thus a pivotal component between the photoperiodic signal and seasonal activation of GnRH neurons. This statement is, however, complicated by results showing a lowered ARC kp expression in LP-breeding Siberian (72, 73) and European (74) hamsters as compared to SP. While these differences in the photoperiodic regulation of ARC kp might be explained by different feedback mechanisms of sex steroids, it also suggests that these neurons are differently implicated in the photoperiodic control of reproduction from one species to another. Therefore, it seems unlikely that kp neurons are solely responsible for mediating the melatonin-dependent seasonal signal onto the reproductive axis. By contrast, increasing results demonstrate that the photoperiodic regulation of RFRP expression within the DMH/VMH is conserved among seasonal species (75), suggesting that RFRP neurons may be potential candidates for integration of the photoperiodic signal.

THE RFRP SYSTEM

RF (Arg–Phe) amide-related peptides were discovered in birds and mammals in 2000 and found to be primarily expressed in neurons located in the paraventricular nucleus (PVN) and in between the DMH and VMH in birds and rats, respectively (76, 77). In birds, the peptide was shown to inhibit gonadotropin secretion from cultured quail pituitaries and thus termed gonadotropin-inhibitory hormone (GnIH) (76). The avian GnIH precursor encodes one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2). GnIH and GnIH-RP-1 contain an LPLRFa motif in the C-teminal, whereas GnIH-RP-2 contains an LPQRFa motif. The mammalian gene named *RFamide-related peptide* (*Rfrp*) encodes RFRP-1, -2, and -3 peptides, of which RFRP-1 (containing an LPLRFa motif) and RFRP-3 (containing a C-terminal LPQRFa motif) are functional peptides (77–79). RFRP-3 and GnIH have been shown to inhibit GnRH neuron activity and gonadotropin release in several seasonal (sheep, hamster, and quail) and non-seasonal (rat and mouse) species (76, 80–87). Moreover, there is evidence of a hypophysiotropic effect of GnIH and RFRP-3 in birds and ewes, respectively, although the effect in ewe is still of controversy since in one study intravenous (iv) infusion of RFRP-3 inhibits pulsatile LH secretion in ovariectomized ewes (81), whereas two other studies find no variation in LH plasma concentrations in neither ovariectomized nor intact ewes injected either intracerebroventricular (icv) or iv with RFRP-3 (88, 89). Two recent studies reported a stimulatory effect of central administration of RFRP-3 in male Syrian and Siberian hamster, indicating that the effects of the peptide are species dependent (6, 7). Strikingly, while RFRP-3 activates the reproductive axis in male Syrian hamsters (6), the avian GnIH inhibits LH secretion in ovariectomized females (84), adding a supplementary sex difference in the effects of the peptides.

Recently, a study of Tena-Sempere and colleagues (86) reported that mouse KO for GPR147, the cognate receptor for RFRPs, does not display strong reproductive phenotypic alterations as compared to wild-type mice. Moderate changes are, however, observed in GPR147-deficient mice, as during pubertal transition, male KO mice exhibit increased LH levels, and in adulthood, FSH levels are higher in both female and male KO mice as compared to wild-type mice. Moreover, litter sizes from KO mice are slightly increased as compared to wild-type litter sizes. Interestingly, the male KO mice moreover show an increased level of kp expression in the ARC, but not in the MPN/AVPV, suggesting that in male mice, RFRP neurons provide a tonic inhibition on ARC kp neurons.

RFRP Modes and Sites of Action

RFRP neurons are mainly found in and around the DMH/VMH from where they project to multiple regions of the rodent brain. RFRP-ir fibers are found especially in the preoptic area/organum vasculosum of the lamina terminalis (POA/OVLT), MPN/AVPV, the anterior part of the SCN, PVN, anterior hypothalamus, VMH, and ARC as well as in the bed nucleus of the stria terminalis, habenular nuclei, and PVN of the thalamus (7, 59, 84). RFRP

terminals make apparent contact to 20–40% of GnRH somas in rodents and sheep (7, 67, 84, 90), and in female mice, around 20% of MPN/AVPV kp neurons and 35% of ARC kp neurons receive RFRP fiber contacts (90, 91). In mice, RFRP-3 application to brain slices inhibits the firing rate of 41% GnRH neurons and stimulates the firing rate of 12% of the GnRH neurons (82), whereas in male Syrian hamster, icv infusion of RFRP-3 induces c-FOS expression in 20–30% GnRH neurons but also in non-kisspeptinergic neurons of the ARC (6).

RFRPs bind preferentially to the G-protein-coupled receptor, GPR147 (also known as NPFF1). GPR147 has been found to couple to both stimulatory and inhibitory G protein subunits *in vitro* (92, 93), and in GPR147-transfected CHO cells, hRFRP-1 induces a maximal inhibition of a forskolin-induced cAMP accumulation, indicating that RFRP-1 might inhibit adenylate cyclase through a Gαi-bound receptor complex (94). However, the exact signaling events occurring downstream of GPR147 in its natural cellular environment still remains unknown. GPR147-encoding mRNA is widely distributed in the brain; however, particular strong expression is observed in hypothalamic regions as the POA/OVLT, MPN/AVPV, SCN, PVN, anterior hypothalamus, VMH, and ARC, and outside the hypothalamus in the posterior part of the bed nucleus of the stria terminalis, habenular nuclei, and the pyramidal cell layer of the hippocampus (59, 95, 96). Interestingly, GPR147 has been shown to be expressed in 15–33% of mice GnRH neurons and a subpopulation of kp neurons in the AVPV (5–16%) and the ARC (25%) (90, 91, 97). Altogether, these studies suggest that RFRPs can act directly on these central neuroendocrine regulators of reproduction.

RFRP: A CRITICAL SWITCH BETWEEN MELATONIN AND THE REPRODUCTIVE AXIS

GnIH and Avian Seasonal Reproduction

Gonadotropin-inhibitory hormone inhibits LH release from cultured quail pituitaries (76), and to further support a direct pituitary effect of GnIH in quail, GnIH-ir fibers have been found to project to the median eminence in this species (98). Also in quail, studies have revealed that GnIH expression and release is directly regulated by melatonin acting on Mel1c receptors specifically expressed in GnIH neurons (99). In contrast to mammalian seasonal species, GnIH expression is increased by melatonin and consequently GnIH-ir expression is increased in SP as compared to LP (99). *In vitro* studies furthermore show that GnIH release has a diurnal rhythm and is increased during nighttime in quail hypothalamic explants (100). In house and song sparrows, GnIH-ir neurons are reported bigger toward the end of the breeding season (101). By contrast in wild Australia zebra finches, there is no variation in neither GnIH-ir nor *GnIH* expression between the breeding and non-breeding states (102).

RFRP in Seasonal Mammalian Species

Photoperiodic Variations in the RFRP System

There are no circadian or day-to-night variations in RFRP mRNA expression in male Syrian (103) and European (74) hamsters, respectively. RFRP expression is however strongly regulated by photoperiod and is downregulated in SP in several seasonal breeders (6, 67, 74, 75, 103, 104). Studies in male Syrian and Siberian hamsters show the SP downregulation to be melatonin dependent (7, 103). Recent findings have revealed that in female Syrian hamsters as well, RFRP expression is downregulated in SP, probably driven by the same mechanisms (59). Interestingly, RFRP expression is similarly downregulated in SP in short-day breeders such as the sheep (67, 96). This suggests that the SP pattern of circulating melatonin displays a conserved inhibition on RFRP expression independently of whether mammals are long- or short-day breeders (**Figure 1**). Importantly, the photoperiodic/melatonin regulation of RFRP expression, in contrast to kp, may not be modulated by the gonadal hormone feed back because, although RFRP neurons express sex steroid receptors (84), neither gonadectomy nor sex-hormone implants alter RFRP expression in male (103) and female (Henningsen et al., unpublished) Syrian hamsters.

We have recently shown that GPR147 mRNA levels in the Syrian hamster's brain also depends on photoperiod being downregulated in SP condition, and interestingly, this downregulation is much stronger and consistent in females as compared to males (59). In Siberian hamsters and sheep, the amount of GnRH neurons receiving RFRP fiber contacts is decreased in SP conditions (7, 81); however, in the Syrian hamster, we did not find any photoperiodic variations in numbers of RFRP-ir fibers projecting specifically to the OVLT or the ARC (59).

Species-Specific Differences in the Effects of RFRP on Seasonal Reproduction

We were the first to show that in male Syrian hamster, RFRP-3 is capable of stimulating the reproductive axis (6). In more details, RFRP-3 was found to stimulate GnRH neuronal activity, LH and FSH release, and testosterone production independently of the photoperiodic condition, although to a lesser extent in SP animals. Moreover, chronic central administration of RFRP-3 in SP-adapted male Syrian hamsters reactivated the reproductive axis *via* an increase in ARC kp expression, despite the animals being kept in SP-inhibitory conditions. The stimulatory effect of RFRP-3 observed in male Syrian hamsters fits well with the high RFRP expression in sexually active LP animals, and our data furthermore indicate that the stimulation of reproductive activity could be mediated *via* the ARC kp neurons. Thus, in the male Syrian hamster, RFRP neurons appear to integrate and transfer the seasonal input toward kp neurons. In another hamster species, the male Siberian hamster, RFRP-3 displays reverse effects depending on the photoperiodic condition, stimulating LH release in SP but decreasing LH levels in LP conditions (7). Although the mechanism underlying such photoperiod-dependent effect of RFRP-3 in this species is unknown, it might help to explain the upregulation of ARC kp in the sexually inactive SP-adapted Siberian hamsters (72, 105, 106). These observations indicate that in the two hamster species, RFRP-3 either has opposite sites of action in LP-adapted animals or is integrated differently along the hypothalamo–pituitary–gonadal axis.

Despite the controversies of RFRP's effect in sheep, one study reported that iv administration of ovine RFRP-3 peptide (also

referred to as GnIH3) inhibits LH release in ovariectomized ewe (107), which indicates that RFRPs might have a hypophysiotropic effect in ovine species, similarly to what is observed in avian species. Indeed, RFRP fibers have been shown to project to the median eminence, and RFRPs are detected in the portal blood of sheep (108, 109). As previous studies have reported, there is no evidence of peripheral effects of RFRPs on hamster's reproduction (6), thus describing another fundamental difference in how the RFRP signal is integrated in the hypothalamo–pituitary axis among mammalian seasonal species. Interestingly, the opposite effects and photoperiodic regulation of RFRP between sheep and hamsters support our hypothesis that a similar neuroendocrine pathway is conserved between LP and SP breeders with the RFRPs playing a pivotal role in adapting reproductive activity to the environment (**Figure 1**). Further analyzes are required to test this hypothesis, in particular whether the inhibitory effect of RFRP-3 account for the lower expression of kp in LP-adapted sexually inactive sheep. The reported effects of RFRP-3 in seasonal mammals are summarized in **Table 1**.

A fascinating issue is to disclose how RFRPs can have opposite seasonal and species-dependent effects. As previously mentioned, GPR147 has been found to couple to both stimulatory and inhibitory G proteins *in vitro* (92, 93), but it remains to be established if there are fundamental species-specific differences in the downstream signaling cascades after activation of the GPR147. Alternatively, it is likely that the cellular response to RFRP is conserved among species, but mediated *via* different targets. In male Syrian hamsters, acute injections of RFRP-3 induce c-Fos expression in a subset of GnRH neurons but also in an unidentified population of neurons in the ARC (6), and one can speculate whether the observed stimulatory effect of RFRP-3 in the male Syrian, but also Siberian hamsters, arises through inhibition of inhibitory interneurons, thus resulting in the stimulatory outcome. Future studies should aim at phenotyping downstream targets of RFRP in order to understand better its various effects on the reproductive axis of seasonal breeders.

TABLE 1 | Overview of the *in vivo* effects of RFRP-3 on reproduction in seasonal mammals.

Species	Sex and status	Effects of RFRP-3	Reference
Siberian hamster	Male	Central administration inhibits LH release in LP Central administration stimulates LH release in SP	(7)
Syrian hamster	Male	Central acute and continuous administration stimulates LH release in LP and SP. No peripheral effect	(6)
	Female OVX	Central and peripheral administration (GnIH) inhibits LH release in LP	(84)
Sheep	Female	Peripheral injection or a 4-h perfusion has no effect on kisspeptin-mediated increase in LH in LP	(89)
		Peripheral administration inhibits LH release in SP	(107)
	Female OVX	Repeated peripheral injection has no effect on pulsatile LH release in LP. 24-h perfusion has no effect on E2-induced LH surge in SP	(89)
		Central and peripheral administration has no effect on LH release in SP and LP	(88)
		Peripheral administration inhibits E2-induced LH surge in SP	(81)
		Peripheral administration inhibits pulsatile LH release in SP	(107)
		Peripheral administration inhibits GnRH-induced LH release. RFRP-3 is detected in the portal blood in SP and LP, with higher conc. detected in LP	(109)

Sex Differences in Seasonal Rodents

In contrast to the stimulatory effect of RFRP-3, we have observed in the male Syrian hamsters (6), central injections of the avian RFRP, GnIH, inhibits LH release in ovariectomized females (84). It should be stressed that GnIH contains a -LPLRFa motif similar to that of the mammalian RFRP-1 and not RFRP-3. However, we recently found that central RFRP-3 administration in the intact female Syrian hamster inhibits LH release similarly to the effects observed with the GnIH ortholog (Henningsen et al., unpublished). These observations add supplementary sex-specific differences in the acute effects of RFRPs, at least in Syrian hamsters. In order to understand such opposite effect of RFRP-3, we explored other potential sex differences in the Syrian hamster RFRP system. We found that the number of RFRP neurons and the intensity of the immunoreactive labeling were markedly higher in females than in males adapted to LP conditions. In SP conditions however, RFRP expression is downregulated to a similar low level in both sexes (59). The number of RFRP-ir fibers projecting specifically to the MPN/AVPV is increased in SP as compared to LP in females, but not in males. Moreover, we found that the overall levels of GPR147 mRNA were higher in females than in males, regardless of photoperiod, and that the SP-induced downregulation of GPR147 mRNA levels were stronger in females than in males (59). A similar sex-specific difference in RFRP expression is also reported in the non-seasonal rat, where RFRP-1 expression is found to be higher in females as compared to males (110). Altogether, these findings point toward a particular importance of the RFRP system in seasonal as well as non-seasonal females.

RFRP and Circadian Changes in Female Reproductive Activity

RFRP neurons project to the MPN/AVPV (59, 84, 90) where kp neurons provide the stimulatory signal onto the GnRH neurons causing the surge of LH and thereby ovulation (111). The surge of LH requests high circulating E2 levels, as an indicator of ovarian maturation, but its timing is also gated by a circadian signal to occur at the end of the resting period (112). It has been suggested that in female mammals, RFRP neurons mediate a SCN-generated circadian output onto the MPN/AVPV kp neurons, thereby modulating the timing and generation of the LH surge (113). Indeed, in the female Syrian hamster, a decrease in RFRP expression occurs around the time of the LH surge (113), and in rat and ewes, RFRP expression is similarly reduced during the preovulatory period

(107, 110). Interestingly, a recent study showed that SCN-derived vasoactive intestinal peptide (VIP)-ergic terminal fibers projections are found in the area of where RFRP neurons are expressed in the female Syrian hamster and more importantly that central VIP administration markedly suppress RFRP cellular activity in the evening, but not in the morning (114). Altogether, these data point toward a specific circadian rhythm in RFRP expression and release in females, adding a supplementary role of RFRP in regulating reproductive activity in seasonal female species.

CONCLUSION

Over the past nearly two decades, RFRPs have been extensively studied for their putative involvement in the regulation of the reproductive axis (87). Initially, RFRPs were thought to act with a similar inhibitory effect as the avian ortholog GnIH in all species and is still widely referred to as GnIH, despite its well-documented stimulatory effect in Siberian and Syrian hamster species (6, 7). These recent findings have challenged the conception of a conserved role of RFRPs throughout species and underline the necessity to delineate its effects in one species, sex, and physiological condition, such as season.

The marked downregulation of RFRP expression observed in seasonal mammals, independently of sex, species, and breeding behavior, provides evidence for a distinct and conserved role of this peptide in the integration of the photoperiodic melatonin signal *via* a TSH-dependent regulation of thyroid hormone locally in the MBH (5, 8, 75) (**Figure 1**).

In the Syrian hamster, RFRP has opposite effects in females and males, and there are strong sex-specific differences in the RFRP system manifested by a higher expression and stronger photoperiodic variations in the RFRP system in females as compared to males. Few studies have investigated the regulation and role of the RFRP system in female reproduction; however, findings so far strongly suggest that RFRP neurons play a critical role in female reproduction in regards to the timing of both daily and seasonal synchronization of their reproductive activity.

AUTHOR CONTRIBUTIONS

JH has written a large part of the review; FG and VS have supervised the writing, corrected the text, and helped in the design of the figure.

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Possible Role of GnIH as a Mediator between Adiposity and Impaired Testicular Function

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The aim of the present study was to evaluate the roles of gonadotropin-inhibitory hormone (GnIH) as an endocrine link between increasing adiposity and impaired testicular function in mice. To achieve this, the effect of GnIH on changes in nutrients uptake and hormonal synthesis/action in the adipose tissue and testis was investigated simultaneously by *in vivo* study and separately by *in vitro* study. Mice were treated *in vivo* with different doses of GnIH for 8 days. In the *in vitro* study, adipose tissue and testes of mice were cultured with different doses of GnIH with or without insulin or LH for 24 h at 37°C. The GnIH treatment *in vivo* showed increased food intake, upregulation of glucose transporter 4 (GLUT4), and increased uptake of triglycerides (TGs) in the adipose tissue. These changes may be responsible for increased accumulation of fat in white adipose tissue, resulting in increase in the body mass. Contrary to the adipose tissue, treatment with GnIH both *in vivo* and *in vitro* showed decreased uptake of glucose by downregulation of glucose transporter 8 (GLUT8) expressions in the testis, which in turn resulted in the decreased synthesis of testosterone. The GnIH treatment *in vivo* also showed the decreased expression of insulin receptor protein in the testis, which may also be responsible for the decreased testicular activity in the mice. These findings thus suggest that GnIH increases the uptake of glucose and TGs in the adipose tissue, resulting in increased accumulation of fat, whereas simultaneously in the testis, GnIH suppressed the GLUT8-mediated glucose uptake, which in turn may be responsible for decreased testosterone synthesis. This study thus demonstrates GnIH as mediator of increasing adiposity and impaired testicular function in mice.

Keywords: GnIH, adipose tissue, testis, GLUT4, GLUT8

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INTRODUCTION

Nutrition has a significant impact on reproductive processes, including steroidogenesis, gametogenesis, early embryonic development, etc. (1, 2). This association is because reproductive activities are energetically expensive, and the brain modulates the reproductive processes according to nutritional availability (3, 4). The reproductive tissues appears to have a number of "nutrient sensing" mechanisms that may link nutrient status to the reproductive system. Glucose is a very important mediator of nutritional effects on reproduction. Blood concentrations are inversely correlated to energy intake (5). Glucose is transported by the family of facilitative glucose transporters (GLUTs), which are

involved in hypothalamic regulation (6, 7). Glucose also plays a major role in providing metabolic substrates to germ cells in the gonads (8, 9). GLUTs in cells act as glucose sensors. Glucose availability influences lutienizing hormone (LH) secretion through gonadotropin-releasing hormone (GnRH) system (10). Insulin is a modulator of the metabolic stimulus and plays crucial roles in the relationship between changes in nutritional levels and reproduction (11). The anabolic actions of insulin on peripheral tissues are well established and plasma insulin also serves as a signal of body fat content to the central nervous system (12). Insulin also amplifies the lipogenesis in adipose tissue (13). Glucose is made available in the body by insulin, which helps to lower the level of circulating glucose by promoting its uptake either in adipose tissue or muscle cells through GLUT4 (14). In the gonads, glucose is essential for maintenance of spermatogenesis *in vivo* (15, 16). The isoforms of GLUTs are expressed in the testis (17). GLUT8 appears to be one of the main GLUTs in the testis (18). Furthermore, it has been demonstrated that adequate amount of GLUT is required for proper testicular activity (18). Insulin has a direct effect at the testis level (16). Insulin receptors (IR) are expressed in both somatic cells, such as Leydig, Sertoli, and peritubular cells, and germ cells in the testis of various vertebrate species (19). IR signal through the IR substrate proteins (IRS) (20, 21) plays a role in regulating fertility under normal fed conditions.

Adipose tissue is the main organ in the body that provides a storage site for triglycerides (TGs) and deals with energy homeostasis. Serum glucose is taken up and stored as fatty acid via lipogenesis in adipocytes, whereas the fall in glucose levels stimulates lipolysis, leading to release of TGs/fatty acid. Mature adipocytes synthesize and secrete numerous hormones called adipokines, which are involved in overall energy homeostasis and also modulate reproductive activities. Recent studies have shown a negative relationship between adiposity and testicular function (22, 23). Although a more recent study suggested a strong association between metabolic disorders and infertility (24), the factors mediating the influence of nutrition on reproduction are currently not clearly known and require detailed investigation.

The neural elements within the brain that control nutritional function and those that control reproduction are interconnected. Thus, studies are required to understand how the neural system that affects food intake may impact on reproductive function. As a generalization, neuropeptides that stimulate reproduction inhibit food intake and *vice versa*. Central (neuroendocrine) regulation of both reproduction and nutritional function provides a meas whereby surplus energy or energy deficit can be perceived, and accordingly, food intake and energy expenditure can be modulated (25). In 2000, a novel hypothalamic neuropeptide that actively inhibits gonadotropin release was discovered in quail and termed gonadotropin-inhibitory hormone (GnIH) (26). GnIH has a C-terminal Arg–Phe–NH_2 (RFamide) motif and acts via GnIH receptor (GnIHR), a new member of G-protein coupled receptor superfamily (GPR147), to inhibit gonadotropin release and synthesis (27, 28). The follow-up studies demonstrated that GnIH acts as a new key player for regulation of reproduction in birds and mammals (27, 28). GnIH is one of the RFamide peptides, and it is also known as RFamide-related peptide (RFRP) in mammals (27, 28). It is known that GnIH acts on gonadotropes in the anterior pituitary and GnRH neurons in the hypothalamus to inhibit gonadotropin release and synthesis via GnIHR (GPR147) in birds and mammals (27–29). Recent evidence further indicates that GnIH operates at the level of the gonads as an autocrine/paracrine regulator of steroidogenesis and gametogenesis in birds and mammals (27–29).

Recent studies suggested an inverse relationship between increasing adiposity with regressive changes in testis (22, 30). The factors responsible for regulating the inverse association between obesity and testicular activity are not yet known. GnIH is shown to have dual function: it suppresses reproductive activity while promoting fat accumulation by acting as an orexigen in birds and mammals (31–33). It is thus possible that GnIH may be the endocrine mediator between nutritional changes associated with adiposity and changes in the reproductive status. If above hypothesis is true, the GnIH should regulate food intake and promote accumulation of fat in adipose tissue; at the same time, it should exert inhibitory effect on testicular function. Therefore, the aim of this study was to determine the role of GnIH as a modulator of both testes (testosterone synthesis) as well as adipose tissue (accumulation of fat) function. This was achieved by investigating the effect of GnIH on changes in nutrients uptake and hormonal synthesis/action in the testis and adipose tissue simultaneously by *in vivo* study and separately by *in vitro* study.

MATERIALS AND METHODS

Animal

Adult Parkes strain male laboratory mice (*Mus musculus*) were obtained from the inbred colony maintained in our animal house. All experiments were conducted in accordance with principles and procedures of the 2002 Animal act, India, and approved by Animal Ethical Committee, Banaras Hindu University. The adult mice (13 weeks old) of nearly equal body weight (approximate body weight = 30 g) were used in this study. Mice were housed under optimum conditions of temperature 24 ± 2°C and humidity 50 ± 5% in a photoperiodically controlled room (12-h light:12-h dark) and were provided with commercial food (Pashu Aahar Kendra, Varanasi, India) and tap water *ad libitum*.

Chemicals

GnIH or mRFRP-3 (SIKPSAYLPLRF-NH2) was synthesized by Son et al. (34). Insulin was obtained from torrent Pharmaceuticals Ltd., Mehsana, India. Antibodies to IR β-subunit, GLUT4, and GLUT8 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and protein kinase B (PKB)/AKT was purchased from GeneScript USA Inc. (Piscataway, NJ, USA). All other chemicals were purchased from Merck, New Delhi, India. The specificity of antibodies is shown in **Table 1**.

In Vivo Study

Mice were injected daily with three different doses (20, 200 ng, and 2 μg/day) of GnIH dissolved in normal saline for 8 days ($n = 10$ per group), intraperitoneally. Mice in the control group received vehicle only. The dose and duration of GnIH was selected based on the previous study (31). Food intake was measured at every

TABLE 1 | Details of antibodies used for Western blot.

Antibody	Target species	Species raised in; monoclonal/polyclonal	Source	Concentration (used for Western blot)
GLUT4	Human	Rabbit; polyclonal	Santa cruz Biotechnology Inc. (H-61, SC 7938)	1:500
GLUT8	Human	Rabbit; polyclonal	Santa cruz Biotechnology Inc. (N-60, SC 30108)	1:500
Insulin receptor β	Human	Rabbit; polyclonal	Santa cruz Biotechnology Inc. (C-19, SC 711)	1:500
AKT	Human	Rabbit; polyclonal	GeneScript Inc. (A00965-40)	1:200
Actin	β-Actin	Mouse; monoclonal	Sigma A2228, 128K4813	1:2000

24 h. The animals were sacrificed by decapitation under a mild dose of anesthetic ether, 24 h after the last injection, and blood was collected. Body mass of each mouse was recorded before killing. Testes and adipose tissue were excised out, cleaned, weighed, and kept at −40°C for immunoblot analysis. The adipose tissue accumulated in the abdominal cavity region of the control and treated mice were excised out and weighed. Serum was collected and stored at −20°C untill testosterone assay, glucose assay, and TG assay.

Food Intake Measurement

All mice were individually housed in standard polypropylene cage with hopper style feeder, for lab blocks keep food waste minimum and wood shavings scattered on the floor. Each mouse was provided with measured amount (25 g) fresh mice feed between 1,000 and 1,100 h daily for 8 days, and the leftover food pellets (excluding fecal matter) were weighed at the end of 24 h. The total food consumed by each mouse every day was calculated by subtracting the weight of leftover food from the total amount of food given. From the individual data, average food consumed by the control and treated group were subsequently calculated. The average food consumption was measured on day first, fourth, and eight of the experiment. Average daily food intake was calculated as sum of the average food intake on day first, fourth, and eight and divided by three. The amount of food intake by each mouse showed not much change up to day third, but from day fourth up to day eighth, mice the treated with GnIH showed marked increase. Generally, the mouse consumed more food during night as comparable to day. The control mouse showed no significant change in food intake throughout the study.

In Vitro Study

Testis Culture

Adult testes ($n = 8$ testes) were quickly dissected out and cleaned of any adhered fat tissue in Dulbecco modified Eagle's medium (DMEM; Himedia, Mumbai, India) containing 250 IU/ml penicillin and 250 mg/ml streptomycin sulfate. The testes were cut into equal pieces (approximate 10 mg in weight) and cultured by the method as described previously (35). Culture medium was a mixture of DMEM (with sodium pyruvate and L-glutamine) and Ham's F-12 (1:1; *v*:*v*) (Himedia, Mumbai, India) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1% bovine serum albumin (BSA; Sigma, St Louise, MO, USA). After initial incubation for 2 h at 37°C, culture medium was discarded, and testicular slices (1 per tube) were finally cultured in 1-ml medium containing 10^{-10} and 10^{-9}M/ml GnIH along with or without 10 and 100 ng/ml luteinizing hormone (LH) in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 24 h at 37°C. The doses of GnIH and LH used for the *in vitro* study were selected from our previous study (31, 36). Testes cultured under these conditions appear healthy and do not show any sign of necrosis. Each treatment group was run in triplicate. This is evaluated by Autospan liquid gold-Lactate dehydrogenase (Surat, Gujarat, India) in culture media after 2 h of incubation at 37°C. Testicular slices were collected at the end of culture, washed several times with PBS, and stored at −40°C for immunoblot study, and media were collected, stored at −40°C until testosterone assay and glucose uptake (35).

Adipocytes Culture

White adipose tissue (WAT) collected from abdominal cavity of adult male mouse ($n = 6$) was used to determine the *in vitro* effects of GnIH with or without insulin on GLUT4, IR, and AKT/PKB protein expression in WAT of male mouse. The dose and duration of RFRP-3 was selected based on previous study (37). We assayed these biochemical markers at three doses of insulin. Culture methods for WAT were adopted according to Roy and Krishna (38). Following collection, WAT was quickly cut into pieces in DMEM (Himedia, Mumbai, Maharashtra, India) containing 250 IU/ml penicillin and 250 mg/ml streptomycin sulfate. Pieces of WAT of equal mass were cultured in a mixture of DMEM (with sodium pyruvate and L-glutamine) and Ham's F-12 (1/1 v/v) (Himedia) containing 100 IU/ml penicillin, 100 mg/ml streptomycin, and 0.1% BSA (Sigma, St Louise, MO, USA). After initial incubation for 2 h at 37°C, the culture medium was discarded, and pieces of WAT were cultured in 1-ml medium containing either 1, 5, or 10 μg/ml insulin or 10 ng/ml GnIH in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 24 h at 37°C. Each treatment group was run in triplicate. After culture, WAT was collected, washed several times with phosphate buffer saline (PBS), and kept frozen at −40°C until immunoblot assay, and media were collected, stored at −40°C until glucose uptake (38).

Immunoblot

The testes and WATs collected at the end of *in vivo* and *in vitro* studies were processed for protein extraction using the method described earlier (39, 40). Western blot analysis was performed as

previously described (38). In short, a 20% homogenate (w/v) of adipose tissue was made in suspension buffer containing 0.1M NaCl, 0.01M Tris–HCl (pH 7.6), 0.001M EDTA (pH 8.0), and 10 μg/ml phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 5,000 *g* and at 4°C for 15 min; the supernatant was extracted with an equal volume of chloroform and the aqueous phase was recovered. Equal amounts of proteins (40 μg) as determined by Folin's method were used for 10% SDS-PAGE. Thereafter, proteins were transferred electrophoretically to a PVDF membrane (Millipore India Pvt. Ltd., Bangalore, Karnataka, India) overnight at 4°C. Membranes were blocked for 1 h with Tris-buffered saline [TBS; Tris 50 mM (pH 7.5), NaCl 150 mM, 0.02% Tween 20] containing 5% fat-free dry milk. The membranes were further incubated with primary antibody (see **Table 1**) for 1 h in blocking solution. Immunodetection was performed with anti-rabbit IgG conjugated horseradish peroxidase (1:1,000) for 4 h. Finally, the blot was washed three times with TBS and developed with an enhanced chemiluminescence (ECL) detection system (Bio-Rad, Hercules, CA, USA). Similarly, a blot was developed for β-actin (Sigma-Aldrich, India) at a dilution of 1:1,000 as a loading control. Immunoreactive bands were later quantified using Image J software (Image J 1.36, NIH, Bethesda, MD, USA) (38).

Testosterone Assay

Testosterone was measured by using ELISA kit purchased from Dia Metra, Giustozzi, Foligno (PG) Italy (LOT No: *DKO002*) as described earlier (31). The 25 μl of standard, control, or sample and 100 μl of diluted conjugate solution were added to each ELISA plate. The ELISA plate was then incubated at 37°C for 1 h with mild shaking. The wells were aspirated and washed several times with distilled water. After adding 100 μl of the tetramethyl benzidine (TMB) chromagen substrate to each well, ELISA plate was incubated at room temperature for 15 min in the dark. Finally, stop solution (100 μl) was added, and absorbance was recorded at 450 nm using a microplate reader. The standard curve ranged from 0.2 to 16 ng/ml (31).

Glucose Assay

Blood glucose was measured by the glucose oxidase method using a commercially available automated glucose analyzer (Span Diagnostics Ltd., Surat, Gujarat, India) with 10 μl of blood.

Serum Triglyceride Assay and Adipose Tissue Triglyceride Content

Triglyceride in blood was measured using a commercially available colorimetric kit (GPO-Trinder) (Span Diagnostics Ltd., Surat, Gujarat, India), and TG in WAT was measured with minor modifications (41, 42). A 20% homogenate (w/v) of WAT was prepared in PBS. Then TG was extracted from the homogenate overnight in heptane:isopropanol (3:2) at 4°C. TG content was measured using a colorimetric kit (GPO-Trinder) from Span Diagnostics Ltd., Surat, Gujarat, India. Results are expressed as milligram TG per milligram protein.

Glucose Uptake Assay: *In Vitro* Study

The media stored at −20°C was used for glucose uptake assay according to Roy and Krishna (38) and Banerjee et al. (16), who described earlier to determine the glucose by quantitative colorimetric method. The glucose concentration of the media was measured at the beginning as well as at the end of culture, and the difference between initial concentration of media and final concentration of media after the 24 h culture was taken as the amount of glucose uptake by the WAT or testis. Each group was run in triplicates. The intra-assay coefficient of variation (CV) was <7.5%.

Statistical Analysis

Data were analyzed using one-way ANOVA followed by Bonferroni's test using SPSS software 16 for Windows (SPSS Inc., Chicago, IL, USA). Correlation studies were performed to compare data from different groups. The differences were considered significant at the level of $p < 0.05$.

RESULTS

Changes in Daily Food Intake

Table 2 shows the effect of *in vivo* administration of GnIH on food intake of adult male mice. Three different doses (20, 200 ng, and 2 μg/day) of GnIH administration caused a dose-dependent increase in food intake. A significant increase ($p < 0.05$) in food intake was noticed in mice treated with a high dose (2 μg/day) of GnIH for 8 days as compared with the control.

Body Mass and Adipose Tissue Mass

The body mass showed significant ($p < 0.05$) increase by the treatment with moderate and high doses (200 ng and 2 μg/day) of GnIH as compared with the control. The adipose tissue mass also changed in response to the treatment with different doses (20, 200 ng, and 2 μg/day) of GnIH. The adipose tissue mass showed no significant change when treated with a low dose (20 ng/day) of GnIH, whereas it increased significantly ($p < 0.05$) in response to moderate and high doses of GnIH treatments as compared with the control (see **Table 3**).

Circulating Triglyceride, Glucose, and Testosterone Levels

The treatment with different doses (20, 200 ng, and 2 μg/day) of GnIH showed significant variation in circulating levels of TG s ($p < 0.05$) and glucose ($p < 0.05$). Both TG s and glucose

TABLE 2 | Effect of GnIH on average daily food intake in mice (*in vivo*).

Doses of GnIH	Daily food intake (g)			Average daily food intake (g)
	Day 1	Day 4	Day 8	
Control	18.4 ± 0.095	18.1 ± 0.170	17.8 ± 0.105	18.1 ± 0.173
20 ng/day	17.08 ± 0.04	16.2 ± 0.138	17.8 ± 0.084	17.05 ± 0.482
200 ng/day	18.01 ± 0.28	22.40 ± 0.44*	24.55 ± 0.183*	21.32 ± 1.69*
2 μg/day	22 ± 0.219*	24.55 ± 0.131*	25 ± 0.002*	23.85 ± 0.93*

Values are mean ± SEM for five animals (daily food intake).
**Significantly different from controls ($p < 0.05$) by one-way analysis of variance (ANOVA) followed by Bonferroni's test.*

TABLE 3 | Effect of GnIH on body mass, WAT mass, adipose tissue TG, testicular glucose, serum TG, and serum glucose mice (*in vivo*).

Doses of GnIH	Body mass (g)	White adipose tissue mass (g)	Tissue		Serum	
			Adipose tissue Triglyceride concentration (TG/mg protein)	Testicular glucose concentration (mg/mg testis wt)	Triglyceride level (mg/dl)	Glucose level (mg/dl)
Control	33.258 ± 0.23	2.121 ± 0.10	442.8 ± 5.34	4.4 ± 0.166	140.38 ± 1.58	43.86 ± 1.68
20 ng/day	33.022 ± 0.56	1.524 ± 0.04	179.8 ± 3.32*	3.8 ± 0.12	229.25 ± 2.01*	85.65 ± 1.49*
200 ng/day	34.236 ± 0.30	2.88 ± 0.08*	256.56 ± 10.86*	3.2 ± 0.176*	196.57 ± 1.43*	65.33 ± 1.85*
2 μg/day	38.89 ± 0.38*	3.22 ± 0.05*	602.65 ± 5.85*	2.5 ± 0.17*	93.36 ± 4.13*	31.65 ± 1.43*

Values are mean ± SEM for five animals.
**Significantly different from controls ($p < 0.05$) by one-way analysis of variance (ANOVA) followed by Bonferroni's test.*

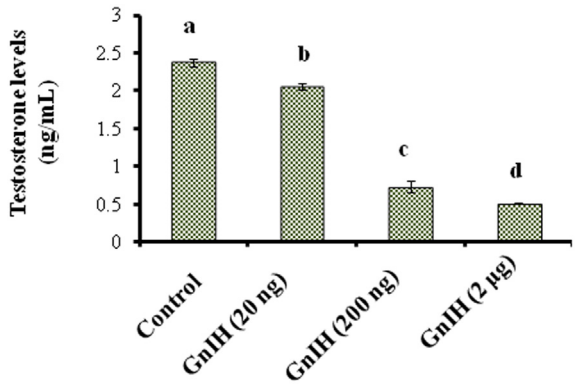

FIGURE 1 | The mice treated with different doses (20, 200 ng, and 2 μg/day) of GnIH on synthesis of testosterone in testis of mice. GnIH showed significantly decreased ($p < 0.05$) synthesis of testosterone levels in dose-dependent manner as compare to control (a, $p < 0.05$).

levels were significantly ($p < 0.05$) increased in response to low and moderate doses (20 and 200 ng/day) of GnIH treatment as compared with the control. However, both TGs and glucose levels were significantly ($p < 0.05$) decreased with a high dose (2 μg/day) of GnIH in comparison to the control (**Table 3**). The treatment with different doses of GnIH showed a significant ($p < 0.05$) dose-dependent decline in circulating testosterone levels as compared with the control (**Figure 1**).

Changes in the Level of Triglycerides and in the Expression of GLUT4 and GLUT8 and IR Proteins in the Adipose Tissue

The treatment with low and moderate doses (20 and 200 ng/day) of GnIH showed significant decrease ($p < 0.05$) in the TG level in the adipose tissue, whereas a high dose (2 μg/day) of GnIH treatment showed a significant increase ($p < 0.05$) in the TGs level in the adipose tissue as compared with the control ($p < 0.05$) **(Table 3)**.

The treatment with low dose (20 ng/day) of GnIH showed significant ($p < 0.05$) increase in the expressions of IR and GLUT8 proteins in the adipose tissue as compared with the control. However, moderate and high doses (200 ng and 2 μg/day) of GnIH showed significant ($p < 0.05$) decreased expressions of IR and GLUT8 proteins as compared with the control (**Figures 2A,B**). A low dose (20 ng/day) of GnIH treatment showed significant ($p < 0.05$) decreased expression of GLUT4 whereas a high dose (2 μg/day) showed significant ($p < 0.05$) increased expression of GLUT4 as compared with the control in the adipose tissue (**Figure 2C**).

Changes in the Level of Glucose and in the Expression of GLUT8 and IR Proteins in the Testis

The treatment with low and moderate doses (20 and 200 ng/day) of GnIH showed significant ($p < 0.05$) decrease in glucose concentrations in the testis as compared with the control. Whereas the treatment with a high dose (2 μg/day) caused a significant ($p < 0.05$) increase in glucose level in the testis as compared to treatment with the moderate dose (**Table 3**).

The treatment with low and moderate doses of GnIH showed significant ($p < 0.05$) decrease in expression of IR protein in the testis as compared with the control, whereas the treatment with a high dose caused a significant ($p < 0.05$) increase in the expression of IR protein as compared with the moderate dose treatment (**Figure 3A**).

The treatment with a moderate dose (200 ng/day) of GnIH showed a significant ($p < 0.05$) decrease in the expression of GLUT8 protein in the testis as compared with the control, whereas the treatment with a high dose (2 μg/day) caused a significant ($p < 0.05$) increase in expression of GLUT8 protein in the testis as compared with the moderate dose treatment (**Figure 3B**).

Effects of *In Vitro* Treatment of GnIH with or without LH in the Testis of Mice

Effect on Glucose Uptake

The *in vitro* treatment with low and high doses (10^{-10} and 10^{-9}M/ml) of GnIH showed a dose-dependent suppression of glucose uptake in the testis. The treatment with low and high (10 and 100 ng/ml) doses of LH alone showed a dose-dependent significant ($p < 0.05$) increase in glucose uptake by the testis. The treatment with a high dose of LH together with a high dose of GnIH showed a significant ($p < 0.05$) increase in uptake of glucose by the testis as compared with the control (**Figure 4A**).

Effect on the Expression of GLUT8 Protein

The *in vitro* treatment with low and high doses (10^{-10} and 10^{-9}M/ml) of GnIH showed a significant ($p < 0.05$) decrease in the expression of GLUT8 protein. However, the *in vitro* treatment

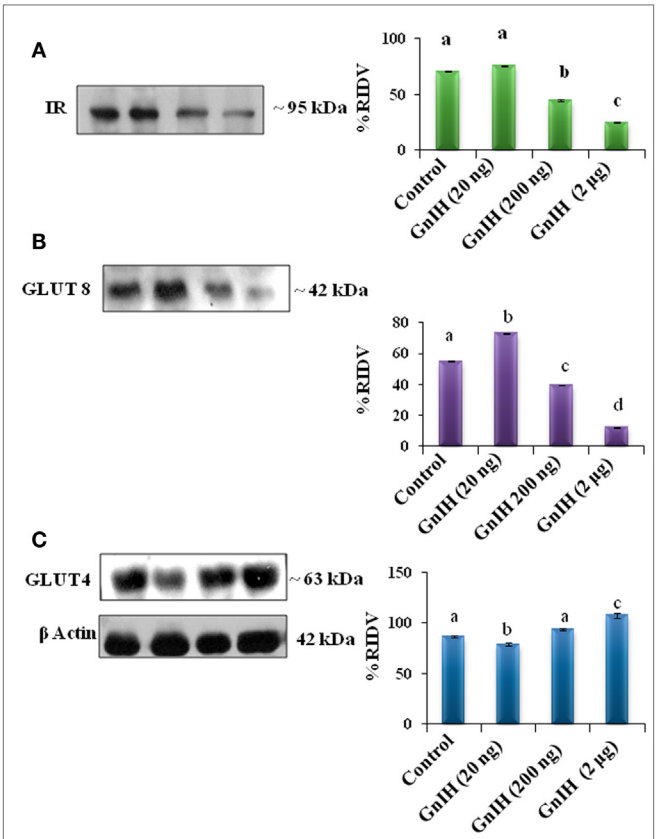

FIGURE 2 | The densitometric analysis of the Western blot showed the treatment of different doses (20, 200 ng, and 2 µg/day) of GnIH on expression of IR, GLUT8, and GLUT4 proteins in adipose tissue of mice. (A) GnIH showed a dose-dependent significant ($p < 0.05$) decline in the expression of IR. Moderate and high doses of GnIH (200 ng and 2 µg/day) showed significant (b and c, $p < 0.05$) decrease in the expression of IR protein as compare with control (a, $p < 0.05$). **(B)** A low dose of GnIH (20 ng/day) showed significant (b, $p < 0.05$) increased in the expression of GLUT8 protein as compared to the control. However, moderate and high doses of GnIH (200 ng and 2 µg/day) showed significant (c and d, $p < 0.05$) decrease in the expression of GLUT8 protein as compare with control (a, $p < 0.05$). **(C)** A low dose (20 ng/day) of GnIH treatment showed significant ($p < 0.05$) decrease in the expression of GLUT4 whereas a high dose (2 µg/day) showed significant ($p < 0.05$) increase in the expression of GLUT4 as compared with the control in the adipose tissue. Values are mean ± SEM.

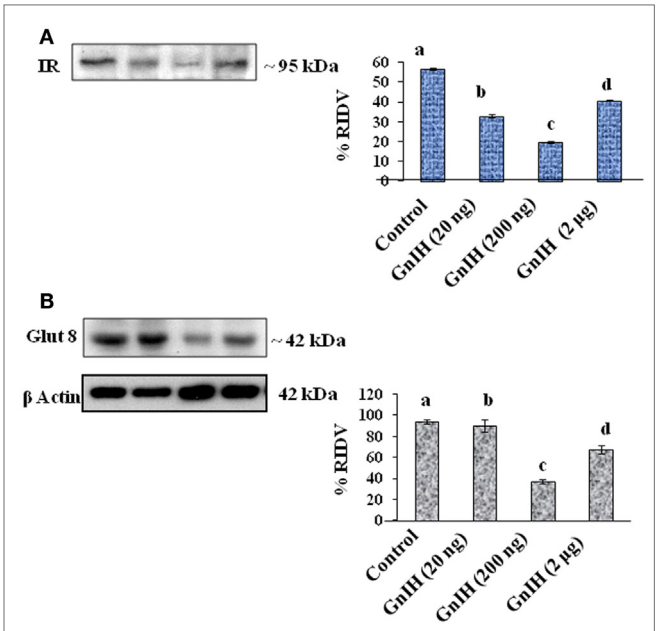

FIGURE 3 | The densitometric analysis of the Western blot showed the treatment of different doses (20, 200 ng, and 2 µg/day) of GnIH on expression of IR and GLUT8 proteins in testis of mice. (A) The treatment with low and moderate doses (20 and 200 ng/day) of GnIH showed significant (b and c, $p < 0.05$) decrease in expression of IR protein as compared with the control, whereas high dose (2 µg/day) caused a significant (d, $p < 0.05$) increase in the expression of IR protein as compared with the control and moderate dose treatment. **(B)** The treatment with a low and moderate dose (20 and 200 ng/day) of GnIH showed a significant (b and c, $p < 0.05$) decrease in the expression of GLUT8 protein as compared with the control, whereas the treatment with a high dose (2 µg/day) caused a significant (d, $p < 0.05$) increase in the expression of GLUT8 protein as compared with the moderate dose treatment. Values are mean ± SEM.

with low and high doses (10 and 100 ng/ml) of LH showed a dose-dependent significant ($p < 0.05$) increase in the expression of GLUT8 protein. The *in vitro* treatment with a high dose of LH with a high dose of GnIH showed a significant increase in the expression of GLUT8 protein in the testis as compared with the control (**Figure 4B**).

Effects of *In Vitro* Treatment of Insulin to the Adipose Tissue of Mice

Effects on the Expressions of IR, GLUT4, and AKT Proteins in the Adipose Tissue

The adipose tissue treated *in vitro* with 1, 5, and 10 µg/ml doses of insulin showed dose-dependent significant ($p < 0.05$) increase in expression of IR, GLUT4 proteins in the adipose tissue as compared with the control (**Figures 5A,B**).

The adipose tissue treated *in vitro* with different doses (1, 5, and 10 µg/ml) of insulin showed dose-dependent significant ($p < 0.05$) increase in the expression of AKT protein in adipose tissue as compare to the control (**Figure 6**).

Effects of *In Vitro* Treatment of GnIH Either Alone Or Together with Insulin to the Adipose Tissue of Mice

The Expression of IR Protein

The adipose tissue treated *in vitro* with different doses (10^{-10} and 10^{-9}M/ml) of GnIH with or without insulin (10 µg/ml) showed a dose-dependent significant ($p < 0.05$) decrease in expression of IR protein as compared with the control. The treatment with a high dose of GnIH together with insulin showed a significant ($p < 0.05$) increase in the expression of IR protein in the adipose tissue as compared with the only GnIH-treated group (**Figure 7A**).

The Expression of GLUT4 Protein

The adipose tissue treated *in vitro* with different doses (10^{-10} and 10^{-9}M/ml) of GnIH with or without insulin (10 µg/ml) showed a

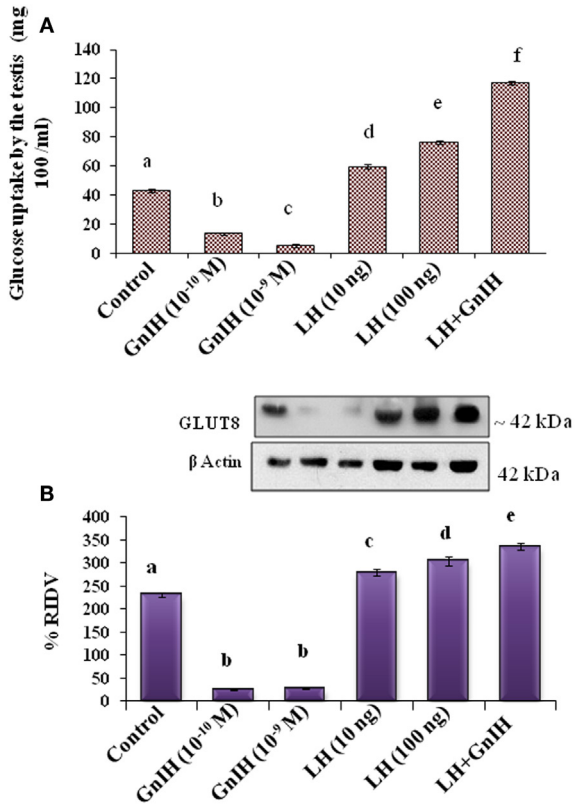

FIGURE 4 | The *in vitro* treatment of different doses (10^{-10} and 10^{-9}M/ml) of GnIH with or without different doses (10 and 100 ng/ml) of LH showed effect on glucose uptake by the testis and expression of GLUT 8 protein. (A) The treatment with low and high doses (10^{-10} and 10^{-9}M/ml) of GnIH showed a dose-dependent suppression of glucose uptake in the testis. The treatment with low and high (10 and 100 ng/ml) doses of LH alone showed a dose-dependent significant (d and e, $p < 0.05$) increase in glucose uptake by the testis. A high dose of LH together with a high dose of GnIH showed a significant (f, $p < 0.05$) increase in uptake of glucose by the testis as compared with the control (a, $p < 0.05$). **(B)** The treatment with low and high doses (10^{-10} and 10^{-9}M/ml) of GnIH showed a significant (b, $p < 0.05$) decrease in the expression of GLUT8 protein. The treatment with low and high doses (10 and 100 ng/ml) of LH showed a dose-dependent significant (c and d, $p < 0.05$) increase in the expression of GLUT8 protein. A high dose of LH with a high dose of GnIH showed a significant (f, $p < 0.05$) increase in the expression of GLUT8 protein in the testis as compared with the control (a, $p < 0.05$).

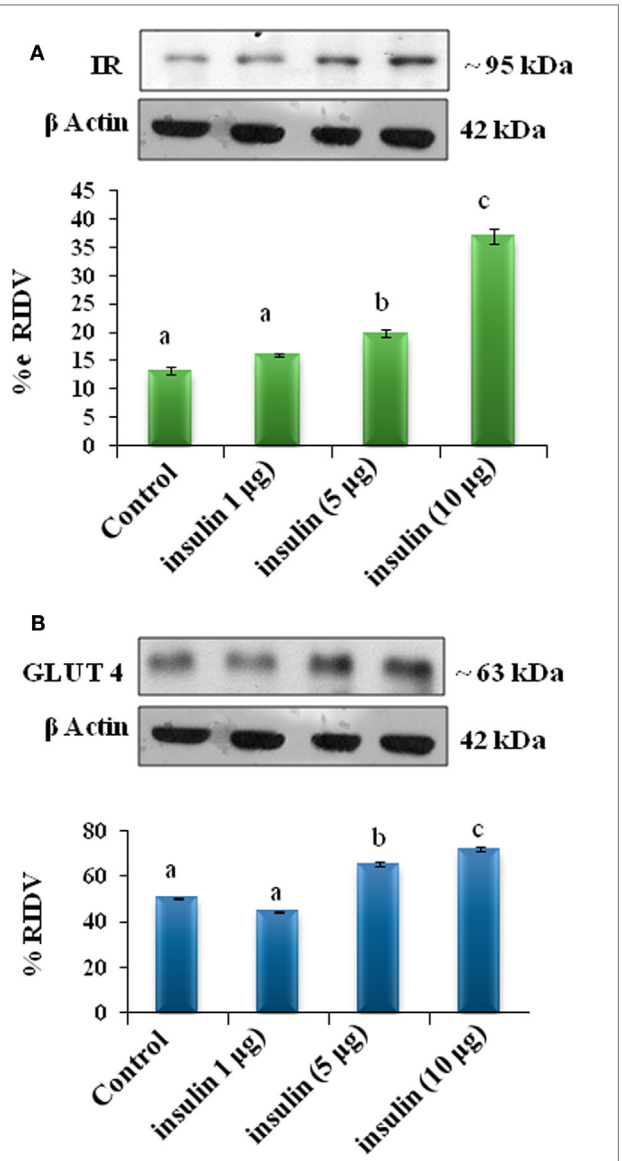

FIGURE 5 | The *in vitro* treatment with different doses of insulin (1, 5, and 10 µg/ml) on expression of insulin receptor and GLUT4 proteins in the adipose tissue. The treatment of insulin showed dose-dependent significant (b, c and d, $p < 0.05$) increase in expression of IR **(A)** and (b and c, $p < 0.05$) GLUT4 **(B)** proteins in the adipose tissue as compared with the control (a, $p < 0.05$).

dose-dependent significant ($p < 0.05$) increase in the expression of GLUT 4 protein as compared with the control. The treatment with the high dose of GnIH together with insulin showed a significant ($p < 0.05$) increase in the expression of GLUT4 protein in the adipose tissue as compared with only GnIH-treated group (**Figure 7B**).

The Expression of AKT/PBK Protein

The adipose tissue treated *in vitro* with different doses (10^{-10} and 10^{-9}M/ml) of GnIH alone showed a dose-dependent significant ($p < 0.05$) decrease in the expression of AKT protein as compared with the control. The treatment with a high dose of GnIH together with insulin showed a significant ($p < 0.05$) increase in the expression of AKT protein in the adipose tissue as compared with the only GnIH-treated group (**Figure 8**).

Correlation Study

The results of correlation study studies are summarized in **Table 4**. A significant ($p < 0.05$) correlation was found between changes in circulating testosterone levels, glucose levels, and TG levels with changes in the rate of expression of GLUT4, GLUT8, and IR in adipose tissue and testis of mice treated with GnIH.

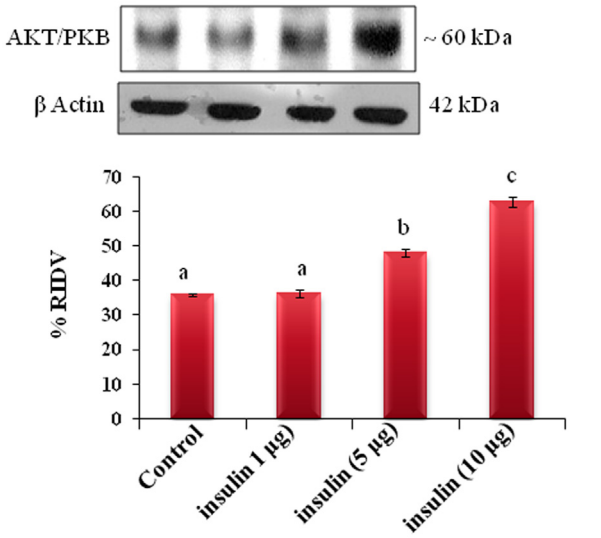

FIGURE 6 | The *in vitro* treatment of adipose tissue with different doses (1, 5, and 10 μg/ml) of insulin showed dose-dependent significant (b, c, and d, $p < 0.05$) increase in the expression of AKT/PKB protein in adipose tissue as compare to the control (a, $p < 0.05$).

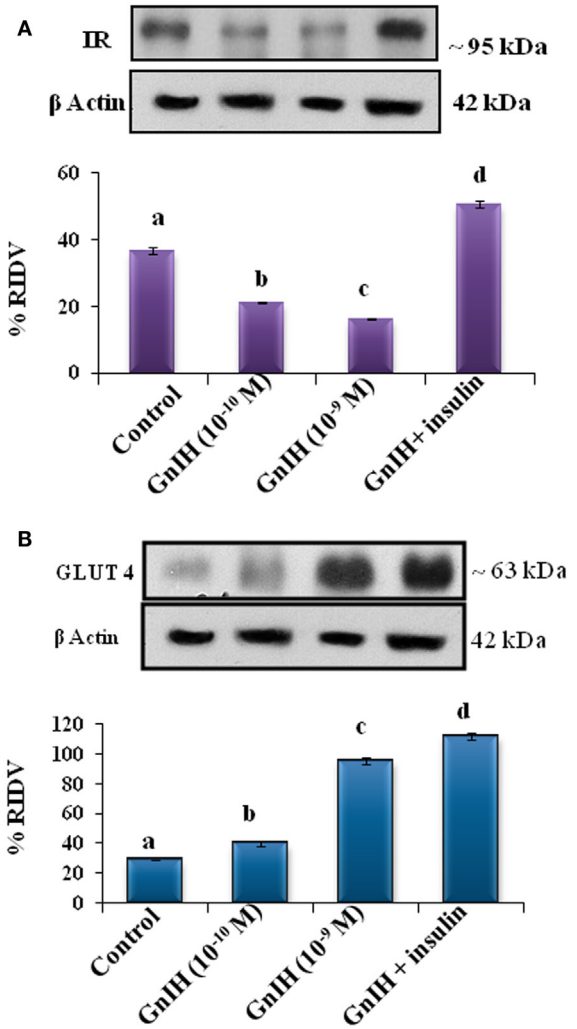

FIGURE 7 | The *in vitro* treatment of adipose tissue with different doses (10^{-10} M and 10^{-9} M/ml) of GnIH with or without insulin (10 μg/ml) showed (A) a dose-dependent (b and c, $p < 0.05$) significant decrease in expression of IR protein as compared with the control with (10^{-10} M and 10^{-9} M/ml) of GnIH. The treatment with a high dose of GnIH together with insulin showed a significant (d, $p < 0.05$) increase in the expression of IR protein in as compared with the only GnIH-treated group. **(B)** The *in vitro* treatment of adipose tissue with different doses (10^{-10} and 10^{-9}M/ml) of GnIH with or without insulin (10 μg/ml) showed a dose-dependent significant ($p < 0.05$) increase in the expression of GLUT 4 protein as compared with the control. The treatment with the high dose of GnIH together with insulin showed a significant (d, $p < 0.05$) increase in the expression of GLUT4 protein in the adipose tissue as compared with only GnIH-treated group.

DISCUSSION

For the first time, the results of present study provide the experimental proof suggesting an active participation of GnIH in increased food intake and glucose and TGs uptake in adipose tissue results in fat accumulation and increased body mass whereas in testis, GnIH suppressed glucose uptake resulting in decreased testosterone synthesis. In the present study, three different doses of GnIH were used, and the results showed dose-dependent changes in majority of the studies. But treatment with the high dose of GnIH (2 μg/day) showed a significant variation as compared with the control, thus the results of high dose of GnIH treatment are generally considered for discussion.

The mice treated *in vivo* with GnIH showed both dose- and duration-dependent increase in food intake. High dose of RFRP-3 (2 μg/day) showed increased food intake on day 1 itself as compared with the control, whereas moderate dose of RFRP-3 (200 ng/day) showed significant increase in food intake on day 4. The lower dose of RFRP-3 did not show any significant increases in food intake until day 8 (**Table 2**). Thus moderate and high doses of GnIH induced a significant increase in food intake as compared to the control, and it is therefore considered that GnIH increases food intake in mice. In addition, the mice treated *in vivo* with GnIH for 8 days showed increased accumulation of adipose tissue and body mass as compared to the control. These findings are in agreement with earlier observations in chicks and rat (32, 43) and suggest that GnIH is one of the orexigenic peptide in mammals. An earlier study has demonstrated the presence of RFRP-3 receptor (GPR147) in the adipose tissue (44). The GnIH containing neurons was shown in contact with the neurons containing neuropeptides, such as neuropeptideY, pro-opio melanocortin, orexin, and melanopeptide, which are known modulators of nutritional changes (28, 45, 46). In an earlier study, where rat was treated with GnRH agonist showed a dose-dependent gain in the body mass (47). It was suggested that GnRH agonist-induced increase in the body mass might be due to neuropeptideY- and/or opioidpeptide-mediated increase in feeding activity in the rats (48). Accordingly, in the present study, GnIH may act as a modulator of energy homeostasis association with other neuropeptides in the mice.

Interestingly, in the present study, *in vivo* treatment of GnIH showed dose-dependent increase in the expression of GLUT4 protein, but significant decline in the expression of GLUT8 and IR proteins in the adipose tissue. GLUT4, a major isoform of GLUT, is normally found in the insulin-responsive tissue, such as the striated muscle and adipose tissue, and plays a crucial role in whole body glucose homeostasis. It is well known that insulin upregulates the expression of GLUT4 protein (49, 50). Besides GLUT4, GLUT8, and GLUT12 are other GLUTs regulated by insulin (51). GLUT8 is mainly expressed in the striated muscle, adipose tissue, testis, blastocyst, brain, liver, and kidney (52, 53). The upregulation of GLUT4 by GnIH in the adipose tissue is unique findings of this study. Despite increased expression of GLUT4, GnIH treatment caused dose-dependent downregulation of GLUT8 and IR proteins in the adipose tissue. In adipose tissue, expression of GLUT4 is under regulation of insulin, but regulation of GLUT8 is not known. This study also showed a significant positive correlation between changes in expression of IR with GLUT8 protein but showed a negative correlation between the changes in expression of IR with GLUT4 protein in the adipose tissue treated *in vivo* with GnIH. This suggests that in adipose tissue, expression of GLUT8 protein changes with IR level. Furthermore, the *in vitro* study confirmed the *in vivo* observations of GnIH-induced upregulation of GLUT4 together with the downregulation of IR proteins in the adipose tissue. These observations thus suggest that GnIH increases glucose uptake in the adipose tissue stimulating expression of GLUT4.

The *in vivo* treatment with high dose of GnIH showed significantly increased synthesis of TGs in adipose tissue. GnIH-induced increased expression of GLUT4 suggests increased uptake of glucose into adipose tissue, which subsequently may get converted into TG and free fatty acid, the storage form of nutrients in the adipocytes. The mice treated with GnIH *in vivo* showed dose-dependent significant decline in the circulating TG and glucose levels. Simultaneously with decline in circulating TG levels, this study showed significant increase in the TG level in the adipose tissue as compared with the control. The significant inverse correlation between changes in TG level in the serum verses adipose tissue suggest that GnIH may be responsible for increased transport of TGs from serum to adipose tissue. This

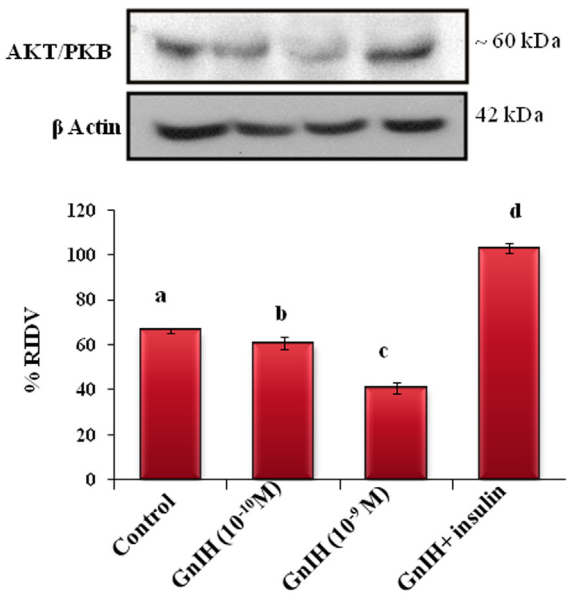

FIGURE 8 | The *in vitro* treatment of adipose tissue with different doses (10^{-10} and 10^{-9}M/ml) of GnIH alone showed a dose-dependent significant (b and c, $p < 0.05$) decrease in the expression of AKT/PKB protein as compared with the control. The treatment with a high dose of GnIH together with insulin showed a significant (d, $p < 0.05$) increase in the expression of AKT/PKB protein in the adipose tissue as compared with the only GnIH-treated group. Values are mean ± SEM.

TABLE 4 | Correlation studies of GnIH treatment between the different parameters in adipose tissue and testis (*in vivo*).

	Adipose tissue TG	Serum TG	Serum glucose	Testicular glucose level	Testosterone level	Adipose tissue			Testis	
						GLUT4	GLUT8	IR	GLUT8	IR
Adipose tissue TG		$r = -0.99$, $p < 0.05$	$r = -0.97$, $p < 0.05$	NS	NS	$r = 0.89$, $p < 0.05$	$r = -0.67$, $p < 0.05$	$r = -0.57$, $p < 0.05$	NS	$r = 0.57$, $p < 0.05$
Serum TG			$r = 0.98$, $p < 0.05$	NS	NS	$r = -0.91$, $p < 0.05$	$r = 0.67$, $p < 0.05$	$r = 0.57$, $p < 0.05$	NS	$r = 0.56$, $p < 0.05$
Serum glucose				NS	NS	$r = -0.93$, $p < 0.05$	$r = 0.63$, $p < 0.05$	NS	NS	$r = -0.56$, $p < 0.05$
Testicular glucose level					$r = 0.94$, $p < 0.05$				$r = 0.63$, $p < 0.05$	NS
Testosterone level									$r = 0.82$, $p < 0.05$	$r = 0.56$, $p < 0.05$
GLUT4							$r = -0.97$, $p < 0.05$	$r = -0.94$, $p < 0.05$	NS	NS
GLUT8								$r = 0.99$, $p < 0.05$	NS	$r = 0.78$, $p < 0.05$

NS, not significant.
Values are significantly different at $p < 0.05$.

consequently may be responsible for increased accumulation of fat in the adipocytes and consequently increase in body mass.

The mice treated *in vivo* with GnIH showed a dose-dependent significant ($p < 0.05$) decline in the level of testosterone as compared with the control. The earlier study on hamster testes revealed the expression of RFRP-receptor (GPR147). Thus, suggesting autocrine or paracrine role of RFRP in testis (54). The GnIH treatment *in vivo* also showed a significant decline in the expression of GLUT8 and IR proteins together with decreased concentration of glucose in the testis. This finding thus suggests that the mice treated with GnIH caused reduced availability of glucose to the testis; this consequently may be responsible for the decreased testosterone synthesis. This finding further confirms our recent study that the decreased availability of glucose to the testis resulted in the decreased steroidogenesis (16). It has earlier been demonstrated that glucose availability also modulates the level of the steroidogenic enzyme nicotinamide adenine dinucleotide phosphate (NADPH) in the testis (55). The *in vivo* treatment of GnIH also caused suppressed expression of IR protein in the testis. Since insulin directly affects testicular steroidogenesis *via* the induction of dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1) in Leydig cells (56), it possible that GnIH may inhibit steroidogenesis by downregulating IR in the testis.

Similar to *in vivo* study, the testes treated *in vitro* with GnIH also showed significant decreased expression of GLUT8 protein, decreased concentration of glucose, and significant decline in the testosterone synthesis. These findings thus suggest that the GnIH-induced decreased uptake of glucose may be directly responsible for the inhibition of testicular steroidogenesis. This study confirmed the earlier finding that Leydig cells culture in the absence of glucose can synthesis testosterone at very low rate (16, 57). The testis treated *in vitro* with LH either alone or together with GnIH showed a stimulatory effect on the glucose uptake together with the increased expression of GLUT8 protein and increased synthesis of testosterone. This study suggests that in presence of physiological concentration of LH, inhibitory effect of GnIH is suppressed. Our earlier studies have shown that LH or hCG increases glucose uptake by increasing GLUT8 expression in the testis (16). Thus in the physiological condition, when LH in sufficient concentration exist in the plasma, GnIH may facilitate glucose uptake by promoting GLUT8 in testis.

To investigate the signaling pathway of GnIH and insulin-mediated glucose uptake, AKT/PKB levels were evaluated in the adipose tissue. The adipose tissue from mice treated with different doses of GnIH with or without insulin *in vitro* showed a significant variation in the expression of AKT/PKB protein together with the expression of GLUT4 and IR proteins. The GnIH-induced changes in GLUT4 levels showed no significant correlation with the changes in AKT/PKB levels, but correlated significantly with the changes in IR protein. Furthermore, the insulin-induced changes in GLUT4 and IR levels correlated significantly with the changes in AKT/PKB levels in the adipose tissue. Although AKT/PKB is involved in many signaling pathways including glucose trafficking (58), this finding suggests that AKT/PKB may function as a signaling molecule in the insulin-induced GLUT4 (59) and IR expression as well as in the GnIH-induced IR expression in the adipose tissue. But AKT/PKB may not function as a signaling molecule in the GnIH-induced changes in the expression of GLUT4 protein in the adipose tissue.

In brief, the results of this study showed differential effect of GnIH-induced changes in nutrient levels of the adipose tissue and testis of mice. The *in vivo* treatment with GnIH showed the increased expression of GLUT4 together with increased uptake of TGs in the adipose tissue, which in turn resulted in the increased accumulation of fat into WAT and increase in the body mass. On the contrary in the testis, GnIH treatment *in vivo* caused downregulation of GLUT8 expression resulting in the decreased uptake of glucose, which in turn resulted in the decreased synthesis of testosterone. The mice treated with GnIH also showed the decreased expression of IR protein in the testis. The decreased level of IR may be responsible for the decreased testicular activity in the mice. The *in vitro* treatment of GnIH to the adipose tissue showed the increased expression of GLUT4 suggesting the increased uptake of glucose, despite decrease in IR. The *in vitro* treatment of GnIH to the testis showed decreased expression of GLUT8 protein resulted in decreased uptake of glucose and testosterone synthesis. These findings thus suggest that GnIH increases nutrients (glucose and TGs) uptake in the adipose tissue resulted in accumulation of fat, whereas in the testis GnIH suppresses the glucose uptake resulted in the decreased testosterone synthesis in mice. This study further showed that the insulin-induced upregulation of GLUT4 expression may be PKB/AKT mediated, whereas GnIH-induced upregulation of GLUT4 is not mediated through PKB/AKT mechanisms. On the basis of present study, GnIH appears to serve an important role in determining the level of fat accumulation in the adipose tissue and accordingly modulating the reproductive strategies to be adopted by the animal. Further studies are required to find out whether the GnIH is associated with decline in testosterone as observed during diabetes mellitus type II, aging, or subfertility in men.

AUTHOR CONTRIBUTIONS

SA executed experimental design and analyze the result and manuscript preparation, AK analyze the result and edited the manuscript, and KT edited the whole manuscript with important suggestions.

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Gonadotropin-Inhibitory Hormone Plays Roles in Stress-Induced Reproductive Dysfunction

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Physical and psychological stressors suppress hypothalamic–pituitary–gonadal axis activity and sexual behavior and consequently induce reproductive dysfunction. Recently, it has been shown that gonadotropin-inhibitory hormone (GnIH), also called RFamide-related peptide 3 (RFRP) in mammals, which is a potent inhibitory regulator of gonadotropin-releasing hormone (GnRH) and gonadotropin, is involved in stress-induced reproductive dysfunction. GnIH/Rfrp (the gene coding RFRP-3) expression and activity are increased by psychological and immune stress, and this alteration suppresses GnRH and gonadotropin secretion. Glucocorticoid acts as a mediator that interacts between stress and hypothalamic GnIH/RFRP-3. GnIH/RFRP-3 also plays important roles in stress-induced suppression of sexual behavior and infertility, and genetic silencing of GnIH/Rfrp completely recovers sexual behavior and fertility. This review summarizes what is currently known about the roles of GnIH in stress-induced reproductive dysfunction.

Keywords: gonadotropin-inhibitory hormone, RFRP-3, Rfrp, stress, hypothalamic–pituitary–gonadal, sexual behavior

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INTRODUCTION

Humans and animals have a finite amount of energy for their activities. Therefore, if any activity has to be energetically prioritized, energy for other activities will be suppressed. Although these changes may play some role in regulation of homeostasis, they occasionally result in negative consequences for normal physiological function and accelerate some diseases. Reproductive functions are often suppressed when large amounts of energy will be used for other physiological functions because such reproductive processes are not essential for individual survival (1). Several kinds of stress, such as infection, psychological burden, and excess of exercise, are thought to be pivotal triggers of reproductive dysfunctions in humans and animals (2–4). Generally, stress activates some endocrine and immune systems, such as the hypothalamic–pituitary–adrenal axis and pro-inflammatory cytokines, to regulate homeostasis. However, such alterations act to suppress reproductive function at the same time.

Reproductive function is mainly regulated by the hypothalamic–pituitary–gonadal (HPG) axis in humans and animals. Physical and psychological stressors suppress HPG activity through inhibition of gonadotropin-releasing hormone (GnRH) in both males and females (5–7), thereby decreasing luteinizing hormone (LH) and follicle-stimulating hormone release from the pituitary (8, 9). When the relationships between stress and reproductive functions are evaluated experimentally, inflammatory stress induced by a Gram-negative bacterial cell wall component, lipopolysaccharide

(LPS), and psychological stress induced by restraint stress are frequently used. Similar to other stressors, these stresses induce some sickness behaviors, and they also suppress HPG activity through inhibition of GnRH synthesis and secretion in several mammals and birds (10–17). In addition, it has been well established that the actions of some stress-related endocrine, neuroendocrine, and inflammatory factors, such as pro-inflammatory cytokines, corticotropin-releasing hormone, and glucocorticoid/corticosterone, are increased by stress and that these alterations act to decrease GnRH and gonadotropin secretion in times of stress (15, 18). However, the results of studies have been slightly controversial, and it has been assumed that some other factors may also be involved in the stress-induced suppression of HPG activity. A breakthrough occurred in the early 21st century when a novel RFamide peptide that directly suppresses GnRH/gonadotropin synthesis and secretion was newly discovered (19). In birds, this neuropeptide was named gonadotropin-inhibitory hormone (GnIH), and later studies have shown that GnIH and its receptor, G-protein-coupled receptor (GPR) 147, have pivotal roles in the regulation of physiological function of the HPG axis, such as GnRH pulses and surges, in many species (20–22). The mammalian orthologous gene and peptide for avian GnIH are called Rfrp and RFRP-3, respectively. It has been gradually shown that GnIH/RFRP-3 and GPR147 also play important roles in the stress-induced suppression of reproductive dysfunction.

This paper presents a review of what is currently known about the roles of GnIH/RFRP-3 in stress-induced reproductive dysfunction in experimental animals. The main focus is on the relationship between GnIH/RFRP-3 and the HPG axis, but the role of GnIH/RFRP-3 in reproductive behavior under stress is also examined.

THE ROLE OF GnIH/RFRP-3 IN STRESS-INDUCED SUPPRESSION OF THE HPG AXIS

In the year 2000, Tsutsui and colleagues discovered a novel neuropeptide that actively suppresses gonadotropin release from cultured bird pituitary (19). Because this was the first demonstration of a hypothalamic factor that suppresses gonadotropin release, this neuropeptide was named GnIH based on its biological action. Thereafter, GnIHs were further identified in other vertebrates, mammals, primates, and humans (21–23). Because the identified neuropeptides possess LPXRFamide (X = L or Q) motif at their C-termini, the mammalian GnIH orthologous gene and peptide are called Rfrp and RFRP-3, respectively. GnIH/RFRP-3 neurons project to the median eminence in birds and female sheep and suppress the secretion and synthesis of gonadotropin *via* GnIH/RFRP-3 receptor GPR147 under both *in vivo* and *in vitro* conditions in male birds, female rats, and female sheep (19, 22, 24, 25). GnIH/RFRP-3 neurons also project to GnRH neurons and inhibit their activity *via* GPR147 in birds and mammals (22, 26, 27). Therefore, GnIH/RFRP-3 inhibits gonadotropin secretion and synthesis through direct and indirect actions on the pituitary. It has been shown that GnIH/RFRP-3 inhibits GnRH-elicited gonadotropin release and decreases LH pulse amplitude in female sheep (22, 24), and GnIH/RFRP-3 activity is decreased by high estradiol (E2) concentrations at the time of the GnRH/LH surge in female hamsters (**Figure 1A**) (20, 22). Similarly, elevated estrogen lowers Rfrp mRNA levels in male and female mice (28). These results show that GnIH/RFRP-3 plays important roles in the regulation of the HPG axis to maintain normal reproductive ability. On the other hand, it has recently been shown that some kinds of stresses induce acute and chronic elevations of the number of GnIH/RFRP-3-immunoreactive cells and GnIH gene expression in the hypothalamus and that these changes disrupt the function of the HPG axis and, consequently, suppress reproductive ability. Kirby et al. found that acute (3 h, measured immediately after stress) and chronic (14 days, 3 h/day, measured 24 h after the end of the last stress) immobilization stresses, common psychological stress models, lead to upregulation of Rfrp mRNA expression levels in the dorsomedial hypothalamic area, and that changes of Rfrp mRNA levels correlate negatively with serum LH levels in male rats (29). In addition, they showed that 53% of RFRP-3 neurons express glucocorticoid receptor (GR) and that adrenalectomy abolishes the chronic immobilization stress-induced increase in Rfrp expression levels (29). Similarly, corticosterone administration increases Rfrp mRNA expression levels *in vitro* in rHypoE23, which is an Rfrp-expressing cell line derived from rat hypothalamus (30). These effects of corticosterone on rHypoE-23 are blocked by a GR antagonist (31, 32). Treatment with cortisol in fish increases GnIH mRNA levels and reduces GnRH mRNA and serum LH levels (33). In addition, dexamethasone exposure during the neonatal period in female mice increases Rfrp mRNA levels, and it reduces GnRH mRNA levels and delays pubertal onset (34). These data indicate that hypothalamic GnIH/RFRP-3 integrates the suppressive effects of glucocorticoid on the HPG axis under psychological stress conditions. Recently, Peragine et al. showed that RFRP-3 suppresses sexual maturation in socially non-dominant female rats living in colonies, where breeding is monopolized by dominant animals (35). This result also indicates that RFRP-3 may be involved in the social stress-induced suppression of reproductive function.

Recently, we evaluated the relationship between immune stress and hypothalamic Rfrp mRNA expression levels in female rats. An injection of a septic dose (5 mg/kg) of LPS in female rats was found to increase hypothalamic Rfrp and GPR147 mRNA levels 6 h after injection, whereas it reduced serum LH levels and hypothalamic GnRH mRNA levels (36). In this condition, Rfrp mRNA levels were negatively correlated with GnRH mRNA and serum LH levels. Interestingly, a lower dose (500 μg/kg) of LPS did not change Rfrp and GPR147 mRNA levels, although it decreased serum LH levels. Similarly, Lopes et al. have shown in birds that an injection of 2 mg/kg of LPS suppressed GnRH mRNA and peptide expressions 2 h after injection, but did not affect GnIH (37). These results suggest that the underlying mechanisms of dysfunction of gonadotropin secretion are changed according to the severity of immune stress, and that changes of some reserve factors, i.e., GnIH/RFRP-3, begin to participate in the suppression of GnRH and gonadotropin under severe conditions. On the other hand, the factors involved in the upregulation of Rfrp in times of immune stress have not been elucidated. Although we think that not only glucocorticoid but also pro-inflammatory cytokines play some roles, further examinations are needed to clarify this

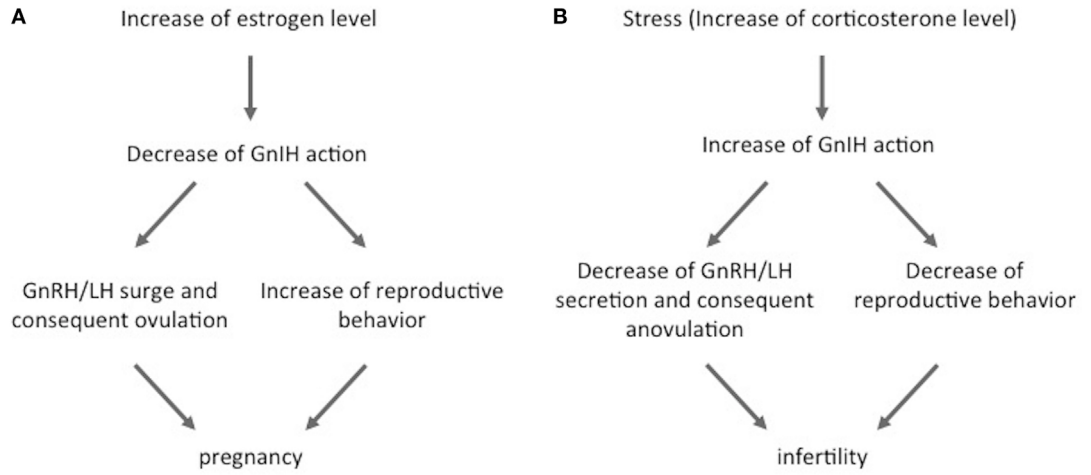

FIGURE 1 | **The role of gonadotropin-inhibitory hormone (GnIH) under physiological and stress conditions**. **(A)** GnIH participates in the control of physiological GnRH/luteinizing hormone (LH) regulation. GnIH activity is decreased by high estrogen concentrations at the time of the GnRH/LH surge. This alteration promotes both ovulation and reproductive behaviors and consequently increases the chance of pregnancy. **(B)** On the other hand, GnIH plays roles in stress-induced reproductive dysfunction. GnIH expression and activity are increased by stress, and this alteration suppresses the hypothalamic–pituitary–gonadal axis action by inhibition of GnRH/LH secretion. It also decreases reproductive behavior and, consequently, decreases the chances of pregnancy and induces infertility. Such stress-induced changes of GnIH may be partially induced by corticosterone secreted from the adrenal gland.

hypothesis. It has also been reported that metabolic challenge, a type of energetic stress, has no effect on Rfrp mRNA levels or RFRP-3 neuronal activity in female mice (38). This result indicates that GnIH/RFRP-3 mediates some kinds of, but not all, stressors.

In summary, GnIH/RFRP-3 plays roles in the suppression of the HPG axis in times of stress (**Figure 1B**). Glucocorticoid may be one of the mediators that transmit the stress signal to hypothalamic GnIH/RFRP-3 neurons. However, it is also possible that the importance of GnIH/RFRP-3 in HPG dysfunction may be changed according to the kind and severity of stressor.

THE ROLE OF GnIH/RFRP-3 IN STRESS-INDUCED SUPPRESSION OF REPRODUCTIVE BEHAVIORS

As well its role in the HPG axis, GnIH/RFRP-3 also plays some roles in the regulation of reproductive behavior in some species. Johnson et al. found that central injection of RFRP-3 decreased sexual behavior in male rats (39), whereas Piekarski et al. reported that it decreased sexual motivation without affecting lordosis behavior in female hamsters (23, 40). Piekarski et al. also showed that administration of RFRP-3 affected neuronal activity in some hypothalamic nuclei, i.e., preoptic area, medial amygdala, and the bed nucleus of the stria terminalis, which are implicated in female sexual behaviors. Therefore, it had been assumed that a stress-induced increase of GnIH/RFRP-3 activity might suppress not only the HPG axis but it may also suppress sexual behavior and, consequently, promote infertility or subfertility. Recently, Geraghty et al. published an excellent report on this matter. In their study, they showed that chronic (3 h/day for 18 days) immobilization stress in female rats led to elevated hypothalamic Rfrp mRNA expression levels both immediately after and 4 days after the end of stress (41). This chronic stress did not affect the estrous cycle, but it decreased sexual behavior and pregnancy rates and increased embryo resorption when they mated 4 days after cessation of stress. The authors further showed that genetic silencing of Rfrp with shRNA during stress completely recovered the sexual behavior, pregnancy rate, and litter size when the females were mated after cessation of stress. These results indicate that stress-induced GnIH/RFRP-3 is related to dysfunction of sexual behavior under stress, including effects on the pregnancy rate and litter size (**Figure 1B**). Therefore, GnIH/RFRP-3 may be one of the clinical targets to prevent stress-induced infertility.

CONCLUSION

Recent studies have shown that GnIH/RFRP-3 plays roles in stress-induced reproductive dysfunction in many species. A stress-induced increase of GnIH/RFRP-3 actions not only suppresses the HPG axis but also disrupts sexual behavior, and these alterations have adverse effects on the pregnancy rate and litter size. These results indicate that GnIH/RFRP-3 may be one of the clinical targets to restore stress-induced infertility. However, because there are only limited data about the roles of GnIH/RFRP-3 in humans, more evaluations in humans would be needed to apply GnIH/RFRP-3 in a clinical setting.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contributions to the work and approved it for publication.

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Early-Life Social Isolation Impairs the Gonadotropin-Inhibitory Hormone Neuronal Activity and Serotonergic System in Male Rats

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Social isolation in early life deregulates the serotonergic system of the brain, compromising reproductive function. Gonadotropin-inhibitory hormone (GnIH) neurons in the dorsomedial hypothalamic nucleus are critical to the inhibitory regulation of gonadotropin-releasing hormone neuronal activity in the brain and release of luteinizing hormone by the pituitary gland. Although GnIH responds to stress, the role of GnIH in social isolation-induced deregulation of the serotonin system and reproductive function remains unclear. We investigated the effect of social isolation in early life on the serotonergic–GnIH neuronal system using enhanced green fluorescent protein (EGFP)-tagged GnIH transgenic rats. Socially isolated rats were observed for anxious and depressive behaviors. Using immunohistochemistry, we examined c-Fos protein expression in EGFP–GnIH neurons in 9-week-old adult male rats after 6 weeks post-weaning isolation or group housing. We also inspected serotonergic fiber juxtapositions in EGFP–GnIH neurons in control and socially isolated male rats. Socially isolated rats exhibited anxious and depressive behaviors. The total number of EGFP–GnIH neurons was the same in control and socially isolated rats, but c-Fos expression in GnIH neurons was significantly reduced in socially isolated rats. Serotonin fiber juxtapositions on EGFP–GnIH neurons were also lower in socially isolated rats. In addition, levels of *tryptophan hydroxylase* mRNA expression in the dorsal raphe nucleus were significantly attenuated in these rats. These results suggest that social isolation in early-life results in lower serotonin levels, which reduce GnIH neuronal activity and may lead to reproductive failure.

Keywords: GnRH, dorsomedial hypothalamus nuclei, serotonin, social stress, dorsal raphe nuclei

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INTRODUCTION

Gonadotropin-releasing hormone (GnRH) and the newly identified neuropeptide gonadotropin-inhibitory hormone (GnIH) are regulators of reproductive activity in vertebrates (1). GnIH neuropeptides contain an Arg–Phe–NH_2 motif [LPXRFamide (X = L or Q) sequence] at their C termini in most vertebrate species. Rat LPXRFamide peptides are known as RFamide-related peptides (RFRPs; RFRP-1 and -3) (2). GnIH-expressing cells are mainly located in the

dorsomedial hypothalamic nucleus (DMN) in rats (3, 4) and send fiber projections to GnRH neurons in the pre-optic area (POA), median eminence, and other areas of the brain in rodents (5–8). GnIH acts on GnRH neurons through its seven-transmembrane domain G protein-coupled receptor (GPR) 147 (7, 9).

Recent studies have demonstrated that GnIH neuronal activity is linked to the hypothalamic–pituitary–adrenal (HPA) axis, since glucocorticoids can stimulate *gnih* mRNA expression (10–12). Our recent study (8) showed that exposure to the glucocorticoid receptor agonist dexamethasone during early life increases GnIH expression, GnIH receptor expression, and the number of fiber projections to the POA in adult female mice. These findings suggest that the GnIH system is sensitive to glucocorticoids, which can influence GnRH neuronal activity and reproduction.

Stress and glucocorticoids modulate the serotonin [5-hydroxytryptamine (5-HT)] system in the brain. Several lines of evidence suggest that 5-HT can modulate GnIH neuronal activity. Cellular localization of 5-HT_{1A} receptors is evident in the DMN, where GnIH neurons reside in the rat brain (13). In fact, 5-HT receptors (5-$HT_{1A, 1B, 1D, 1F, 2A, 2B, 3A, 5A, 5B, 6, \text{and } 7}$) are co-expressed in GnIH neurons in the DMN (14). Acute glucocorticoid treatment can cause 5-HT to accumulate in the DMN (15, 16) and treatment with the antidepressant citalopram can stimulate the GnIH system in male mice (14). The 5-HT system of the brain modulates sexual behavior, sexual arousal, and motivation in rodents (17); thus, 5-HT–GnIH signaling may participate in the negative regulation of reproductive activity, including sexual behavior.

Social isolation, a passive stress, activates the HPA axis (18, 19), causes imbalances in 5-HT turnover in rats (20) and decreases the number of 5-HT neurons, expression of 5-HT receptors, number of 5-HT fiber projections in the hippocampus (21–23) and binding activity of the 5-HT_{1A} receptor (24). Indeed, social isolation in early life impairs 5-HT-associated functions of the brain, which include the control of anxiety, depression, aggression (22, 25–27), and sexual behavior in adult male rats (28–30). Furthermore, social isolation results in a significantly higher level of plasma testosterone (31, 32) and increased testis weight (32) in male rats. Social isolation in early life may affect the production of sex steroids and related behaviors, such as sexual behavior and aggression. The neuronal mechanism underlying early-life social isolation-induced reproductive failure through the deregulation of 5-HT and the GnIH system remains unknown.

We examined the activity of GnIH neurons in our newly created enhanced green fluorescent protein (EGFP)–GnIH transgenic rats. We measured 5-HT fiber juxtapositions to and c-Fos protein expression in GnIH neurons using immunohistochemistry and mRNA levels of the 5-HT associated genes, *serotonin transporter* (*sert*) and *tryptophan hydroxylase 2* (*tph2*), using real-time polymerase chain reaction (PCR) in male rats socially isolated in early life. The examination of this model of social isolation in early life may improve our understanding of mental disorders, as well as sexual dysfunction caused by passive stress, in young adults.

MATERIALS AND METHODS

Animals

Male transgenic Wistar rats expressing enhanced green fluorescent protein (EGFP) under rat GnIH promoter (GnIH–EGFP transgenic rats) (4), after weaning (3 weeks of age), were randomly assigned to group housing (2–4 male littermates per cage) ($n = 63$) or individual housing (isolated) condition ($n = 65$) up to 9 weeks of age. The animals were maintained under a controlled 12 h light/dark cycle (lights on from 12:00 a.m. to 12:00 p.m.) with temperature maintained at 22°C in the SPF animal facility for 6 weeks prior to sampling. Autoclaved water and food were available *ad libitum* to the rats. Body weight of each rat was measured once a week. All aspects of animal welfare and experiments were in accordance with the guidelines and authorization of Monash University Animals Ethics Committee, AEC (MARP/2012/140, MARP/2013/041).

Anxiety and Depression-Like Behavior Tests

The open field test (OFT) was conducted in an open field area made up of a black box (width: 1.2 m, length: 1.2 m, and height: 0.30 m) for use with white rats of 9 weeks old. A handheld camcorder (Sony Corp., Japan) was used to record the movement of the rat in the arena. The OFT experiments were performed in light (white lighting, 10:00 a.m.–12:00 p.m.) and dark conditions (red lighting, 3:00–6:00 p.m.). The video file from the camcorder was analyzed using an automated motion detector software (Lolitrack v2.0, Loligo Systems, Denmark) to track the movements of the rat. Control rats (group housed; $n = 11$/light phase and $n = 9$/night phase) and isolated rats ($n = 12$/light phase and $n = 13$/night phase) were subjected to OFT for 30 min in order to observe anxiety-like behavior in the dark phase and light phase. The total distance traveled (cm), total time of activity (s), total number of center intrusions, and total time spent in center (s) were measured.

Forced swimming test (FST) was carried out using an automated behavior analytical system (MicroAct system, Neuroscience, Inc., Tokyo, Japan). FST was carried out twice; a pre-test as a habituation session and the actual test was performed 24 h later. The FST apparatus consisted of a glass cylinder (height: 45 cm and diameter: 20 cm) which was surrounded by round coil. Prior to the FST, the glass cylinder was filled with water (25 ± 1°C) to a depth of 30 cm, and a magnet (diameter: 1 mm and length: 3 mm) was taped to each front paw of the rats. Individual rat was gently lowered into the water-filled glass cylinder for a 7 min swim test. Electrical currents were generated in the coils corresponding to the movements of the magnet taped to the front paws. The currents were amplified, transformed into voltage, and recorded by the system. The duration of immobility was detected automatically using the *MicroAct*™ Scratch software (Neuroscience, Inc., Tokyo, Japan). Twenty-six male rats were randomly allocated to group-housing conditions ($n = 17$) or isolated conditions ($n = 9$) and used in the FST.

The sucrose preference test (SPT) was conducted over a period of 5 days. Prior to the test, the rats were habituated to

the presence of two water bottles in their cages for a minimum of 5 days beforehand. The rats undergoing the experiment were provided with two water bottles: one containing sucrose water (sucrose powder diluted in distilled water, 200 mL) and the other containing only distilled water (200 mL). The concentration of the sucrose water was increased every day over a period of 5 days (0, 0.25, 0.5, 1.0, and 2.0%). Water consumption was measured at 12:00 p.m. everyday by weighing the water bottles to determine consumption by weight. The position of the water bottles was swapped daily at the time of measurement to reduce preference bias in the results. The data for percentage of sucrose consumed against total water consumed was calculated from the results and used as an indicator for sucrose preference. A repeated measures test was used for statistical analysis in order to confirm any significant difference between group-housed and isolated rats in their pattern of sucrose preference over the 5-day test period. Thirty-one male rats were randomly allocated to group-housing conditions ($n = 14$) or isolated conditions ($n = 17$) at 9 weeks of age. We divided the control and the post-weaning social isolation rats each into two groups for three behavioral tests. Group I was used for OFT and FST and group II was tested for SPT.

Polymerase Chain Reaction

Control ($n = 6$) and socially isolated rats ($n = 6$) at 9 weeks of age were deeply anaesthetized with an intraperitoneal injection of ketamine xylazine (4.5 mg/kg/BW) followed by rapid removal of the brain and immediately dissected by 1 mm rat brain slicer (Neuroscience, Inc., Japan). The POA (bregma +1.2 to −0.12) and dorsal raphe (bregma −6.96 to −8.16) areas were collected with a sterile blade. Total RNA from these tissues was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and transcribed using High Capacity Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. Quantitative real-time PCR (ABI 7300, Applied Biosystems Foster City, CA, USA) was performed using primers for *gnrh*, *gnih*, *sert*, *tph*, and *IMPDH2* (0.2M, Table S1 in Supplementary Material) in a final volume of 10 µl of 2X Power SYBR Green PCR mix (Applied Biosystems). The house keeping gene, *IMPDH2* is listed as a reference gene in real-time PCR to show geometric average expression level (33). The resulting PCR products were validated using an ABI PRISM 310 Genetic Analyzer and Sequence Analysis Software (Applied Biosystems) and ran on a 2.5% agarose gel with ethidium bromide used for visualization.

Immunohistochemistry

Immunocytochemistry for cFos, 5-HT, and 5-HT_{2A} was performed on the DMN sections obtained through coronal sectioning (30 µm). The perfusion fixed (4% PFA) brain tissue sections were washed with 0.1M PBS, in an incubation chamber for 10 min at room temperature and gently shaken at 60 rpm. The sections were then incubated in a blocking solution (40 µL normal goat serum (NGS), 10 µl 0.5% Triton-X, and 1950 µl PBS in 2 mL/well) for 1 h in the same conditions as above. After washing, the sections were incubated with polyclonal rabbit anti-c-Fos antiserum diluted 1:600 (sc52, Santa Cruz Biotechnology, Inc., USA), goat anti-5HT antiserum diluted 1:1000 (20079, Immunostar Inc., WI, USA), rabbit anti-5HT_{2A} antiserum diluted 1:200 (24288, Immunostar Inc., WI, USA) in 2 mL 0.1M PBS containing 2% NGS, 0.5% Triton-X/well for 24 h at 4°C for c-Fos, 5-HT and 5-HT_{2A} respectively at 4°C. Next, the sections were washed in 0.1M PBS incubated in biotinylated anti-rabbit immunoglobulin G (IgG) or biotinylated anti-goat IgG (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA) for 45 min. Subsequently, the sections were incubated with avidin-biotinylated horseradish peroxidase complex for 45 min (Vectastain ABC Elite kit, Vector laboratories, Burlingame, CA, USA). Sections were visualized with Alexa Fluor 594 streptavidin conjugates (S32356, Invitrogen Corporation, USA) and pasted on microscope slides (Superfrost PLUS, Fisher Scientific, Pittsburgh, PA, USA). Mounting medium was applied (VectaShield, Vector Laboratories) followed by coverslips. The number of immunoreactive EGFP–GnIH cells within the DMN were determined using the laser scanning confocal microscope (C1si, Nikon, Tokyo, Japan), equipped with NIS-Element 4.0 Advance software. The specificity of both c-Fos and 5-HT antibody was tested using the rat brain from previous study (4). We divided the control and the post-weaning social isolation rats each into two groups. Group I was used for c-Fos, 5-HT (control: $n = 20$, isolated condition: $n = 17$) and group II was tested for 5-HT_{2A} ($n = 3$). Double-labeled images of c-Fos and GnIH staining, viewed under the red channel were converted to magenta. The brightness and the contrast were adjusted using Adobe Photoshop CS2 (Adobe, San Jose, CA, USA).

Confocal Analysis of c-Fos Expression and 5-HT Fiber Juxtapositions to GnIH Neurons

The procedure for confocal analysis of c-Fos expression in GnIH neurons has been described previously (4). Briefly, immunoreactive c-Fos positive GnIH neurons were visualized using digitized images captured with a Nikon-30 confocal microscope (C1si, Nikon Instruments Inc., Tokyo, Japan). The total number of GnIH neurons and immunoreactive c-Fos positive GnIH neurons were determined using 0.225 µm Z-steps in 10–15 sections which included all EGFP–GnIH neurons in the DMN. To confirm the co-localization of c-Fos in GnIH neurons, the Z-steps were carefully inspected with 3D image rotation using NIS Elements AR Version 4.0 (Nikon Instruments Inc.). We then calculated the percentage of c-Fos positive GnIH neurons. Only cells with visible nuclei were counted. The procedure for confocal analysis of fiber projections to GnIH neurons has been described previously (4). Briefly, 5-HT fiber juxtapositions were captured with a confocal microscope at 0.225 µm Z-steps using 60× water immersion objective lens, 4× digital zoom function to cover the entire depth of the neuron (ECLIPSE 90i, Nikon instruments Inc., Japan). Scans of 488 and 543 nm excitation wavelength were also performed sequentially across optical sectioning to avoid bleed-through between the channels. The number of GnIH neurons with intimate 5-HT fiber juxtapositions was determined in 10–15 sections to include all EGFP–GnIH neurons in the DMN. To confirm close juxtapositions between 5-HT fibers and GnIH neurons, the Z-steps were carefully inspected with 3D rotation image using NIS Elements AR Version 4.0 (Nikon Instruments Inc.). GnIH neurons with 5-HT fiber juxtapositions on the cell soma or dendrites were

counted. A contact was scored only if 5-HT fiber varicosity was in direct contact with the GnIH neuron. The percentage of GnIH neurons with visible nuclei in the DMN and with at least one close juxtapositions with 5-HT fiber was calculated.

Statistics

Data are presented as means ± SEM in all bar graphs. Behavioral data were analyzed by two-way apposition using SPSS 20 (IBM, Chicago, IL, USA). SPT was analyzed by a univariate repeated measures using SPSS 20. Immunohistochemistry and gene expression results were analyzed using the Student's *t*-test. Significance was set as $p < 0.05$.

RESULTS

Social Isolation, Anxiety, Depression, and the Serotonin System

After 6 weeks of social isolation, we conducted three behavioral tests and took samples of brain tissues for biological study (**Figure 1A**). Although the total distance traveled (cm), total time

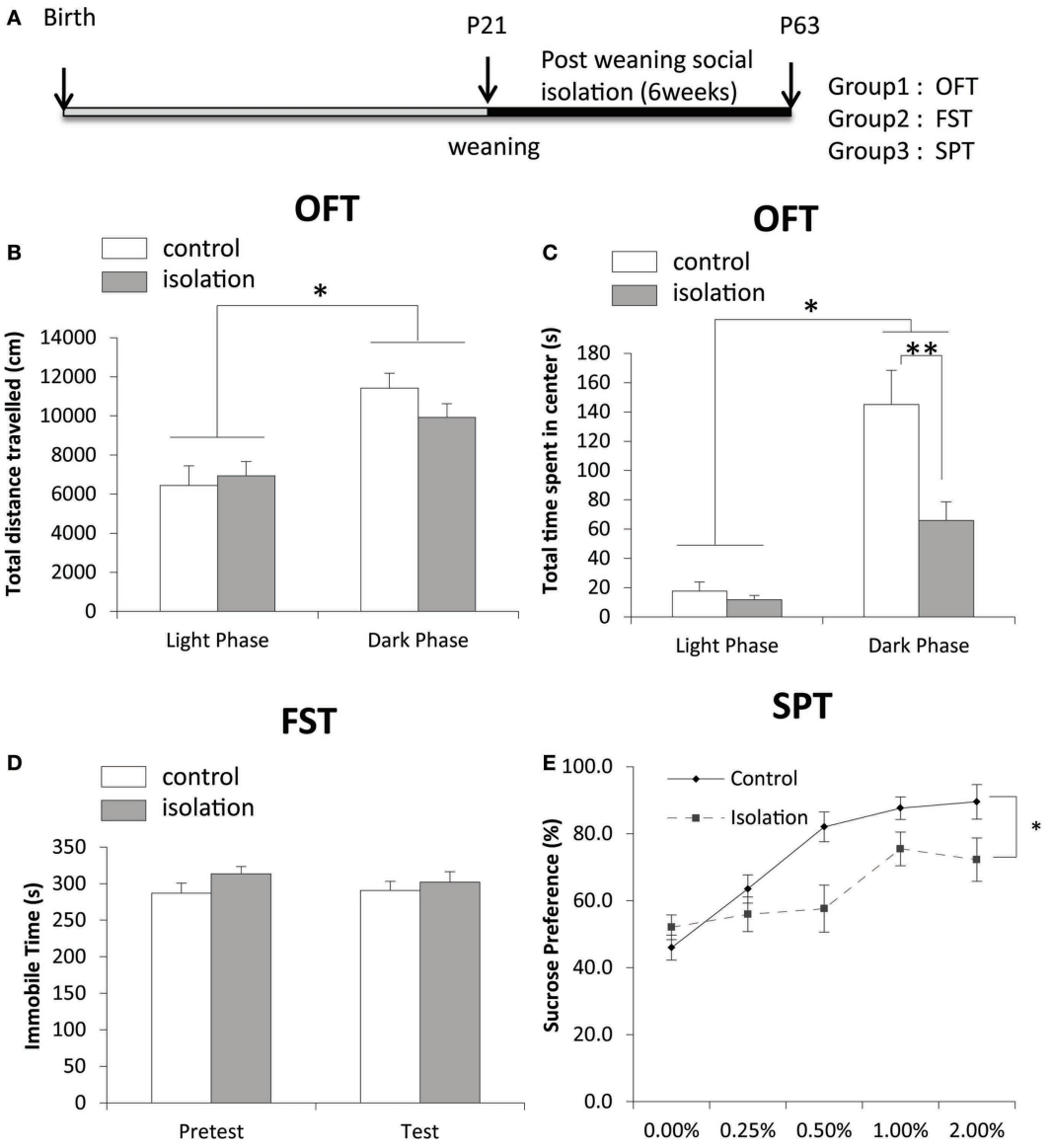

FIGURE 1 | The effect of post-weaning social isolation on behavior. **(A)** Experimental timeline of social isolation and sampling for four experiments. **(B)** Total distance traveled in an open field test (OFT) in the light (CT8–10) and dark (CT15–18) phases in control and socially isolated rats (light-phase control: $n = 11$, light phase isolated: $n = 12$, dark-phase control: $n = 9$, dark-phase isolated: $n = 13$). **(C)** Total time spent in the center during the OFT (s) in the light and dark phases in control and socially isolated rats. **(D)** Time spent immobile during a forced-swim test for 7 min (control: $n = 9$ and isolated: $n = 17$). **(E)** Comparison of sucrose preference was expressed as a percentage of consumption in control ($n = 14$) and isolated ($n = 17$) rats. Data are presented as mean ± SEM for each set. $^*p < 0.05$ and $^{**}p < 0.01$.

of activity (s), total number of intrusions into the center, and total time spent in the center (s) were not significantly different between control and isolated rats, both control and isolated rats traveled more and were active for longer durations in the dark phase compared with the light phase (**Figure 1B**). No significant difference in activity in the light phase was evident between control and isolated animals (**Figure 1B**). However, the total time spent in the center by socially isolated rats was significantly shorter in the dark phase than in controls (control: 145.11 ± 23.28 s, isolated: 65.85 ± 12.73 s, $p < 0.05$; **Figure 1C**). Control ($n = 17$) and isolated ($n = 9$) rats were subjected to a forced-swim test for 7 min to measure time spent immobile. A pre-test was conducted 24 h earlier for habituation. No significant difference was observed in time spent immobile between control and isolated rats during the pre-test or test sessions (**Figure 1D**). The difference in sucrose consumption between groups was measured over 5 days. Using a univariate repeated measures test for analysis, a significant difference was observed in sucrose preference between control and isolated rats [control: $F(1,31) = 6.168$, $p < 0.05$; **Figure 1E**].

Social Isolation and Reproduction

There was no difference in body weight post-weaning between controls and socially isolated male rats (**Figure 2A**). The level of *gnrh* mRNA in the POA was significantly lower in socially isolated rats compared with controls (control: 1 ± 0.2 and isolated: 0.29 ± 0.2, $p < 0.05$; **Figure 2B**). However, there was no difference in the expression of *gnih* mRNA in the hypothalamus between socially isolated and control rats (**Figure 2B**).

Social Isolation and Gonadotropin-Inhibitory Hormone Neuronal Activity

EGFP–GnIH cell bodies were visible in the DMN, which comprised the central, ventral, and dorsal portions of the DMN, and in the dorsal tuberomammillary nucleus (DTM). There was no significant difference in the total number of EGFP–GnIH cells in the entire DMN and DTM between isolated males and group-housed control males [control: 1561.43 ± 156.72 ($n = 14$) and isolated: 1457.55 ± 244.48 ($n = 11$); **Figure 3A**]. To study the effect of social isolation on GnIH neuronal activity, c-Fos immunoreactivity was analyzed in GnIH neurons using a laser scanning confocal microscope. The percentage of GnIH cells exhibiting c-Fos immunoreactivity in the entire DMN was significantly decreased in isolated males compared with group-housed control males (control: 7.01 ± 2.2% and isolated: 1.68 ± 0.77%, $p < 0.05$; **Figures 3B,C**).

Social Isolation and Serotonergic Regulation of Gonadotropin-Inhibitory Hormone Cells

5-HT_{2A}-positive cells were evident in the DMN. Some EGFP–GnIH neurons co-expressed 5-HT_{2A} (**Figures 4A–C**). In addition, close juxtapositions between 5-HT-immunoreactive fibers and GnIH cell bodies were observed in the DMN (**Figures 4D–G**). To study the effect of social isolation on the serotonergic regulation of GnIH cells, close juxtapositions between 5-HT-immunoreactive fibers and GnIH cell bodies was determined and analyzed using a laser scanning confocal microscope. The percentage of 5-HT-immunoreactive fibers in close juxtapositions to GnIH cells in the entire DMN and DTM was significantly decreased in isolated males compared with group-housed control males (control: 13.96 ± 3.05% and isolated: 5.6 ± 0.7%, $p < 0.05$; **Figures 5A,B**). However, 5-HT fiber density per unit area in the DMN was the same in control and isolated rats (**Figure 5C**). Expression of the 5-HT-related genes *sert* and *tph2* in the dorsal raphe nucleus (DR) was measured using quantitative real-time PCR. There were no differences in the levels of *sert* mRNA expression between control and socially isolated rats. However, *tph2* mRNA expression was significantly lower in socially isolated rats ($n = 6$) compared with controls ($n = 6$; control: 1 ± 0.22 and isolated: 0.3 ± 0.11, $p < 0.05$; **Figure 5D**).

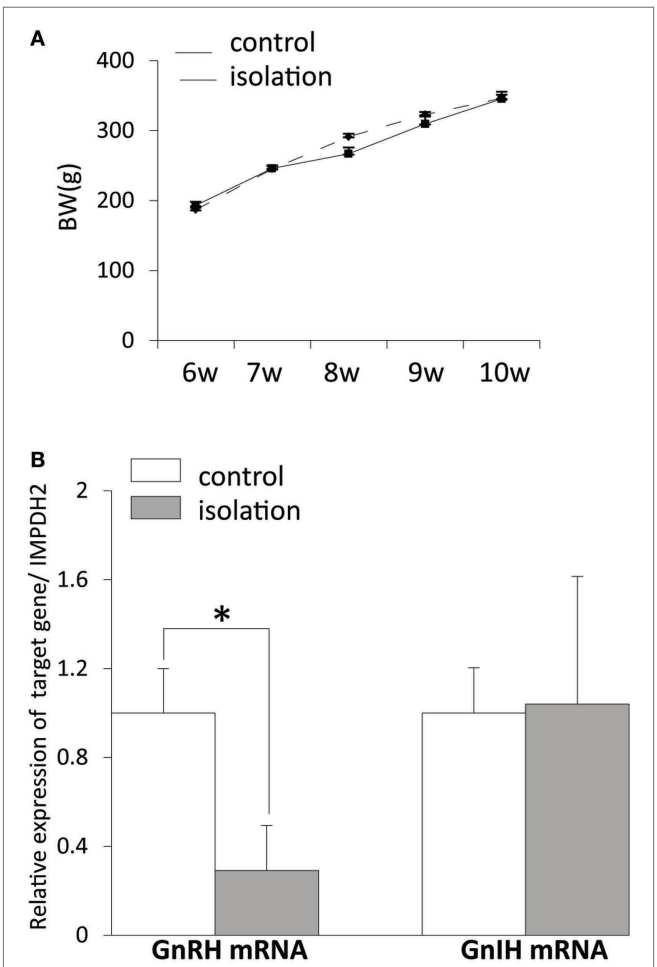

FIGURE 2 | The effect of post-weaning social isolation on body weight and reproductive neuropeptide gene expression in the brain. **(A)** Body weight changes during post-weaning social isolation ($n = 6$/housing). **(B)** Post-weaning social isolation decreased the levels of *gonadotropin-releasing hormone* mRNA in the pre-optic area ($n = 6$/housing) but did not change the levels of *gonadotropin-inhibitory hormone* mRNA in the hypothalamus ($n = 6$/housing). The relative mRNA expression levels were normalized to that of *inosine 5′-monophosphate dehydrogenase 2* mRNA. All data are presented as mean ± SEM. Significant differences were determined using the Student's *t*-test for unpaired values; significance was set at $^*p < 0.05$.

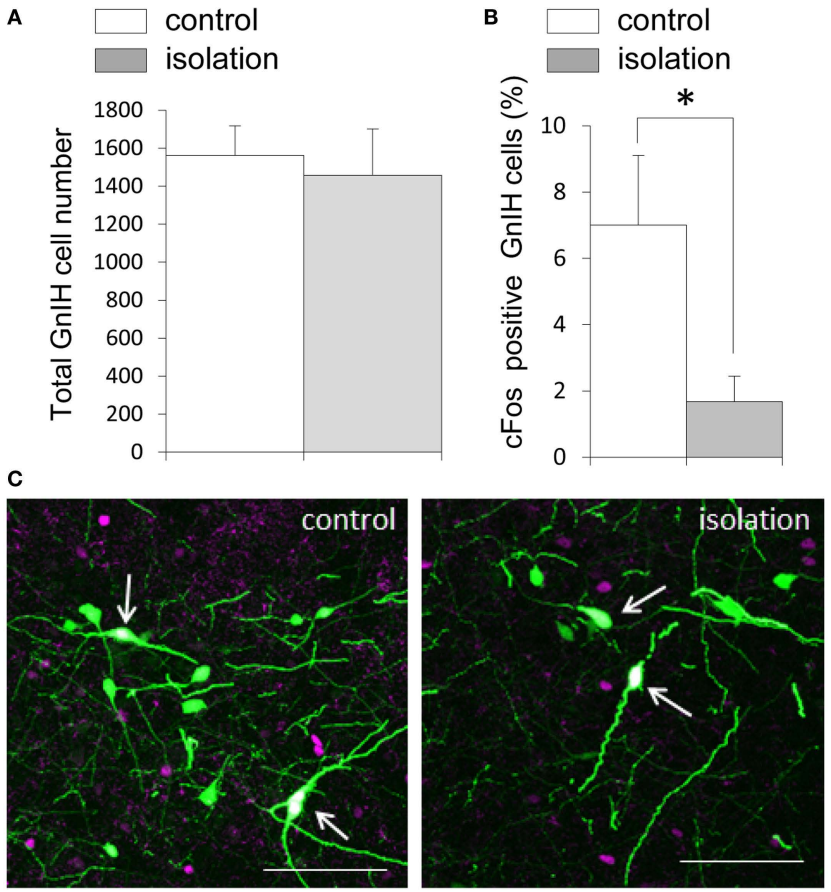

FIGURE 3 | The effect of post-weaning social isolation on enhanced green fluorescent protein–gonadotropin-inhibitory hormone neurons in the dorsomedial hypothalamic nucleus. **(A)** Total number of gonadotropin-inhibitory hormone (GnIH) cells in the dorsomedial hypothalamic nucleus (DMN) of control and isolated male rats (control = 14 and isolated = 11). **(B)** Percentage of c-Fos-positive GnIH neurons in the DMN of control and isolated male rats. Data represent the mean ± SEM for each group. *$p < 0.05$. **(C)** Confocal images of enhanced green fluorescent protein (EGFP)–GnIH cells expressing c-Fos protein (white, indicated by arrows), EGFP–GnIH neurons (green), and red c-Fos protein (magenta) in control (left panel) and isolated (right panel) male rats. Scale bar: 100 μm.

DISCUSSION

In this study, we showed that post-weaning social isolation impairs GnIH neuronal activity and the serotonergic system in the DMN, which may contribute to the deregulation of GnRH neuronal activity in the POA and sexual dysfunction.

Social Isolation, Behavior, and the Serotonin System

It is established that post-weaning isolation for 6 weeks from postnatal day (P) 21 (the time of weaning) has serious consequences for brain development, causing alterations in neurotransmission and behavioral abnormalities (aggression, anxiety, and depression) in rodents (20, 34, 35). This suggests that social stimuli received after weaning are critical to the development of social behaviors and related neuronal circuits. We observed a daily variation in anxiety-like behavior in both group-housed and socially isolated rats. Importantly, during the dark phase, anxiety-like behaviors were observed in socially isolated rats. However, total locomotor activity was unaffected by group or socially isolated housing. These data suggest that the anxiogenic effect of post-weaning social isolation could depend on light conditions and their effect on circadian rhythm. Indeed, disrupted sleep patterns are evident in socially isolated rats (36).

The total time spent immobile (a parameter of depressive-like behavior) is unaffected by post-weaning social isolation in rats (37). Several studies have shown that social isolation increases despair-like immobility (38–40) and immobile time in male rats (41). These conflicting results may be explained by differences between rat strains and the duration of social isolation. Furthermore, in this study, post-weaning isolation resulted in a decreased sucrose intake and reduced preference for sucrose. This anhedonia-like phenotype induced by social isolation can be reversed by treatment with the antidepressant imipramine in male rats (30), suggesting that it is mediated by the 5-HT pathway. For the first time, we show that post-weaning social isolation specifically decreases 5-HT fiber projections to the DMN; this may, in turn, cause the downregulation of *sert* gene

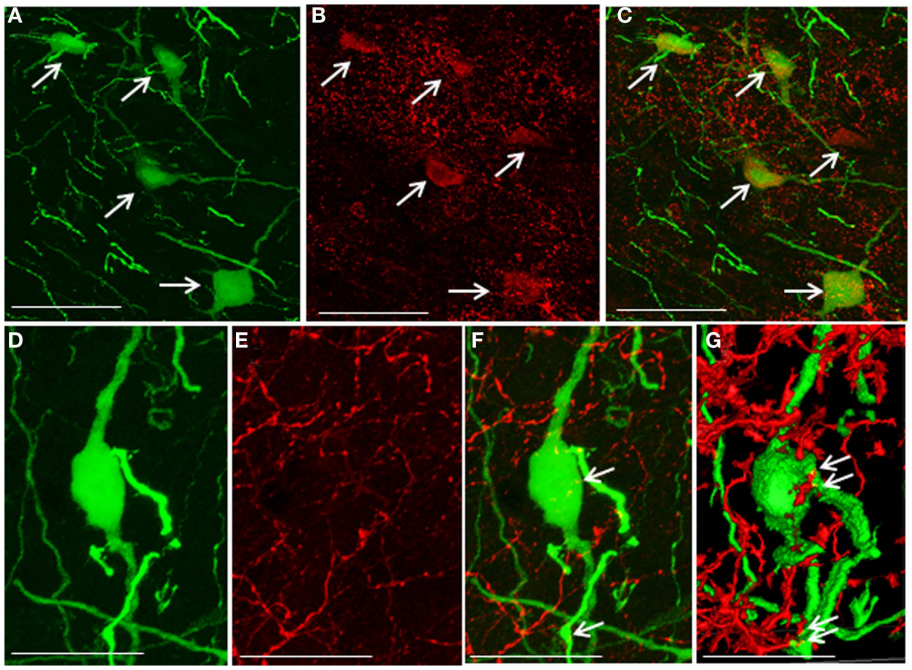

FIGURE 4 | Co-localization of 5-hydroxytryptamine fibers and the 5-hydroxytryptamine$_{2A}$ receptor in enhanced green fluorescent protein–gonadotropin-inhibitory hormone cells in the dorsomedial hypothalamic nucleus. **(A)** Enhanced green fluorescent protein (EGFP)–gonadotropin-inhibitory hormone (GnIH) cells and fibers (green), **(B)** 5-hydroxytryptamine $_{2A}$ (5-HT$_{2A}$)-immunostained neurons (red), and **(C)** EGFP–GnIH cells expressing 5-HT$_{2A}$ (yellow/orange) and 5-HT$_{2A}$-immunostained neurons (red) in the dorsomedial hypothalamic nucleus (DMN). **(D)** EGFP–GnIH neurons (green), **(E)** 5-hydroxytryptamine (5-HT)-immunostained fibers (red), and **(F)** 5-HT in close juxtapositions in EGFP–GnIH neurons or fibers (yellow dot, indicated by arrows). **(G)** 3D images of 5-HT in close juxtapositions in EGFP–GnIH neurons or fibers (yellow dot, indicated by arrows) in the DMN. Scale bars: **(A–C)**, 50 μm; **(D–G)**, 20 μm.

expression in the DR, where 5-HT neurons are primarily located. This is supported by the decrease in central 5-HT and 5-HT receptors evident in socially isolated animals during episodes of increased anxiety (27, 42). Although evidence of serotonergic and 5-HT receptor activity in the DMN remains inconclusive, serotonergic projections to DMN neurons have been reported (43). Indeed, in this study, we found that 5-HT$_{2A}$ is co-localized in GnIH neurons in rats. Although the magnitude of changes in 5-HT$_{2C}$ in GnIH neurons in socially isolated rats is unknown, antagonists of 5-HT$_{2C}$ receptors reportedly increase sucrose preference (44). Several 5-HT receptor types, including 5-HT$_{2C}$, are expressed in GnIH neurons in the DMN of female mice (8). Therefore, the alteration of serotonergic signaling in the DMN may underlie the reduced preference for sucrose in socially isolated rats. Therefore, GnIH neurons and other neurons in the DMN may be targets of the circuitry for anxiety and anhedonia that mediates serotonergic activity following post-weaning social isolation.

Social Isolation and Reproduction

Reproductive senescence can be caused by factors related to the social environment. Stress in early life delays pubertal onset, lowers GnRH expression, lowers testosterone synthesis, and impairs sexual behavior, all of which eventually lead to sexual dysfunction in mammals (31, 32, 45–47). Post-weaning social isolation impairs male sexual behavior, as indicated by an increased latency of ejaculation during adulthood (30, 48). GnRH expression and release from the POA is a key regulator of gonadotropin release and reproductive behavior. Our results show that post-weaning social isolation decreases the expression of GnRH mRNA in male rats, which could lead to sexual dysfunction.

Social Isolation and Gonadotropin-Inhibitory Hormone Neuronal Activity

This study is the first to demonstrate GnIH neuronal activity following post-weaning social isolation. Although it is established that GnIH inhibits GnRH neuronal activity (7, 9) and GnRH induced-LH release by the pituitary gland (5), we found that post-weaning social isolation decreases both GnRH mRNA expression and GnIH neuronal activity. Thus, both the GnRH and GnIH systems are down regulated following post-weaning social isolation. In rats, neural activity and the release of GnRH are increased just before and after puberty in the POA (49), which coincides with pubertal processes, such as changes in γ-aminobutyric acid (GABA) and glutamate levels (50). Morphological changes, such as structural remodeling of the dendrites of GnRH neurons, are the key change during puberty (51). Although the timing of the formation of inhibitory GnIH neuronal inputs to GnRH neurons during the post-natal period remains unknown, a lack of social stimuli pre- and post-puberty may have an impact on

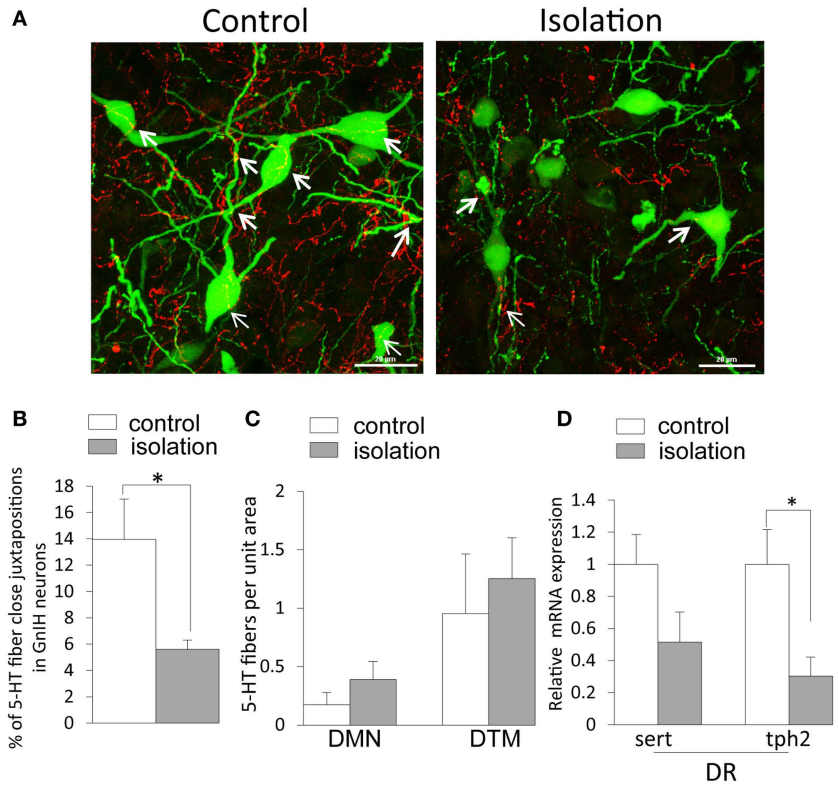

FIGURE 5 | The effect of post-weaning social isolation on 5-hydroxytryptamine projections in enhanced green fluorescent protein–gonadotropin-inhibitory hormone neurons in the dorsomedial hypothalamic nucleus and 5-hydroxytryptamine-related gene expression in the dorsal raphe nucleus. **(A)**. Confocal images of gonadotropin-inhibitory hormone (GnIH) cells expressing 5-hydroxytryptamine (5-HT) fiber juxtapositions (enhanced green fluorescent protein–GnIH neurons, green; 5-HT, red; fiber juxtapositions; yellow indicated by arrows) in control (left panel) and isolated (right panel) male rats. Scale bar: 20 μm. **(B)** Percentage of 5-HT fibers in close juxtapositions in GnIH cells in the dorsomedial hypothalamic nucleus (DMN) of control and isolated male rats (control = 6 and isolated = 6). **(C)** 5-HT immunostaining density per unit area in the DMN and dorsal tuberomammillary nucleus of control and isolated male rats. **(D)** Relative *serotonin transporter* and *tryptophan hydroxylase 2* mRNA expression was normalized to that of the housekeeping gene *inosine 5′-monophosphate dehydrogenase 2* mRNA in the dorsal raphe nucleus of control and socially isolated rats (n = 6/housing). Data are presented as mean ± SEM for each group. $^*p < 0.05$.

the formation of GnIH inputs to GnRH neurons. Post-weaning social isolation may disturb normal GnIH–GnRH neuronal signaling during pubertal development, which may result in reduced expression of GnRH and GnIH in the brain.

Accumulated evidence from recent studies (8, 10, 12) shows that stress increases GnIH expression, with an associated suppression of the hypothalamic–pituitary–gonadal axis suggesting that the inhibitory effect of stress on reproductive function may be mediated by the GnIH system. Post-weaning social isolation causes hypofunction of the HPA axis in adult rats, suggesting that the HPA axis becomes desensitized to stressful stimuli (52). GnIH neurons are sensitive to stress (10–12); thus, in socially isolated rats, they may also become desensitized, as implied by their low neuronal activity. Long-term social isolation lowers GnRH mRNA expression in the POA during adulthood; therefore, inhibitory GnIH signaling may be reduced as a result of short-loop negative feedback from GnRH neurons. Post-weaning social isolation may disrupt the normal development and balance of the GnIH–GnRH neuronal pathway for reproductive activity.

We did not observe any erroneous positioning of EGFP–GnIH neurons in the DMN of socially isolated rats. Likewise, social isolation had no effect on the total number of EGFP–GnIH cells. During development, GnIH expression starts at embryonic days (E) 13–14. GnIH neurons migrate to the dorsal and ventral regions of the third ventricle at E16–17 and establish their positions in the medial hypothalamus by E18 (53, 54). GnIH neurons send ascending and descending projections to other regions of the brain by P1 and the GnIH neuronal system is almost completely formed by P28 (3, 53). Our study shows that the development and positioning of GnIH neurons in the DMN during the prenatal period is not altered by post-weaning (after P21) social isolation.

Social Isolation and the Serotonergic Regulation of Gonadotropin-Inhibitory Hormone Neurons

5-HT-immunoreactive fibers form close juxtapositions to GnIH neurons in the DMN of the rat brain. The DMN receives 5-HT fibers and terminals through the medial forebrain bundle from 5-HT nerve cell bodies of the DR (55). Our findings related to 5-HT_{2A}, and those of our previous study (14), show that 5-HT

receptors are co-expressed in GnIH neurons in female mice. Additionally, administration of citalopram increases the number of GnIH neurons in the DMN and the density of GnIH fibers in the POA (14), supporting the concept that GnIH is under direct serotonergic control. The significant decrease in c-Fos expression evident in GnIH neurons, combined with the decreased 5-HT innervation of GnIH neurons in socially isolated animals, suggest a reduction in GnIH neuronal activity. Early environmental manipulations impair long-term synaptic potentiation (56). The decrease in 5-HT fiber juxtapositions to GnIH neurons reflects neuroanatomical adaptation of hypothalamic neuronal circuits in response to a deprived social environment in early life. 5-HT synaptogenesis is important for brain 5-HT concentration during the critical period of brain development (57). Moreover, 5-HT receptor complements are established between P30–50 in rats (58, 59). Thus, the reduction in 5-HT fiber juxtapositions to GnIH neurons following post-weaning social isolation may delay post-natal maturation, which may weaken the strength of existing synapses.

CONCLUSION

In this study, we showed that post-weaning social isolation enhances anxiety-like behavior and an anhedonia-like phenotype that is related to altered *sert* expression in the serotonergic system. Furthermore, we demonstrated that post-weaning social isolation reduces GnIH neuronal activity and decreases 5-HT fiber juxtapositions to GnIH neurons, suggesting that serotonergic regulation may participate in GnIH signaling to accomplish normal GnRH neuronal activity and reproductive function. Although a complex molecular and neuronal mechanism is involved in post-weaning social isolation-induced reproductive dysfunction, altered serotonergic activity may be one factor that mediates GnIH–GnRH signaling in the brain. Our findings characterize the long consensus on the negative effects of post-weaning social isolation and provide insights into the neuronal and molecular mechanisms underlying the serotonergic regulation of GnIH.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2015.00172

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Social Isolation Modulates CLOCK Protein and Beta-Catenin Expression Pattern in Gonadotropin-Inhibitory Hormone Neurons in Male Rats

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Postweaning social isolation reduces the amplitude of the daily variation of CLOCK protein in the brain and induces lower reproductive activity. Gonadotropin-inhibitory hormone (GnIH) acts as an inhibitor in the reproductive system and has been linked to stress. Social isolation has been shown to lower neuronal activity of GnIH-expressing neurons in the dorsomedial hypothalamus (DMH). The exact mechanism by which social isolation may affect GnIH is still unclear. We investigated the impact of social isolation on regulatory cellular mechanisms in GnIH neurons. We examined *via* immunohistochemistry the expression of CLOCK protein at four different times throughout the day in GnIH cells tagged with enhanced fluorescent green protein (EGFP-GnIH) in 9-week-old adult male rats that have been raised for 6 weeks under postweaning social isolation and compared them with group-raised control rats of the same age. We also studied the expression of β-catenin—which has been shown to be affected by circadian proteins such as Bmal1—in EGFP-GnIH neurons to determine whether it could play a role in linking CLOCK in GnIH neurons. We found that social isolation modifies the pattern of CLOCK expression in GnIH neurons in the DMH. Socially isolated rats displayed greater CLOCK expression in the dark phase, while control rats displayed increased CLOCK expression in the light phase. Furthermore, β-catenin expression pattern in GnIH cells was disrupted by social isolation. This suggests that social isolation triggers changes in CLOCK and GnIH expression, which may be associated with an increase in nuclear β-catenin during the dark phase.

Keywords: stress, dorsomedial hypothalamus, gonadotropin-releasing hormone, reproduction, diurnal rhythmicity

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INTRODUCTION

Social isolation is defined as a state of being where individuals are unable to contact or communicate other members of their community, whether by choice or by circumstance. Social isolation impacts many different species, but detrimental influence is more clearly observed on communal-minded organisms such as humans. Indeed, a 50% increase in the mortality rate has been observed in socially isolated individuals (1, 2). Severe social withdrawal is a rising phenomenon amongst youths (3) who are subsequently diagnosed with mood disorders including depression (4). Physiologically, social isolation beginning from the age of 5 in children may lead to greater mental difficulties (5).

Animal studies have shown the effect of social isolation on various neuronal systems in the rat brain, such as a dysregulated hypothalamic–pituitary–gonadal (HPG) axis (6–8), which results in sexual dysfunction.

In the HPG axis, gonadotropin-inhibitory hormone (GnIH) acts as an inhibitor of gonadotropin-releasing hormone (GnRH) and gonadotropins in mammals including humans (9–13). Discovered from the Japanese quail (14), GnIH is linked to stress-related disorder *via* the hypothalamic–pituitary–adrenal (HPA) axis. Stressful stimuli would induce GnIH neurons in the dorsomedial hypothalamus (DMH) to increase expression of c-Fos, indicating increased activity within those neurons when exposed to stress (15). The presence of glucocorticoid receptors, the major actors of the HPA axis, has been shown on GnIH neurons (16). Glucocorticoids have been shown to enhance transcription of GnIH mRNA (17, 18) and immunostained GnIH cells (19). In addition, social isolation impairs negative feedback regulation of the HPA axis (20) and fail to suppress corticosterone responses under acute stress (21). It is possible that social isolation increases sensitivity to stressful stimuli, thus making it easier for heightened glucocorticoid levels to induce an increase in GnIH mRNA transcription. However, the cellular mechanisms underlying social isolation-related changes in GnIH neurons remain to be fully elucidated.

In a previous study, alteration of the serotonergic system in the dorsal raphe of socially isolated rats caused an increase in serotonin receptors in GnIH (22) and serotonin fibers in GnIH neurons (23), suggesting serotonergic regulation of GnIH neurons and their possible involvement in the event of serotonergic dysfunctions such as major depressive disorder. C-fos expression in GnIH neurons was observed to be lower in cases of social isolation, indicating reduced GnIH neuronal activity (23). Socially isolated rats were also noted to behave in a more anxious manner while conducting an open field test during the dark phase (23). Social isolation can desynchronize diurnal rhythms (24), and a disruption of the day/night cycle has been indicated as both possible cause and effect of depression in humans (25). This disparity in behavior between light and dark phases suggest a possible diurnal component involved in the role of GnIH on social isolation-induced depressive behavior.

The serotonergic system is related to phase shifts in the circadian clock (26, 27), and so it is possible that social isolation-induced serotonergic dysfunction may result in circadian alterations to GnIH activity. CLOCK is a vital circadian protein that forms a heterodimer with another circadian protein, BMAL1, before binding to an E-box (CACGTG) site on a gene's promoter region where it acts as a transcription factor (28). CLOCK has demonstrated rhythmic circadian oscillation, while BMAL1 does not within the DMH (29). This suggests that DMH neurons are regulated by circadian rhythm *via* clock expression. To investigate the possibility of a diurnal cycle existing within the rat GnIH neurons of the DMH, we focused on the CLOCK protein. GnIH mRNA expression has been demonstrated to be sensitive to photoperiodism in a study involving long-day and short-day photoperiods (30, 31). GnIH neurons of female rats have also demonstrated positive receptivity to melatonin, an important factor in the circadian clock (32, 33).

From a circadian perspective, β-catenin has also been associated with the circadian system in several ways, as it can alter circadian clock gene expression by inducing PER2 degradation (34), which in turn prevents the inhibition of the CLOCK-BMAL1 complex (35). β-catenin has a dual function as both adhesion protein and in gene transcription and is an important component of the canonical Wnt signaling pathway (36–38). In the cell, β-catenin presence is regulated by Wnt; in the absence of Wnt, β-catenin is targeted by GSK-3β for degradation, but the activation of the signaling pathway by Wnt reduces β-catenin degradation, causing it to accumulate and translocate into the nucleus where it activates TCF/LEF1 transcription factors that bind to Wnt target genes (39). β-catenin is also a protein that has been suggested to play a role in the molecular pathophysiology of stress, as sufferers of major depressive disorder exhibit decreased β-catenin mRNA levels in the prefrontal cortex (40). More importantly, the presence of β-catenin in the nucleus accumbens has been shown to reduce susceptibility to social isolation-induced depression in rodents *via* mediating an increase in the production of microRNAs related to stress resilience (41).

There is an emerging view that GnIH neuronal activity may be influenced by social isolation and subsequently play a role in the display of social isolation-related sexual dysfunction and stress-related physiological changes. This has led us to investigate whether a day/night cycle is a component of that role and to seek out potential models for GnIH activity that may involve diurnal action. First, we asked whether CLOCK protein is present in GnIH neurons and whether it exists, whether its expression is influenced by the day/night cycle. We then examined the effect of social isolation on the expression of CLOCK protein in GnIH neurons of the DMH. Next, as a potential link for CLOCK in GnIH neurons, we explored whether β-catenin is expressed in GnIH neurons and whether both social isolation and diurnal phases affect that expression. We also studied the effect of β-catenin activity on GnIH neuronal activity.

MATERIALS AND METHODS

Animals and Housing Conditions

EGFP-GnIH transgenic rats (42) were randomly allocated to group-housing conditions (2–3 males/cage, $n = 32$) or isolated conditions (1 male/cage, $n = 32$). The grouped rats were housed in standard cages (dimensions: 276 mm × 445 mm × 204 mm, CLEA Japan, Inc., Tokyo, Japan), while isolated rats were housed in single cages (dimensions: 225 mm × 338 mm × 140 mm, CLEA Japan, Inc., Tokyo, Japan) that were wrapped in aluminum foil on all sides to prevent visual contact with other cages. Each single cage was placed on a separate shelf of an animal cage shelf with a blower unit attached to avoid olfactory social cues from other rats. Allocation of the rats was performed postweaning, 21 days after birth, and the rats were housed up till 9 weeks of age. The rats were reared under a 12-h light/dark cycle (lights on from 12:00 a.m. till 12:00 p.m.), and the temperature of the rooms were maintained at 22 ± 1°C and constant humidity for the duration of the housing prior to sampling. Food and water was made available *ad libitum* for the animals. Animal welfare

and experimental ethics in BRIMS SPF animal facility were followed in line with the authorized guidelines laid out by Monash University Animal Ethics Community (MARP/2012/140, MARP/2017/021).

Immunocytochemistry

Brain samples were collected at four periods in the day: 6:00 a.m. (ZT6), 12:00 p.m. (ZT12, commencement of the dark phase), 6:00 p.m. (ZT18) and 12:00 a.m. (ZT24, commencement of the light phase). The process was performed on male adult rats ($n = 64$, control: ZT6 $n = 6$, ZT12 $n = 9$, ZT18 $n = 11$, ZT24 $n = 6$; isolation: ZT6 $n = 6$, ZT12 $n = 8$, ZT18 $n = 12$, ZT24 $n = 6$, 9 weeks old) following the perfusion and fixation protocol that has been described in a previous experiment (42). Immunocytochemistry for CLOCK and β-catenin was performed on the DMH sections obtained through sectioning. Sectioning of the rat brain took place in a cryostat chamber (Leica CM1900, Leica Biosystems, Heidelberg, Germany) with its temperature set to −20°C. Each section was sliced along the coronal plane at a thickness of 30 µm, starting from the beginning till the end of the DMH (bregma −0.26 to −4.16 mm), with approximately 80 tissue sections obtained per brain. The sections were placed in an antifreeze solution (30% ethylene glycol, 20% glycerol in 0.1 M PB solution) and stored at −20°C in a 12-well plate. Alternating sections were divided and stored in separate wells to provide two sets of samples for analysis. The tissue sections were washed twice with ice-cold 0.1 M PBS, with each repetition incubated in an incubation chamber for 10 min at room temperature and gently shaken at 60 rpm. The washing step would be repeated in between each major incubation step of the procedure. The sections were then incubated in a blocking solution [40 µL normal goat serum (NGS), 10 µL 0.5% Triton-X and 1950 µL PBS in 2 mL/well for CLOCK, with the NGS being replaced by normal horse serum (NHS) for β-catenin] for 1 h in the same conditions as above. After washing, the sections were incubated with a rabbit anti-CLOCK antibody diluted 1:400 [5.0 µL clock antiserum (H-276) sc-25361, Santa Cruz Laboratories, USA; 40 µL NGS, 10 µL 0.5% Triton-X and 1945 µL 0.1 M PBS in 2 mL/well] or with a mouse anti-β-catenin antibody diluted 1:400 [beta-catenin antiserum (12F7), ab22656, Abcam Inc., MA, USA] for 1 h in the incubation chamber before being transferred to 4°C storage for overnight incubation of 24 h. The antibody immunogen sequences can be found in the supplementary material (Table S1 in Supplementary Material). Subsequently, the sections were incubated in the corresponding secondary antibody solution [10 µL biotinylated anti-rabbit immunoglobulin-G (IgG) for CLOCK, 40 µL NGS, 1,950 µL 0.1 M PBS in 2 mL/well and 10 µL biotinylated anti-mouse IgG for β-catenin, 40 µL NHS, 1,950 µL 0.1 M PBS in 2 mL/well (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA)] for 45 min, followed by incubation with A-B complex [40 µL avidin–biotinylated horseradish peroxidase complex (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA), 1,960 µL 0.1 M PBS in 2 mL/well] for 45 min and finally Streptavidin Alexa Fluor 594 [3.5 µL (S32356, Invitrogen Corporation, USA), 1,996.5 µL 0.1 M PBS in 2 mL/well] 30-min incubation for the purposes of visualization. The sections were pasted on microscope slides (Superfrost PLUS, Fisher Scientific, Pittsburgh, PA, USA) and mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA) for fluorescence preservation before being stored at 4°C.

CLOCK and β-catenin antibody specificity was tested using a blank buffer test where one specimen would be incubated with primary antibody solution (1:400 clock antiserum or beta-catenin antiserum) (in 40 µL NGS, 10 µL 0.5% Triton-X, and 1,945 µL 0.1 M PBS in 2 mL/well), while the other specimen would have the primary antibody omitted (40 µL NGS, 10 µL 0.5% Triton-X, and 1,950 µL 0.1 M PBS in 2 mL/well), with the remainder of the steps as per the immunocytochemistry procedure detailed above (Figure S1 in Supplementary Material). The CLOCK antibody utilized in this experiment has been previously used successfully for other published papers (43, 44). The specificity of the β-catenin antibody was also tested by absorption tests (1:400) with human β-catenin peptide (5 µg/mL beta-catenin peptide, ab16377, Abcam Inc., MA, USA). Incubation of the β-catenin antibody with the β-catenin peptide inhibited the staining in the rat hypothalamus (Figure S1 in Supplementary Material).

Cell Counting and Colocalization

The Leica laser-scanning microscope (Leica Microsystems, Germany) was used for counting of immunoreactive GnIH cells and determining immunoreactive clock and β-catenin activity in the fluorescent GnIH neurons. The EGFP and Alexa Fluor 594 fluorophores were excited by lasers emitting at wavelengths of 488 and 543.5 nm respectively. By using the Leica Application Suite X software, the Z-steps were obtained at an interval of 1.25 µm *via* depth scanning with the use of a 10× objective lens for CLOCK immunoreactive specimens and 20× objective lens for β-catenin immunoreactive specimens. A higher magnification was chosen for the latter to better distinguish the differences in β-catenin staining morphology. Maximum intensity projections were utilized to portray the full gamut of fluorophore expression in the sample. Each of the 1.25-µm Z-steps was examined carefully to confirm that the neurons counted were fully present in the observed section. All EGFP-GnIH neurons in the DMH region were counted. The presence of CLOCK or β-catenin (red fluorescence) in association with GnIH neurons (green fluorescence) were marked as colocalization, appearing yellow on the images captured, and the percentage of GnIH neurons expressing CLOCK or β-catenin were recorded. For β-catenin, colocalization is further divided into nuclear and cytoplasmic depending on the primary area of β-catenin localization observed. 51 samples were counted for CLOCK immunoreactivity (control: $n = 24$ and isolation: $n = 27$, $n = 6$ for each of control at ZT6, ZT12 and ZT18, and ZT24, and $n = 6$ for isolation at ZT6, $n = 7$ for isolation at ZT12, $n = 8$ for isolation at ZT18, and $n = 6$ for isolation at ZT24), and 30 samples were counted for β-catenin immunoreactivity (control: $n = 15$ and isolation: $n = 15$, $n = 6$ for each of control and isolation at ZT12, $n = 9$ for each of control and isolation at ZT18). Approximately 30 DMH sections were counted for each brain, with the total GnIH cells counted per brain approximating 1,000.

EFGP-GnIH Intensity in GnIH Neurons

The intensity of each EGFP-GnIH-expressing neuron was measured using the inbuilt software of the Leica laser-scanning microscope (Leica Microsystems, Germany), using the same images that have been captured for the cell counting procedure above. Each EGFP-GnIH neuron was manually designated as a single region

using the software. The software would then record the intensity value of the fluorescent green expressed by each neuron. The data were tabulated and manually analyzed to determine the average intensity of the GnIH neuron in the samples. The intensity value is measured as a grayscale number ranging from 0 to 255, with 0 being the darkest (black) and 255 being the brightest (white). It is defined as the average brightness of all the pixels in the selected region, which would constitute a single neuron. 12 samples were measured for EGFP-GnIH intensity (control: $n = 6$ and isolation: $n = 6$ at ZT18). The total number of GnIH cells counted per brain approximated 1,000.

DAPI Staining

DAPI staining was carried out to confirm colocalization of β-catenin within the nucleus of the GnIH neurons. After completion of the Alexa Fluor 594 staining following the immunocytochemistry procedure as detailed above, the sections were then incubated for 15 min in DAPI dihydrochloride solution [2.5 µL DAPI dihydrochloride (28718-90-3, Sigma-Aldrich, MO, USA), 1,997.5 µL 0.1 M PBS in 2 mL/well] for nuclear staining before being pasted on slides as per the aforementioned protocol (DAPI-stained specimens, $n = 2$). The Eclipse 90i Nikon fluorescent microscope (Nikon Instruments, Tokyo, Japan) equipped with a Nikon DXM 1200C camera and NIS-Element 3.0 software was used to capture images of the β-catenin and DAPI-stained sections. The EGFP, Alexa Fluor 594, and DAPI fluorophores were excited by lasers emitting at wavelengths of 488, 543.5, and 358 nm, respectively. Fluorescent microscope images were captured at a resolution of 4,116 × 3,072 pixels and at a magnification of 20×. Each fluorophore was captured separately at the same focal point before being merged into a single picture using NIS-Element 3.0 software.

Statistics

Data are presented as means ± SEM in all bar graphs. Immunohistochemistry and EGFP-GnIH intensity results were analyzed using two-way ANOVA to determine the significance of the effect of time, housing, and the interaction between the two, before applying *t*-tests for further analysis. The change in CLOCK expression across time points was analyzed by a univariate repeated measures test using SPSS 20 (IBM, Chicago, IL, USA). Significance was set as $p < 0.05$.

RESULTS

CLOCK Expression in GnIH Neurons

To identify any possible effects of social isolation on CLOCK rhythms, we selected samples from four different time points; ZT6, ZT12 and ZT18, and ZT24 for our measurements. The primary focus for the cell counting was the GnIH population of the DMH region (**Figures 1A,B**). All GnIH neurons and GnIH neurons expressing CLOCK proteins were counted. CLOCK colocalization with GnIH neurons was observed in the nucleus of the cells (**Figures 1C–E**).

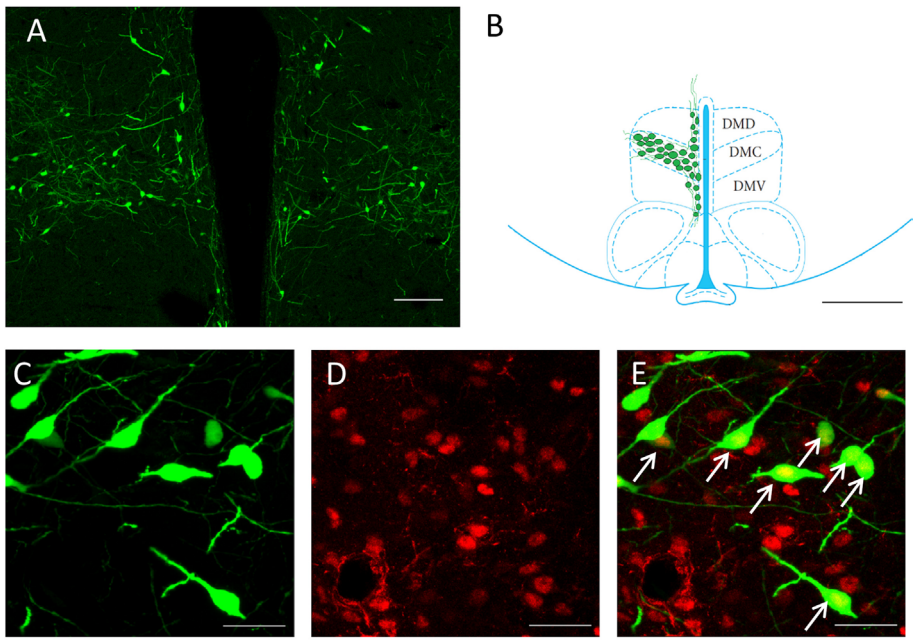

FIGURE 1 | Expression of CLOCK protein within GnIH neurons in the DMH region. **(A)** An overview of GnIH-expressing neurons in the DMH at 10× magnification. **(B)** Primary area of GnIH neuron expression mapped out on the rat brain atlas. Bregma of image is at −3.12 mm. Sectioned areas were taken from a bregma of −0.26 to −4.16 mm. Scale bar = 100 µm **(A)** and 200 µm **(B)**. **(C)** EGFP-GnIH-expressing neurons (green), **(D)** CLOCK-immunostained neurons (red), and **(E)** combined EGFP-GnIH (green) and CLOCK (red) images at 40× magnification. Scale bar = 20 µm **(C–E)**. White arrows indicate the presence of CLOCK localization. DMC, dorsomedial hypothalamic nucleus, compact; DMD, dorsomedial hypothalamic nucleus, dorsal; DMV, dorsomedial hypothalamic nucleus; ventral; GnIH, gonadotropin-inhibitory hormone; DMH, dorsomedial hypothalamus.

The number of GnIH cells in the DMH did not vary significantly both between control and isolation and between time points (**Figure 2A**). We found that the interaction between housing and time was a significant factor in the change of GnIH neuronal fluorescent intensity, as was time itself (control: $n = 12$, isolation: $n = 12$, $F[1, 24] = 13.61$, $p < 0.01$). The average intensity of GnIH neurons was observed to be higher in socially isolated animals at ZT12 in comparison to control rats at the same time point [control ZT12: 92.94 ± 8.89 ($n = 6$) and isolation ZT12: 125.31 ± 4.61 ($n = 6$), $p < 0.05$; **Figure 2B**]. A significant difference was also observed comparing the intensity of isolated rats at ZT12 to similarly isolated rats at ZT18 [isolation ZT12: 125.31 ± 4.61 ($n = 6$) and isolation ZT18: 65.51 ± 12.62 ($n = 6$), $p < 0.01$; **Figure 2B**]. This difference in intensity can be observed in control (**Figure 2C**) and isolation groups (**Figure 2D**) in the light phase, with higher intensity for isolation groups. There was no significant difference to be found in intensity measurements for control rats at both time points.

The expression of CLOCK within the DMH, and its colocalization with GnIH neurons, appears to rise and fall as we travel across each time point in the control group (**Figure 3A**), starting at ZT6 (**Figure 3A**, i), peaking at ZT12 (**Figure 3A**, ii) before dropping down at ZT18 (**Figure 3A**, iii), and beginning to rise again by ZT24 (**Figure 3A**, iv). Within the isolated group, the changes in expression levels appear to increase (**Figure 3B**), with ZT6 (**Figure 3B**, i) and ZT12 demonstrating lower CLOCK levels than (**Figure 3B**, ii) ZT18 (**Figure 3B**, iii) and ZT24 (**Figure 3B**, iv).

The interaction between housing and time was indicated as a significant factor for the changes in colocalization of CLOCK and GnIH neurons throughout a 24-hour period (control: $n = 24$, isolation: $n = 27$, $F[1, 51] = 4.25$, $p < 0.01$). Using the repeated measures test, the percentage of expression of CLOCK protein in GnIH neurons for the control group demonstrated a significant fluctuation in values when comparing the difference between each of the time points (control: $F[1,24] = 5.57$, $p < 0.01$; **Figure 3C**), while any difference in CLOCK expression within the GnIH neurons from the isolated group was found to be insignificant when compared in the same fashion.

Subsequently, we grouped the specimens into light (ZT6 and ZT12) and dark (ZT18 and ZT24) phases and compared the colocalization between the two groups. We discovered a strong correlation between housing and phase interaction with CLOCK colocalization (control: $n = 24$, isolation: $n = 27$, $F[1, 51] = 9.83$, $p < 0.01$). CLOCK protein colocalization was significantly higher in the light phase compared to the dark phase for control group rats, while the reverse was observed by time for the isolation-housed rats [control ZT6 + ZT12: 18.09 ± 2.21 ($n = 12$) and control ZT18 + ZT24: 12.02 ± 1.36 ($n = 12$), $p = 0.029$; isolation ZT6 + ZT12: 13.49 ± 1.45 ($n = 13$) and isolation ZT18 + ZT24: 19.76 ± 2.44 ($n = 14$), $p < 0.05$; **Figure 3D**]. Comparing between groups, while we did not observe any significant differences

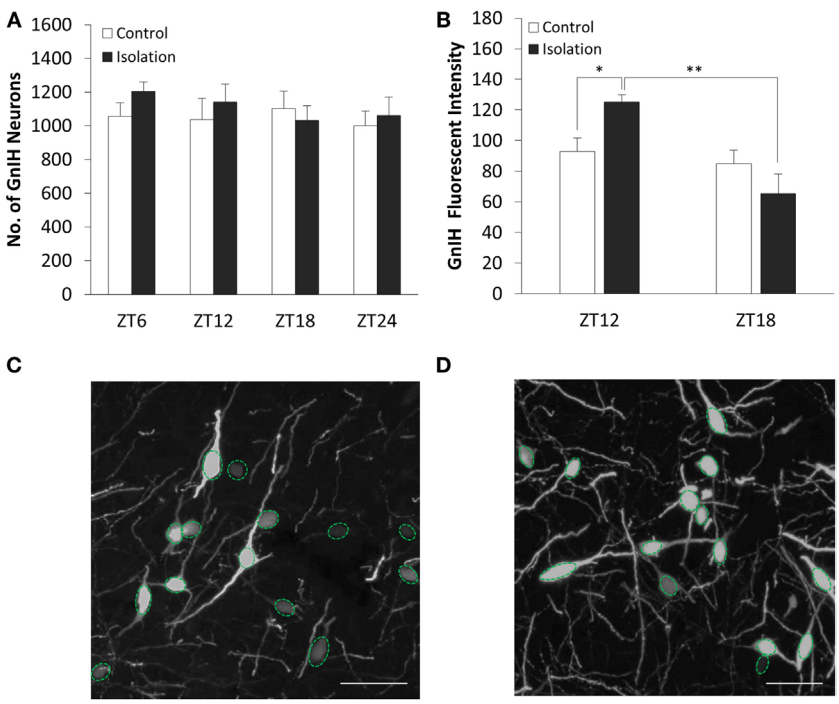

FIGURE 2 | The effect of social isolation on CLOCK expression in gonadotropin-inhibitory hormone (GnIH) neurons in the dorsomedial hypothalamus (DMH) region. **(A)** Total GnIH cell numbers aggregated by ZT time, for control and isolated groups in the DMH (control: ZT6 $n = 6$, ZT12 $n = 6$, ZT18 $n = 6$, ZT24 $n = 6$ and isolation: ZT6 $n = 6$, ZT12 $n = 7$, ZT18 $n = 8$, ZT24 $n = 6$). **(B)** Average intensity of GnIH neurons of control and isolated rats at ZT12 and ZT18 (control: ZT12 $n = 6$, ZT18 $n = 6$ and isolation: ZT12 $n = 6$, ZT18 $n = 6$). **(C)** Image of DMH GnIH neurons at 40× magnification for control (ZT12) and **(D)** isolation (ZT12). Scale bar = 50 μm **(C,D)**.

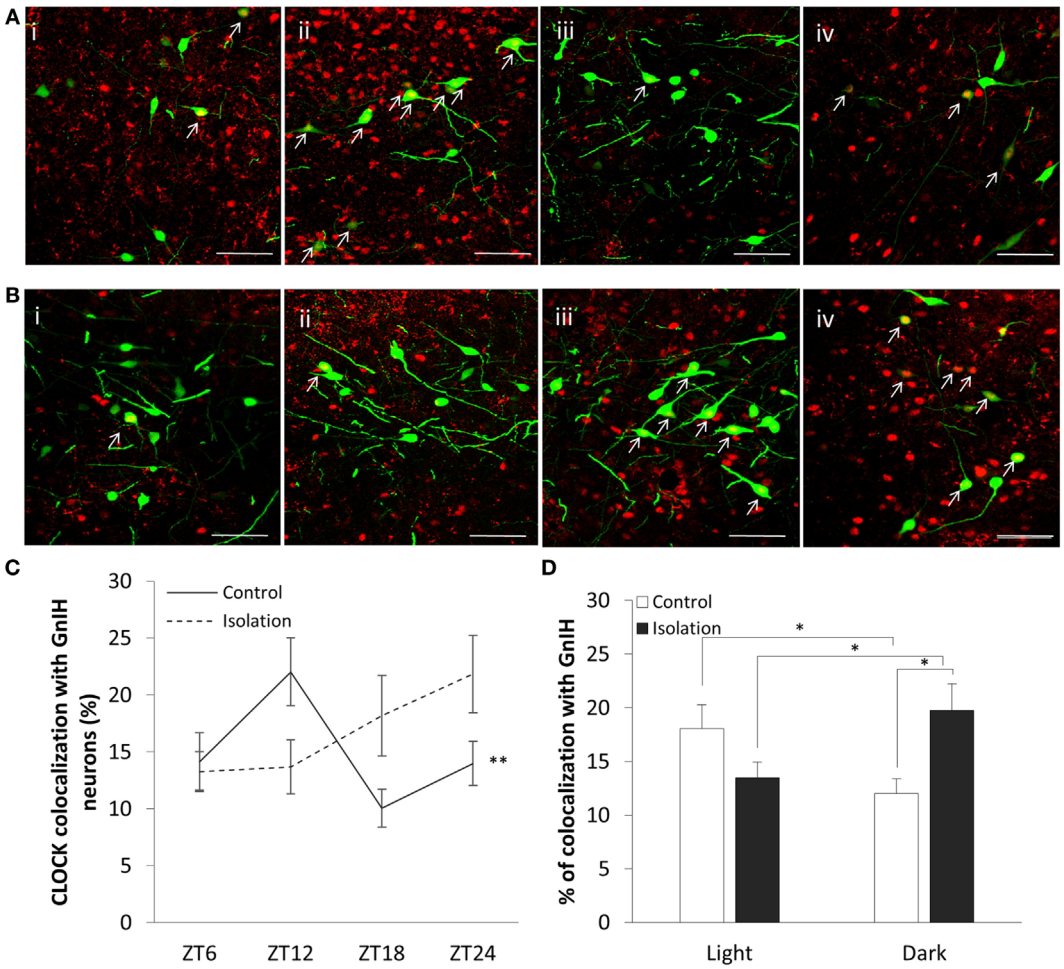

FIGURE 3 | Colocalization of CLOCK protein within gonadotropin-inhibitory hormone (GnIH) neurons in the dorsomedial hypothalamus (DMH) region across different time points. **(A)** EGFP-GnIH neurons (green) and CLOCK-immunostaining (red) in the DMH of group-housed rats sampled at ZT6 (i), ZT12 (ii), ZT18 (iii), and ZT24 (iv). **(B)** EGFP-GnIH neurons (green) and CLOCK-immunostaining (red) in the DMH of isolation-housed rats sampled at ZT6, ZT12, ZT18, and ZT24. White arrows indicate colocalization between GnIH neurons and CLOCK protein. Scale bar = 50 μm. **(C)** Comparison of CLOCK colocalization percentage in the GnIH neurons of control and isolated rats over time (ZT 24 = lights on, ZT 12 = lights off, control: ZT6 $n = 6$, ZT12 $n = 6$, ZT18 $n = 6$, ZT24 $n = 6$ and isolation: ZT6 $n = 6$, ZT12 $n = 7$, ZT18 $n = 8$, ZT24 $n = 6$). **(D)** Comparison of CLOCK colocalization percentage in the GnIH neurons of control and isolated rats, grouped by light (ZT6 and ZT12) and dark (ZT18 and ZT24) phases (control: light $n = 12$, dark $n = 12$ and isolation: light $n = 13$, dark $n = 14$). Data are presented as means ± SEM for each set. Significance was set at $p < 0.05$.

between control and isolation in the light phase, isolated animals demonstrated higher colocalization for CLOCK in the dark phase [control ZT18 + ZT24: 12.02 ± 1.35 ($n = 12$) and isolation ZT18 + ZT24: 19.76 ± 2.45 ($n = 14$), $p < 0.05$; **Figure 3D**].

β-Catenin Expression in GnIH Neurons

β-catenin localization could be observed in GnIH neurons. We spotted two distinct morphologies; one where colocalization staining of β-catenin and nuclear DAPI staining expression of β-catenin within the cell demonstrated staining of the cytoplasm (**Figures 4A,B**) and one where β-catenin is localized primarily in the nucleus (**Figures 4C,D**).

To determine whether β-catenin expression in GnIH can be influenced by temporal and housing factors, we selected specimens from ZT12 and ZT18, so chosen because they represented the highest and lowest points in CLOCK expression as observed earlier. We also focused on neurons where β-catenin is co-localized in the nucleus as an indicator of the protein's activity in its role as a transcription factor. Analysis of the data of cytoplasmic colocalization of β-catenin pointed to time points as the influencing factor (control: $n = 15$, isolation: $n = 15$, $F[1, 30] = 4.70$, $p < 0.05$). Control-housed specimens demonstrated higher cytoplasmic colocalization of β-catenin when comparing ZT12 to ZT18 [control ZT12: 31.10 ± 2.35 ($n = 6$) and control ZT18: 23.83 ± 1.79 ($n = 9$), $p < 0.05$; **Figure 5A**]. We also observed that nuclear colocalization was conversely affected by housing and not time points (control: $n = 15$, isolation: $n = 15$, $F[1, 30] = 7.75$, $p < 0.01$), and we were able to identify a significant elevation in nuclear colocalization for isolated rats in ZT18 compared to the control [control ZT18: 6.91 ± 0.99 ($n = 9$) and isolation ZT18:

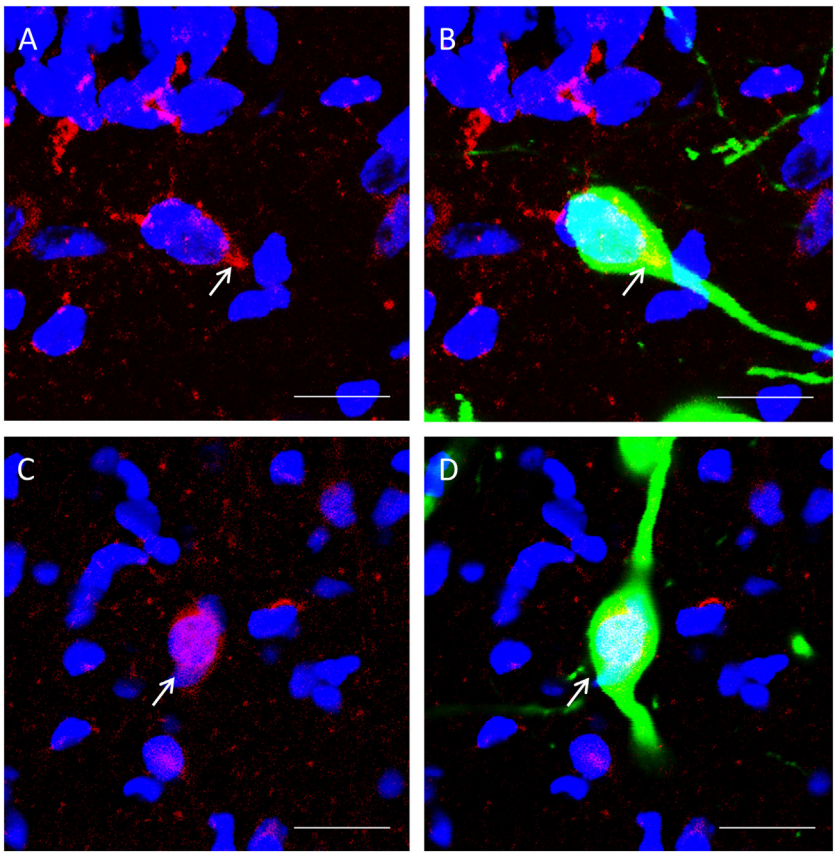

FIGURE 4 | The colocalization of β-catenin immunostaining with DAPI within gonadotropin-inhibitory hormone (GnIH) neurons in the dorsomedial hypothalamus (DMH) region. **(A)** β-catenin immunostaining (red) and DAPI cytoplasmic staining (blue) and **(B)** colocalization with GnIH neuron (green). **(C)** β-catenin immunostaining (red) in the cytoplasm with DAPI nuclear staining (blue) and **(D)** colocalization with GnIH neuron (green). Scale bar = 20 μm. White arrows indicate the presence of β-catenin colocalization with GnIH neurons.

12.22 ± 1.98 ($n = 9$), $p < 0.05$; **Figure 5B**]. No difference was observed for the GnIH neurons of isolated rats.

Expression of β-catenin within the nuclei of GnIH neurons in control rats (**Figures 6A,B**) was less prevalent than isolated rats in ZT18 (**Figures 6C,D**).

DISCUSSION

In this study, we observed daily variation of CLOCK expression in GnIH neurons. Compared to the control group, which displayed a fluctuation in CLOCK levels throughout the day, the isolated animals did not exhibit significant difference in CLOCK expression. CLOCK expression was significantly heightened in the dark phase in isolated rats compared to control rats. Looking at β-catenin, we found that they displayed a difference between ZT12 and ZT18 in both cytoplasmic and overall colocalization, increasing at ZT12 and decreasing at ZT18. We also observed higher EGFP intensity in isolated rats compared to control rats at ZT12.

CLOCK Expression in GnIH Neurons

CLOCK protein was observed within GnIH neurons in the DMH. Counting only the CLOCK-positive neurons that were also co-localized with GnIH expression, CLOCK was found to be expressed more highly in the light phase in control animals and more highly in the dark phase for isolated animals. Isolated animals also saw reduced differences in CLOCK expression between each time point measured.

In control rats, the peak of CLOCK expression was in the light phase compared to the dark, which supports the findings of Wyse and Coogan (29) where they determined the rhythmic nature of CLOCK in the DMH. Prior studies have demonstrated fluctuations in GnIH peptide and mRNA levels according to photoperiods, demonstrating a link between GnIH and the diurnal cycle in multiple species (30, 32, 45). A recent study performed on Syrian hamsters demonstrated differential action of vasointestinal peptide on GnIH cellular activity depending on the time of day (33). The same study also observed a rhythmic expression of PER1 protein in GnIH neurons (33), supporting our findings that GnIH neurons maintain their own internal clock. The presence of CLOCK and its rhythmicity suggests that the functionality of GnIH neurons in the DMH may be influenced by circadian rhythms.

In particular, the observed reduction of differences in CLOCK expression across time in GnIH cells in isolated animals leads

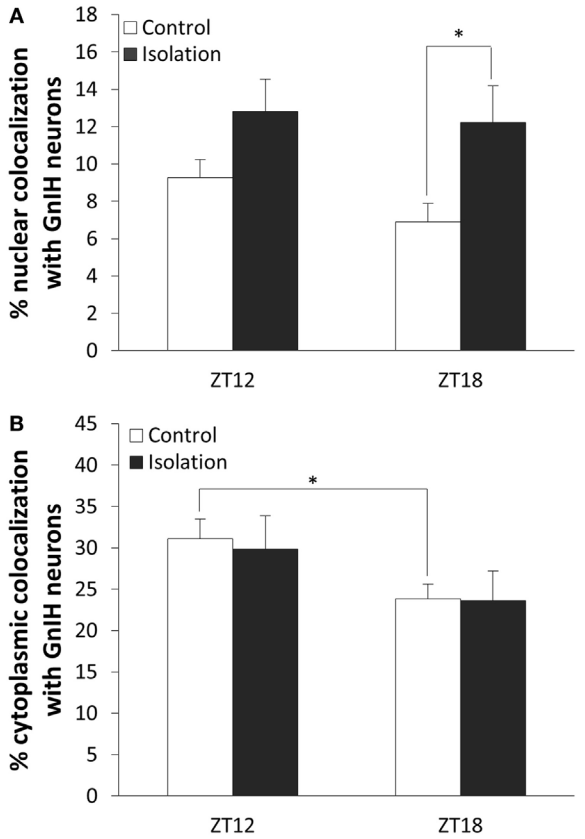

FIGURE 5 | The effect of postweaning social isolation on β-catenin colocalization with gonadotropin-inhibitory hormone (GnIH) neurons in the dorsomedial hypothalamus region. **(A)** Comparison of β-catenin nuclear colocalization within GnIH neurons of the control and isolated rats at ZT12 and ZT18 (control: $n = 6$ and isolation: $n = 6$ for ZT12 and control: $n = 9$ and isolation: $n = 9$ for ZT18). **(B)** Comparison of β-catenin cytoplasmic colocalization in GnIH neurons of control and isolated rats in ZT12 and ZT18 (control: $n = 6$ and isolation: $n = 6$ for ZT12 and control: $n = 9$ and isolation: $n = 9$ for ZT18). Data are presented as means ± SEM for each set. Significance was set at $p < 0.05$.

us to view the impact of social isolation as weakening the cyclic pattern of CLOCK expression. This is similar to a symptom of depression that has been observed in humans; a comprehensive postmortem study was conducted on patients suffering from major depressive disorder, discovering that circadian genes in depressed patients displayed a weaker cyclic pattern and that the expression of those genes exhibited less synchronicity between each other—in the normal scheme of things, the rise of a set of cyclic circadian genes would be linked to the fall of another set of circadian genes, but in depressed patients, the expression of the genes lose that synchronicity (46). In our study, we observed that the expression of CLOCK is heightened in the dark phase for isolated animals, in relative terms, compared to the regular CLOCK expression in control animals. Previous behavior experiments have indicated a more pronounced effect of social isolation on anxiety-like behavior in the dark (23). As those behaviors are linked to GnIH activity, the desynchronicity of CLOCK and its relative overexpression during the dark phase may be a factor. However, the cell counts performed show that GnIH neurons do not observably increase in number, which indicates that this link may be through the neuronal activity of existing neurons or the release of GnIH to related areas to control anxiety, rather than proliferation of GnIH neurons.

As EGFP is tagged to the GnIH promoter (42), increased activation of the promoter would result in a higher intensity value. Isolated rats displayed elevated intensity of GnIH neurons at ZT12 in comparison to their control counterparts. This indicates that the isolation process may have introduced a change in the basal activity patterns of GnIH neurons in the DMH, with increased activity during the light phase and reduced activity during the dark. The correlation appears to be inverse to that of the pattern observed in CLOCK, whereas social isolation has reduced the variation in CLOCK expression in GnIH neurons, it has also induced a variation in GnIH promoter activity across light and dark phases.

As under social isolation, both GnIH and GnRH expressions are reduced (23), it is likely GnIH activity is normally low since it acts mainly as a counter measure to GnRH activity. We conjecture that while social isolation may exert a suppressive effect on GnIH neurons at a basal level, it also renders them more sensitive to stressful stimuli, causing them to react in a pronounced way upon exposure to stress (21).

β-Catenin Expression in GnIH Neurons

This study is the first to observe the expression of β-catenin within GnIH neurons. Between 30 and 40% of GnIH neurons counted expressed observable levels of β-catenin, and different morphologies of β-catenin staining was observed in the cells, where it may be present in the cytoplasm, where the amount of β-catenin in the cytoplasm is in the process of increasing until it has saturated the binding sites, or the nucleus, where β-catenin has translocated from the saturated cytoplasm into the nucleus. This appears to match the role of β-catenin in the Wnt signaling pathway; namely its accumulation in the cytoplasm and subsequent translocation into the nucleus. β-catenin's presence in the nucleus would indicate its activity as a transcription factor. This would further suggest that β-catenin and the Wnt signaling pathway is active within GnIH neurons.

Nuclear β-catenin levels in GnIH neurons in the dark phase for isolation-raised rats are increased over that of the control group. This appears to suggest that social isolation may elevate β-catenin activity particularly in its role as a nuclear transcription factor. It is also possible that expression of β-catenin, at least within the DMH, may change according to the time of the day. In accordance with that, we found that in the control group, β-catenin expression in the cytoplasm was observed to fluctuate according to the phase, with a higher expression at ZT12 compared to ZT18. These time points match the peak and trough of CLOCK expression in the control group. In the isolated group, this fluctuation appears to be reduced, similarly to CLOCK under the same circumstances. This suggests a possible link between CLOCK and β-catenin expression. It also suggests that the Wnt signaling pathway may be influenced by the circadian cycle, as an

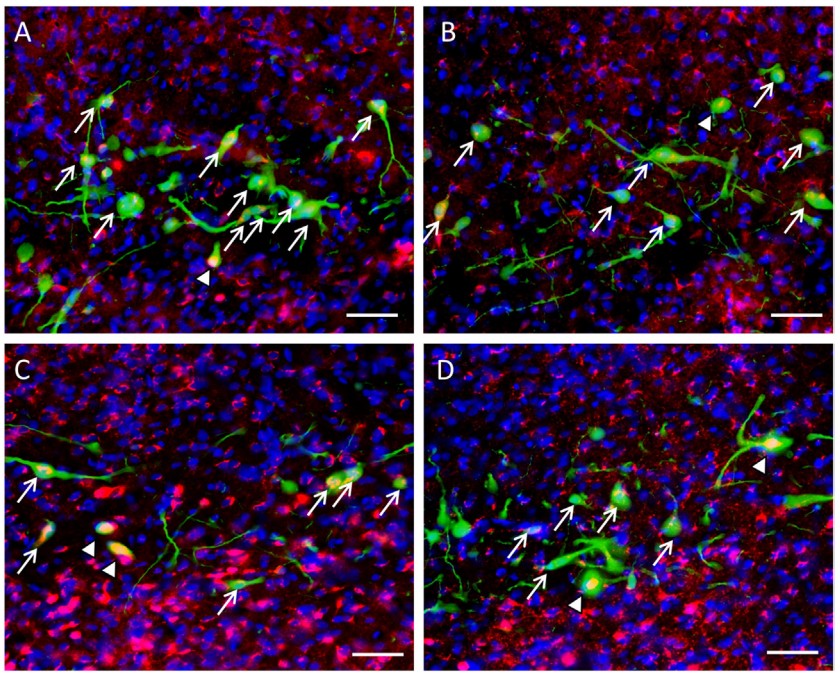

FIGURE 6 | Colocalization of β-catenin within gonadotropin-inhibitory hormone (GnIH) neurons in the dorsomedial hypothalamus (DMH) region across different time points. β-catenin immunostaining (red) was observed within GnIH neurons (green) in **(A)** control at ZT12, **(B)** control at ZT18, **(C)** isolation at ZT12, and **(D)** isolation at ZT18. Scale bar = 25 μm. White arrows indicate cytoplasmic colocalization, while white arrowheads indicate colocalization within or around the nucleus of GnIH neurons.

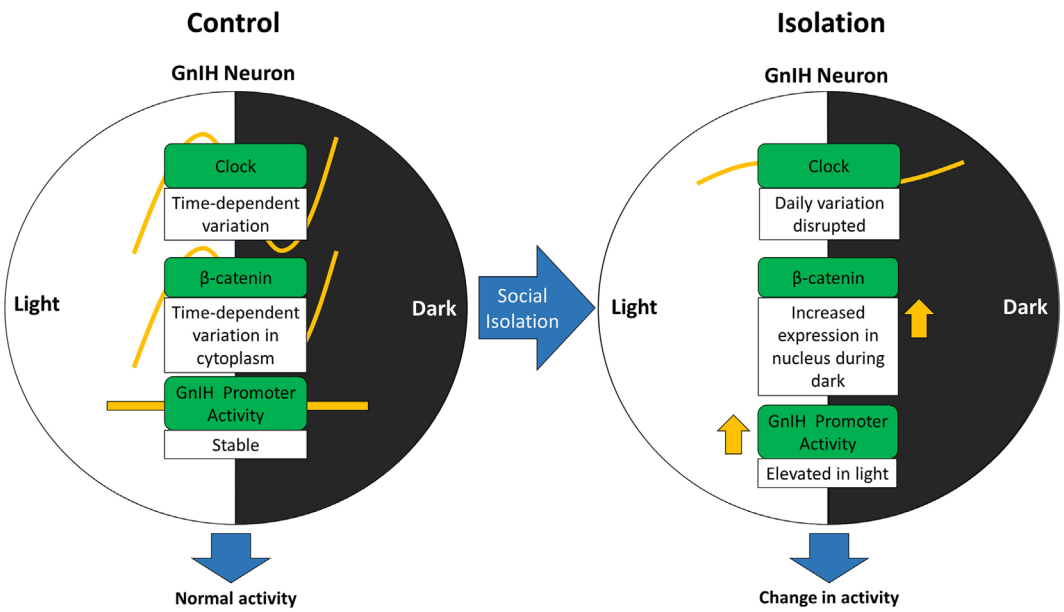

FIGURE 7 | Alteration of gonadotropin-inhibitory hormone (GnIH) neuronal activity may involve change in β-catenin activity due to disturbances in CLOCK expression stemming from social isolation.

increase of β-catenin levels in the cytoplasm is a prerequisite step for nuclear translocation. It may be possible that the heightened nuclear β-catenin levels in isolated rats during the dark phase may correspond to the increased CLOCK expression levels that we have described in the results section pertaining to CLOCK (**Figure 3D**).

β-catenin cytoplasmic colocalization varies according to the time of day, which points to the presence of a diurnal component in the potential interactions of β-catenin in GnIH neurons. The altered light/dark phase of CLOCK may affect the expression of β-catenin.

The relation between CLOCK and β-catenin in neuronal cells has not been elucidated by any other studies. Given that β-catenin has been predicted to have an E-box (CACGTG) binding site on its promoter, it is certainly possible that CLOCK may drive β-catenin expression directly *via* binding to the E-box in liver cells (47). This appears to explain the reduction of β-catenin fluctuations in relation to the reduction of CLOCK fluctuations as observed in our results, as well as the increase in CLOCK expression corresponding to a rise in nuclear β-catenin levels.

The potential link between β-catenin and GnIH neuronal activity appears to be clearer. Although the GnIH promoter is predicted to express a binding site for TCF/LEF1, the reduced neuronal activity in conjunction with increased β-catenin activity makes it less likely that β-catenin directly promotes activation of the GnIH gene *via* the Wnt signaling pathway. In their article regarding the role of β-catenin in preventing depression, Dias et al. (41) made specific mention of β-catenin's control of Dicer1, a gene highly involved in the production of microRNAs as a factor in stress resilience. Similarly microRNAs may also play a part in β-catenin's role within GnIH neurons. Two microRNAs, mir-155 and mir-7b, in particular, have been shown to decrease c-Fos protein levels by suppressing the translation of its mRNA transcript into protein (48, 49). Both microRNAs are regulated by Dicer1.

It is possible that the increased expression of β-catenin in isolated rats is related to decreased GnIH neuronal activity through this pathway. Expression of nuclear β-catenin may in this way increase the expression of Dicer1, which in turn promotes the production of microRNAs that reduce GnIH neuronal activity through the suppression of c-Fos translational activity. Alterations of β-catenin activity in the brain in response to long-term physiological changes have been observed elsewhere, notably in aging, where nuclear β-catenin was increased with age and demonstrated a resistive effect against age-related neural degeneration (50). These findings indicate that β-catenin plays a role in neuro protection. We theorize that the change in nuclear β-catenin expression patterns within GnIH neurons in the DMH under isolation conditions suggests the presence of a possible response mechanism to chronic stress, which promotes translocation of β-catenin into the nucleus as a neuroprotective measure.

We propose that β-catenin activity can be altered due to changes in CLOCK expression stemming from social isolation and that this signaling might be regulated by social stress (**Figure 7**). The increase in nuclear β-catenin translocation in the GnIH neurons of isolated rats indicates that social isolation may affect β-catenin in its capacity as a transcription factor within those neurons. To address this, we plan to use luciferase assays to investigate the changes in β-catenin expression in relation to inducible GnIH promoter activity, in the future.

CONCLUSION

In this study, we demonstrated the colocalization of CLOCK and β-catenin in GnIH neurons of the DMH region. We also showed that social isolation appears to invert CLOCK expression patterns in GnIH neurons across time. Furthermore, we also noted the effect of social isolation on modifying the expression of cytoplasmic β-catenin in GnIH neurons, which could be associated with heightened GnIH promoter activity in the light phase *via* measuring EGFP fluorescent intensity. Given that we also noted elevated nuclear translocation of β-catenin in isolated rodents during the dark phase, it is possible that CLOCK, β-catenin, and GnIH are connected in a pathway that culminates in the control of GnIH neuronal activity. As GnIH has been indicated to have a role in stress, the role of CLOCK and β-catenin in concert with GnIH neuronal activity may point toward a circadian component in the maintenance of regular neuronal activity under chronically stressful conditions, such as that induced by social isolation. Our findings continue to support the long-held consensus on the negative effects of social isolation and provide new insights into circadian regulation of GnIH neuronal activity.

ETHICS STATEMENT

Animal welfare and experimental ethics in our institute. SPF animal facility was followed in line with the authorized guidelines laid out by Monash University Animal Ethics Community (MARP/2012/140, MARP/2017/021).

AUTHOR CONTRIBUTIONS

TS and IP designed this research. CT conducted all experiments and did analysis together with TS and IP. CT prepared a manuscript and TS and IP edited it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2017.00225/full#supplementary-material.

FIGURE S1 | Specificity test for CLOCK and β-catenin antibodies. Blank buffer test was conducted for CLOCK and β-catenin. **(A)** Result of CLOCK-immunostaining. **(B)** The result of the same immunostaining process with the CLOCK antibody absent. **(C)** Demonstrates β-catenin antibody staining, **(D)** with the antibody absent and (E) with the peptide absorption test. Scale bar = 50 μm.

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REVIEW
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A Journey through the Gonadotropin-Inhibitory Hormone System of Fish

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Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide that belongs to the RFamide peptide family and was first identified in the quail brain. From the discovery of avian GnIH, orthologous GnIH peptides have been reported in a variety of vertebrates, including mammals, amphibians, teleosts and agnathans, but also in protochordates. It has been clearly established that GnIH suppresses reproduction in avian and mammalian species through its inhibitory actions on brain GnRH and pituitary gonadotropins. In addition, GnIH also appears to be involved in the regulation of feeding, growth, stress response, heart function and social behavior. These actions are mediated *via* G protein-coupled GnIH receptors (GnIH-Rs), of which two different subtypes, GPR147 and GPR74, have been described to date. With around 30,000 species, fish represent more than one-half of the total number of recognized living vertebrate species. In addition to this impressive biological diversity, fish are relevant because they include model species with scientific and clinical interest as well as many exploited species with economic importance. In spite of this, the study of GnIH and its physiological effects on reproduction and other physiological processes has only been approached in a few fish species, and results obtained are in some cases conflicting. In this review, we summarize the information available in the literature on GnIH sequences identified in fish, the distribution of GnIH and GnIH-Rs in central and peripheral tissues, the physiological actions of GnIH on the reproductive brain-pituitary-gonadal axis, as well as other reported effects of this neuropeptide, and existing knowledge on the regulatory mechanisms of GnIH in fish.

Keywords: LPXRFa, gonadotropin-inhibitory hormone, reproduction, teleosts, GnRH, gonadotropins, gonads, behavior

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INTRODUCTION

Gonadotropin-inhibitory hormone (GnIH) is a neuropeptide that was first identified in the Japanese quail (*Coturnix japonica*) brain and exhibited inhibitory actions on gonadotropin secretion both *in vitro* (1) and *in vivo* (2). Following on from pioneer research in avian species, subsequent studies performed in mammals demonstrated that GnIH could also inhibit the reproductive process in this group of vertebrates (3–7). In the last 17 years, GnIH orthologs have been identified not only in tetrapod vertebrates, but also in fish and protochordates (8–10). The ancestral form of GnIH, which has a C-terminal PQRF-amide structure, emerged in the

amphioxus, a protochordate species (11). However, this ancestral form of GnIH was duplicated into two paralogous genes, GnIH and NPFF, by chromosome duplication that occurred at the beginning of vertebrate evolution (12, 13). NPFF is also expressed in the brain of vertebrates and discussed when relevant in this review.

Fishes, which represent around half of all living vertebrate species, are one of the most successful radiations in the long evolutionary history of vertebrates (14). Almost all ray-finned fishes are teleosts, which represent the dominant vertebrates inhabiting marine and freshwater ecosystems. Fishes include most commercially important species from fisheries and aquaculture, but also several model organisms for genomics, developmental biology and clinical studies. Despite their impressive biological diversity, key phylogenetic position, economic and scientific importance, the study of GnIH and its physiological effects have been approached in only a few fish species. In addition, the action of GnIH on gonadotropin secretion and reproduction has conflicting results in fish. For example, Amano et al. (15) reported that goldfish GnIH stimulated gonadotropin release from cultured sockeye salmon (*Oncorhynchus nerka*) pituitary cells. On the other hand, Zhang et al. (16) reported that intraperitoneal administration of zebrafish GnIH to goldfish (*Carassius auratus*) inhibited serum gonadotropin levels. The reason for this discrepancy may be partially because the endogenous GnIH peptides of the fish were not used to show their physiological effects. The nature of GnIH effects seems to be dependent on the species, as well as on the sex of the animals, the physiological status, dose, the route and the time elapsed after administration of the GnIH peptide. For example, *in vivo* stimulatory effects of intraperitoneally injected tilapia GnIH (tiGnIH)-2 on FSH and LH secretion have been reported in female tilapia, *Oreochromis niloticus* (17) whereas intracerebroventricular (icv) administration of sea bass GnIH (sbGnIH)-2 inhibited *fshβ* and *lhβ* expression and LH plasma levels in male European sea bass, *Dicentrarchus labrax* (18). In goldfish, there are remarkable differences in reproductive responses to GnIH at different gonadal maturation stages, which reinforce the idea that seasonal reproductive influences are important modulators of GnIH actions (19, 20). Therefore, more efforts on fish GnIH research appear necessary to obtain a clear picture on the role of this neuropeptide on reproduction and other physiological processes in this important group of vertebrates. This review aims at synthesizing the most relevant information regarding the forms, brain distribution, actions and regulation of fish GnIH reported up to date in the literature.

COMPARISON OF TELEOST FISH GnIH WITH OTHER VERTEBRATE GnIH ORTHOLOGS

The comparison of GnIH precursor and GnIH peptide sequences in various teleost fish species as well as the spotted gar, the coelacanth *Latimeria chalumnae*, the Japanese quail, and humans are presented in **Figure 1**. In order to identify in this review if the differences in protein or peptide sequences respond to a taxonomic pattern, taxonomic information (Class, Order, Family) of the species analyzed in this figure is provided in **Table 1**. The mature structure of quail GnIH that was identified by immunoaffinity chromatography and mass spectrometry is SIKPSAYLPLRFamide (1). The cDNA sequence that encodes the quail GnIH precursor polypeptide was cloned and was found to encompass two further peptide sequences besides GnIH that have -LPXRFamide (X = L or Q) sequences at the C-temini. These LPXRFamide peptides were named GnIH-related peptide (RP)-1 and GnIH-RP-2 (21). These three LPXRFamide peptides, GnIH-RP-1, GnIH, GnIH-RP-2 are encoded in the quail GnIH precursor polypeptide in this order (**Figure 1**). The mature peptide sequence of GnIH-RP-2, which is SSIQSLLNLPQRFamide, was also identified by immunoaffinity chromatography and mass spectrometry (21) (**Figure 1**). Later in the year 2000, a cDNA encoding human LPXRFamide peptide precursor polypeptide was found in the gene database (22). Human LPXRFamide peptide precursor encompasses one C-terminal—LPLRFamide peptide and one—LPQRFamide peptide in this order, and these were named human RFamide related peptide (RFRP)-1 and -3, respectively. There is also an LPXRFamide-like peptide sequence named human RFRP-2 which has a C-terminal—LPLRSamide sequence in between human RFRP-1 and -3 in the precursor polypeptide (**Figure 1**). The structures of the mature human RFRP-1 and -3 peptides were identified to be MPHSFANLPLPFamide and VPNLPQRFamide by immunoaffinity chromatography and mass spectrometry (23). The alignment of GnIH precursor polypeptides by the European Bioinformatics Institute (EMBL-EBI) Clustal Omega showed that human RFRP-1 and RFRP-2 align with quail GnIH-RP-1 and GnIH, respectively (**Figure 1**). Human RFRP-3 aligns with an LPXRFamide-like peptide in the quail GnIH precursor, which has a C-terminal—LSNRSamide sequence (**Figure 1**).

The coelacanth is a lobe-finned fish that is closely related to tetrapods. Three LPXRFamide peptides and one LPXRFamide-like peptide are encoded in the coelacanth (*Latimeria chalumnae*) GnIH precursor, and they align to human RFRP-1/quail GnIH-RP-1, human RFRP-2/quail GnIH, human RFRP-3, and quail GnIH-RP-2 (**Figure 1**). The spotted gar (*Lepisosteus oculatus*) is a ray-finned fish that diverged from teleosts before teleost specific genome duplication, and therefore, it is regarded as a good biomedical model (24). Three LPXRFamide peptides are encoded in the gar GnIH precursor polypeptide and they align to human RFRP-1/quail GnIH-RP-1, human RFRP-2/quail GnIH, and human RFRP-3 (**Figure 1**). GnIH precursor polypeptides of spotted green pufferfish (*Tetraodon nigroviridis*), torafugu (*Takifugu rubripes*), tongue sole (*Cynoglossus semilaevis*), and European sea bass encode only two LPXRFamide-like peptide sequences, which have C-terminal—MPMRFamide and—MPQRFamide sequences, which align to human RFRP-1/quail GnIH-RP-1 and human RFRP-2/quail GnIH, respectively (**Figure 1**). GnIH precursor polypeptides of Nile tilapia, princess cichlid (*Neolamprologus brichardi*), and Lake Victoria cichlid *Pundamilia nyererei* encode one LPXRFamide-like and one LPXRFamide peptide sequence, which have C-terminal—MPLRFamide and—LPQRFamide sequences, which align to human RFRP-1/quail GnIH-RP-1 and human RFRP-2/quail GnIH, respectively (**Figure 1**). The GnIH precursor of the other teleost fish species encodes three LPXRFamide or LPXRFamide-like peptides and

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Homo sapiens                --MEIISS-KLFILLTLATSSLLT--SNIFC------ADELVMSNLHSKENYD-KYSEPR
Coturnix japonica           --MEIIST-QKFILLTLATVAFLT--PHGAC------LDELMKSSLESREDDDDKYYEIK
Latimeria chalumnae         ----------------------------------------MPISEPPTIEVVTKVFQEAS
Lepisosteus oculatus        --MTP-NS-CWPVLLLLGCSVLQT--PAAQS------ADERPLSADQD-----RLDPDAT
Tetraodon nigroviridis      ----MLVTLFLAMLLMIAGLGKAA-VSDLQVTGKVN---DRTLGSREGRH-NMRK--ELH
Takifugu rubripes           ----MLVTAFLAMLLMIAGIGGAA-ETDLQVNGKLN---DRTLSSREGRH-NVRK--QLR
Cynoglossus semilaevis      ----MLSTVFLSVLLMLGGPGGAAAAADFQVYGKSAYSDKSLPSSEEGRH-TVRR--QPL
Thalassoma bifasciatum      ----MSITVFLPVLLLLGALLGTV-TTNQQVLEKSVPGGKSLLSSGDGSP-TMRK--HLH
Oryzias latipes             ----MLTMMMLSVLLVLGGLGGAA-ASDLHVFGKSFHGDDPLESSHDSQLNMLRK--QLH
Aphyosemion striatum        ----MLTTVTLLALLMLGGLRGAA-ASDFHVFGKSIHNDETLQSSNDNRY-SIRK--LPR
Nothobranchius kuhntae      ----MLTTVTLLALLMLGGLGGAA-AYDFRVFGKSIHNDETLQSSNGNRY-SIRK--LPR
Nothobranchius furzeri      ----MLTTVTLLALLMLGGLGGAA-AYDFRVFGKSIHNDETLQSSNGNRY-SIRK--LPR
Nothobranchius pienaari     ----MLTTVTLLALLVLGGLRGAA-AYDFRVFGKSIHNDETLQSSNGNRY-SIRK--LPR
Kryptolebias marmoratus     ----MLTMVILLALLMLGGLGGAA-ASDFHVFGKSIHDDETLQSSNDDRH-SIRK--QPR
Austrofundulus limnaeus     ----MLTTVIMLALLMLGGLRGAA-ASDFHVFGKSIHDDETLQSSSSDRI-LLRK--QPR
Cyprinodon variegatus       ----MLAAAILSVLLTLGGLGGAA-ASDLHIFGKSFYNDGTLRSSSDDRY-TVRK--QQS
Fundulus heteroclitus       ----MFTAAVLSVLLMMGGLGGAA-ASDFHVFGKPFHNDEALRSSSDDRH-SVRK--QPR
Xiphophorus maculatus       ----MLTAVILSALLMMGGLGGVA-ASDFHVFGKSLHNGEALRSSIDDRH-SVRK--QPR
Poecilia latipinna          ----MLTAAILSALLMMGDLGGAA-ASDFHVFGKSFHNDETLRSSVDDRH-SVRK--QPR
Poecilia reticulata         ----MLTAAILSALLMMGGLGGAA-ASDFHVFGKSFHNDETLRSSIDDRH-SVRK--QPR
Poecilia Formosa            ----MLTAAILSALLMMGGLGGAA-ASDFHVFGKSFHNDETLRSSVDDRH-SVRK--QPR
Poecilia mexicana           ----MLTAAILSALLMMGGLGGAA-ASDFHVFGKSFHNDETLRSSVDDRH-SVRK--QPR
Oreochromis niloticus       ----MLVTMILSALLMLRGLGG----SDVHVFGKSVRSGKTLLSSNDGTY-SVRK--QPH
Neolamprologus brichardi    ----MLVTMILSALLMLRGLGG----SDVHVFGKSVRSGKNLLSSNDGTY-SVRK--QPH
Pundamilia nyererei         MVVNMLVTMILSALLMLRGLGG----SDVHVFGKSVRSGKNLLSSNDGTY-SVRK--QPH
Dicentrarchus labrax        ----MLTTVFLSTLLMLGGLGGAA-VSDLQVYGKSIHSDKTLLSSDDGRH-TVRK--QPH
Stegastes partitus          ----MLTTMMLSALLMLWGLGA----FDLQVYGKSIHSDKTLLISNDGKH-SARK--QPH
Anguilla japonica           -----MNRFAASLLLAAGIIGRFA-PRTVTCVG----GHGSAATFAQRAG-RLPV--YPE
Salmo salar                 -----MSM-FTLVILGCLGLQGVM-ASDSRVYRMSMTNDNDGHTTSQ------RQ--HPQ
Ictalurus punctatus         ------MS-CSALSLTLVILSSLV-FQDVTTVKLPLTGNNNINTVTR------RM--FLK
Pygocentrus nattereri       ------MP-CSTLSLTLGILSSLL-FQDVGAVRLPLTGDDDSNRIIN------GI--FSE
Danio rerio                 -----MSY-FALLSLALGILSSFM-LSEVTALRLPLSGERDLNGFTW------GQ--FSE
Carassius auratus           -----MSY-FTLVFLALGTLSSFM-LREVTALRWPLPDDSDPDRFTW------GQ--FLE
Cyprinus carpio             -----MSY-FTLLSLAFGILSSFM-LREVTALRLPLPDDRDPNRFTW------GQ--FPE
Sinocyclocheilus rhinocerous -----MSY-FTLLFLAFGILSSFM-LREVTALRLPLPDDSDPDRFTW------GQ--FPE
Sinocyclocheilus grahami    -----MSY-FTLLFLAFGILSSFM-LREVTVLRLPLPDDSDPDRFTW------GQ--FPE

                                                                    RFRP-1/GnIH-RP-1
Homo sapiens                --GYPKGERSLNFEELKDWGPKNV-IKMSTPAVNKMP-------HSFANLPLRFGRNVQ-
Coturnix japonica           DSILEEKQRSLNFEEMKDWGSKNF-MKVNTPTVNKVP-------NSVANLPLRFGRSNP-
Latimeria chalumnae         QESLEERQRSLSNEQLKEWEPKAT-IKMKTPIISKFS-------NSVINLPLRFGRAFP-
Lepisosteus oculatus        HENMQEELRSIELERIQDILPSVT-RKIDVPTIQKLY-------HSVTNLPLRFGRA---
Tetraodon nigroviridis      HQVKNNILRSLDLESLNIHVSPTS--KISFPTIIRLYPPTPQPPLRHINMPMRFGRNSFH
Takifugu rubripes           HQIKSNILRSLDMERINIQVSPTS-GKVSLPTIVRLYPPTLQPRHQHVNMPMRFGRDGVQ
Cynoglossus semilaevis      QQAKAVTRRSLDLERLNMRVTPTA-SKSSLPTIIKLYPPTVNP-HIHANMPMRFGREVEP
Thalassoma bifasciatum      QQTKSEWRRSLDFNSFNIHSTPT--SKIRLPSIIKLYPPTVQPAYLHPNMPLRFGRQSGS
Oryzias latipes             QQTKRGIRRSLDLESFNIRVTPTS-SKLNLPTIIKLYPPTAKPLHMHANMPLRFGRESSA
Aphyosemion striatum        QQLTSGIRRSLDLESFKIHVTPTT-SKISLPTIIKLYPPTAKPLHMHANMPLRFGRDI--
Nothobranchius kuhntae      QQLMSGICRSLDLESFKIHVTPTT-SKISLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Nothobranchius furzeri      QQLMSGIRRSLDLESFKIHVTPTT-SKISLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Nothobranchius pienaari     QQLMSGIRRSLDLESFKIHVTPTT-SKISLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Kryptolebias marmoratus     QQMASGIRRSLDLESFKIHVTPAT-SKISLPTIIKLYPPTAKPLHMHANMPLRFGRES--
Austrofundulus limnaeus     QQMTSGIRRSLDLESFKLHVTPAT-SKISLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Cyprinodon variegatus       QQMASGIRRSLDLESFKLHMTPAT-SRMSLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Fundulus heteroclitus       QQVPSGIRRSLDLESFKLHMTPAT-SKMSLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Xiphophorus maculatus       QQIPSGLRRSLDLESFKLHVTPAT-SRNSLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Poecilia latipinna          QQVPSGLRRSLDLESFKLHVTPAT-SRNSLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Poecilia reticulata         QQVPSGLRRSLDLESFKLHVTPAT-SRNSLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Poecilia formosa            QQAPSGLRRSLDLESFKLHVTPAT-SRNSLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Poecilia mexicana           QQAPSGLRRSLDLESFKLHVTPAT-SRNSLPTIIKLYPPTAKPLHMHANMPLRFGRDS--
Oreochromis niloticus       QETKNEIHRSLDLESFNIRVAPTT-SKFSLPTIIRFYPPTVKPLHLHANMPLRFGRQS--
Neolamprologus brichardi    QETKNEIHRSLDLKSFNIHVAPTT-SKFSLPTIVRFYPPTVKPLHQHANMPLRFGRQSDP
Pundamilia nyererei         QETKNKIHRSLDLDSFNIHVAPTT-SKFSLPTIVRFYPPTVKPLHQHANMPLRFGRQSHP
Dicentrarchus labrax        QQAKGEIRRSLDLESFNIHVSPTT-SKISLPTIIRLYPPTAKPLHLHANMPMRFGRDSNP
Stegastes partitus          QQIKGEIRRSLDLESYNIHVSPTT-SKHSLPTIIRIYPPTSIPRHLHANIPMRFGRDSDP
Anguilla japonica           AMQESERARSLEMDDFKVQEVPDD-TMGSTPIILKLYPPVAKPALLHANLPLRFGRSSSR
Salmo salar                 --TSNEIPRSIEVDDFKINVVPTSGKVILAPTMVRLYPPPVKTSHLHANLPLRFGRDSLS
Ictalurus punctatus         --SNEDLPRSLEMEDSTLNMAPTSSSRVNSPTILRLHPLLAKSVHTHANLPLRFGRGSAH
Pygocentrus nattereri       --NSQDIPRSLEMEEFAFNVVPTS-GRASSPTILRLHPISAKPSHLHANLPLRFGREAY-
Danio rerio                 --NAQEIPRSLEIQDFTLNVAPTS-GGASSPTILRLHPIIPKPAHLHANLPLRFGRDAQP
Carassius auratus           --NAQEIPRSLEIEDFTLNVAPTS-GRVSSPTILRLHPKITKPTHLHANLPLRFGRDTEN
Cyprinus carpio             --NTQEIPRSLEIEDFSLNVAPTS-SRVSSPTILRLHPIIKKPTHLHANLPLRFGRDAQT
Sinocyclocheilus rhinocerous --NNQEIPRSLEIEDFTLNVAPTS-SRVSSPTILRLHPIIKKPTHLHANLPLRFGRDAQT
Sinocyclocheilus grahami    --NTQEIPRSLEMEDFTLNVAPTS-SRVSSPTILRLHPIIKKPTHLHANLPLRFGRDAQM
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FIGURE 1 | Continued

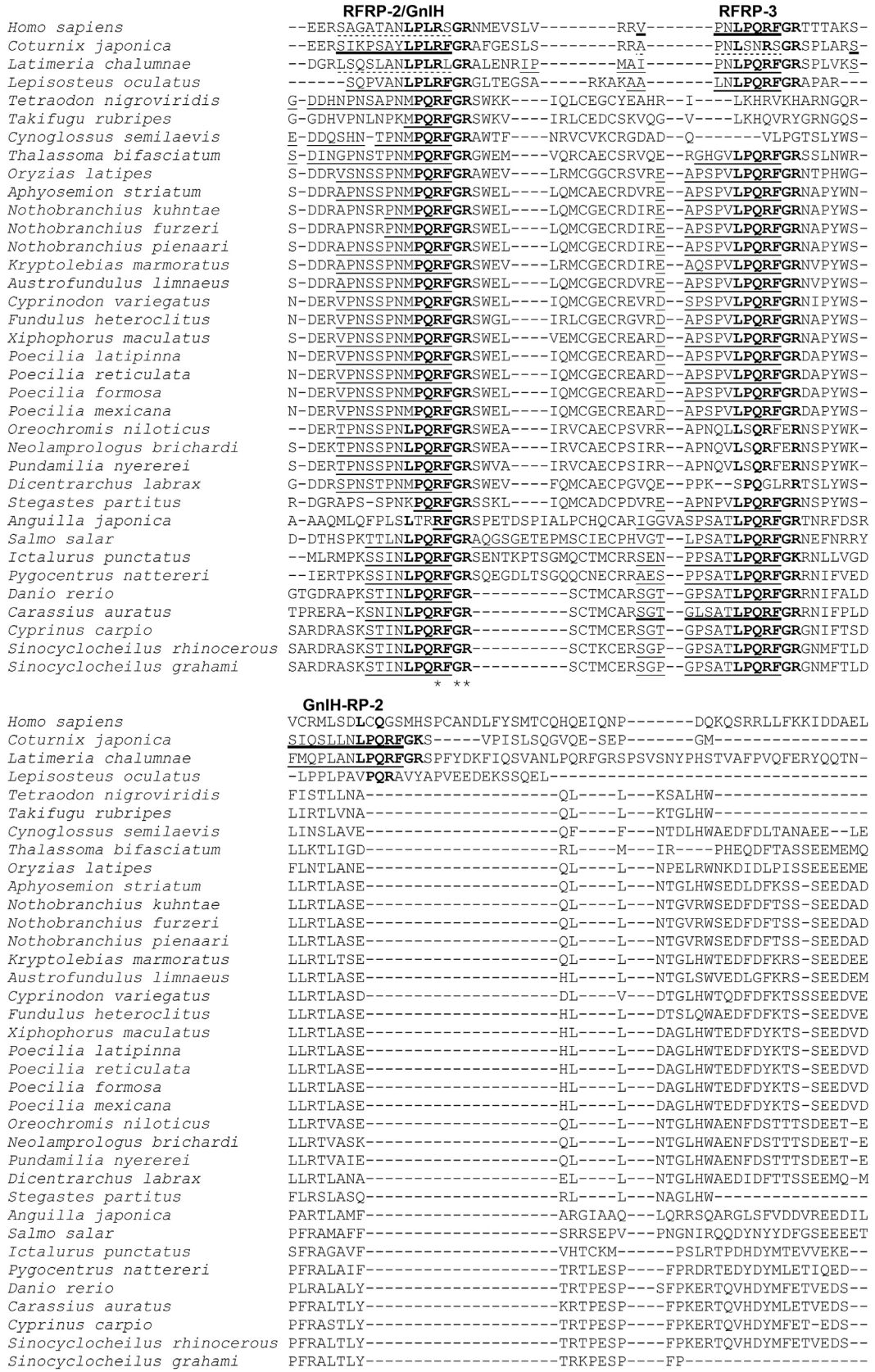

FIGURE 1 | Continued

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Homo sapiens                KQEK----------
Coturnix japonica           ----------
Latimeria chalumnae         ----------
Lepisosteus oculatus        ----------
Tetraodon nigroviridis      ----------
Takifugu rubripes           ----------
Cynoglossus semilaevis      MERTSFKG----------
Thalassoma bifasciatum      --E---------
Oryzias latipes             GKDIYNMK----------
Aphyosemion striatum        MEDKSFKG----------
Nothobranchius kuhntae      MEEKTFKG----------
Nothobranchius furzeri      MEVKTFKG----------
Nothobranchius pienaari     MEEKTFKG----------
Kryptolebias marmoratus     VEEKNL----------
Austrofundulus limnaeus     EEKTFKE----------
Cyprinodon variegatus       TEEKAFKG----------
Fundulus heteroclitus       TEEKSFKG----------
Xiphophorus maculatus       TDEKTFKG----------
Poecilia latipinna          TNEQTFKG----------
Poecilia reticulata         TDKKTFKG----------
Poecilia formosa            TDEKTFKG----------
Poecilia mexicana           TDEKTFKG----------
Oreochromis niloticus       LEEKKSKE----------
Neolamprologus brichardi    LEEKKSK----------
Pundamilia nyererei         LEEKKSK----------
Dicentrarchus labrax        -QEKTFNG----------
Stegastes partitus          ----------
Anguilla japonica           NIPKDIEASSSPASD---
Salmo salar                 -REKTLKSSTLDLLLWNS
Ictalurus punctatus         --DKALKSKTYIDLEI--
Pygocentrus nattereri       --DKALKSKKYID-----
Danio rerio                 --EETVKNTDYTALD---
Carassius auratus           -VEETVKNKDYTVLD---
Cyprinus carpio             --EETVKRTDYTALD---
Sinocyclocheilus rhinocerous --EETVKRTDYTALD---
Sinocyclocheilus grahami    ----------
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FIGURE 1 | The alignment of gonadotropin-inhibitory hormone (GnIH) precursor polypeptides of human, Japanese quail, coelacanth, spotted gar, and various teleost fish species. The GnIH precursor polypeptide sequences were aligned by using EMBL-EBI Clustal Omega Multiple Sequence Alignment software. The characteristic amino acid sequence of the GnIH peptide, Leu (L), Pro (P), Leu (L) or Gln (Q), Arg (R), Phe (F) with Gly (G) as an amidation signal followed by Arg (R) or Lys (K) as an endoproteolytic basic amino acid at the C-termini are shown in bold. Identified mature GnIH peptide sequences in human (*Homo sapiens*), Japanese quail (*Coturnix japonica*), and goldfish (*Carassius auratus*) are underlined with thick lines. Possible mature GnIH peptide sequences that may be cleaved after the first N-terminal basic amino acids (R or K) are underlined with thin lines. Possible mature GnIH-like peptide sequences that may be cleaved after the first N-terminal basic amino acids (R or K), which have C-terminal—LPLRSamide (human), -LSNRSamide (Japanese quail), and LPLRLamide sequences (*Latimeria chalumnae*, coelacanth) are underlined with broken lines. Accession numbers of GnIH precursor polypeptide sequences in US National Center for Biotechnology Information database are *Homo sapiens* (NP_071433.3), *Coturnix japonica* (XP_015709159.1), *Latimeria chalumnae* (XP_005993154.1), *Lepisosteus oculatus* (XP_015213317.1), *Tetraodon nigroviridis* (BAF34880.1), *Takifugu rubripes* (NP_001092115.1), *Cynoglossus semilaevis* (AMB48604.1), *Thalassoma bifasciatum* (ANV28067.1), *Oryzias latipes* (XP_004073896.1), *Aphyosemion striatum* (SBP35361.1), *Nothobranchius kuhntae* (SBQ91527.1), *Nothobranchius furzeri* (XP_015811406.1), *Nothobranchius pienaari* (SBR89569.1), *Kryptolebias marmoratus* (XP_017278134.1), *Austrofundulus limnaeus* (XP_013866639.1), *Cyprinodon variegatus* (XP_015229614.1), *Fundulus heteroclitus* (XP_012729657.1), *Xiphophorus maculatus* (XP_005802819.1), *Poecilia latipinna* (XP_014884496.1), *Poecilia reticulate* (XP_008419875.1), *Poecilia formosa* (XP_007562706.1), *Poecilia mexicana* (XP_014852162.1), *Oreochromis niloticus* (NP_001298256.1), *Neolamprologus brichardi* (XP_006788138.1), *Pundamilia nyererei* (XP_013765199.1), *Dicentrarchus labrax* (CEK03537.1), *Stegastes partitus* (XP_008290012.1), *Anguilla japonica* (BAV18007.1), *Salmo salar* (XP_013998456.1), *Ictalurus punctatus* (XP_017336524.1), *Pygocentrus nattereri* (XP_017549097.1), *Danio rerio* (NP_001076418.1), *Carassius auratus* (BAC06473.1), *Cyprinus carpio* (AML83913.1), *Sinocyclocheilus rhinocerous* (XP_016370559.1), and *Sinocyclocheilus grahami* (XP_016150344.1). Refer to **Table 1** for the common names, class, order, and family of these species. *Indicates positions which have a single, fully conserved amino acid residue, and symbols : and . indicate conservation between groups of strongly (:) and weakly (.) similar amino acid properties, respectively.

they align to human RFRP-1/quail GnIH-RP-1, human RFRP-2/quail GnIH, and human RFRP-3 (**Figure 1**). The C-terminal sequence of the LPXRFamide-like peptides that aligns to human RFRP-1/quail GnIH-RP-1 is—MPLRFamide in Labriformes, Beloniformes, Cyprinodontiformes, and Cichliformes (**Figure 1**; **Table 1**). The C-terminal—LPLRFamide peptides align to human RFRP-1/quail GnIH-RP-1 in Anguilliformes, Salmoniformes, Siluriformes, Characiformes, and Cypriniformes (**Figure 1**; **Table 1**). The C-terminal sequence of the LPXRFamide-like peptides that aligns to human RFRP-2/quail GnIH is—MPQRFamide in Tetraodontiformes, Pleuronectiformes, Labriformes, Beloniformes, Cyprinodontiformes, and some Perciformes (**Figure 1**; **Table 1**). The C-terminal LPQRFamide peptides align to human RFRP-2/quail GnIH in Cichliformes, Salmoniformes, Siluriformes, Characiformes, and Cypriniformes (**Figure 1**; **Table 1**). The C-terminal LPQRFamide peptides align to human RFRP-3 in Labriformes, Beloniformes, Cyprinodontiformes, Anguilliformes, Salmoniformes, Siluriformes, Characiformes, Cypriniformes, and some Perciformes (**Figure 1**; **Table 1**).

In summary, most of the teleost GnIH precursor polypeptides encode three LPXRFamide or LPXRFamide-like peptides, which align to human RFRP-1/quail GnIH-RP-1, human RFRP-2/quail GnIH, and human RFRP-3. The C-terminal sequences are mostly conserved in the peptide sequences that align to human RFRP-1/quail GnIH-RP-1, followed by those that align to human RFRP-2/quail GnIH. However, the C-terminal sequence of the peptides

TABLE 1 | Scientific name, common name, class, order, and family of the species analyzed.

Scientific name	Common name	Class	Order	Family
Homo sapiens	Human	Mammalia	Primates	Hominidae
Coturnix japonica	Japanese quail	Aves	Galliformes	Phasianidae
Latimeria chalumnae	Coelacanth	Sarcopterygii	Coelacanthiformes	Coelacanthidae
Lepisosteus oculatus	Spotted gar	Actinopterygii	Semionotiformes	Lepisosteidae
Tetraodon nigroviridis	Spotted green pufferfish	Actinopterygii	Tetraodontiformes	Tetraodontidae
Takifugu rubripes	Torafugu	Actinopterygii	Tetraodontiformes	Tetraodontidae
Cynoglossus semilaevis	Tongue sole	Actinopterygii	Pleuronectiformes	Cynoglossidae
Thalassoma bifasciatum	Bluehead wrasse	Actinopterygii	Labriformes	Labridae
Oryzias latipes	Japanese medaka	Actinopterygii	Beloniformes	Adrianichthyidae
Aphyosemion striatum	Red-striped killifish	Actinopterygii	Cyprinodontiformes	Nothobranchiidae
Nothobranchius kuhntae	Beira killifish	Actinopterygii	Cyprinodontiformes	Nothobranchiidae
Nothobranchius furzeri	Turquoise killifish	Actinopterygii	Cyprinodontiformes	Nothobranchiidae
Nothobranchius pienaari	Black rachovii	Actinopterygii	Cyprinodontiformes	Nothobranchiidae
Kryptolebias marmoratus	Mangrove rivulus	Actinopterygii	Cyprinodontiformes	Rivulidae
Austrofundulus limnaeus	Annual killifish	Actinopterygii	Cyprinodontiformes	Rivulidae
Cyprinodon variegatus	Sheepshead minnow	Actinopterygii	Cyprinodontiformes	Cyprinodontidae
Fundulus heteroclitus	Mummichog	Actinopterygii	Cyprinodontiformes	Fundulidae
Xiphophorus maculatus	Southern platyfish	Actinopterygii	Cyprinodontiformes	Poeciliidae
Poecilia latipinna	Sailfin molly	Actinopterygii	Cyprinodontiformes	Poeciliidae
Poecilia reticulata	Guppy	Actinopterygii	Cyprinodontiformes	Poeciliidae
Poecilia formosa	Amazon molly	Actinopterygii	Cyprinodontiformes	Poeciliidae
Poecilia mexicana	Shortfin molly	Actinopterygii	Cyprinodontiformes	Poeciliidae
Oreochromis niloticus	Nile tilapia	Actinopterygii	Cichliformes	Cichlidae
Neolamprologus brichardi	Princess cichlid	Actinopterygii	Cichliformes	Cichlidae
Pundamilia nyererei	Lake Victoria cichlid	Actinopterygii	Cichliformes	Cichlidae
Dicentrarchus labrax	European sea bass	Actinopterygii	Perciformes	Moronidae
Stegastes partitus	Bicolor damselfish	Actinopterygii	Perciformes	Pomacentridae
Anguilla japonica	Japanese eel	Actinopterygii	Anguilliformes	Anguillidae
Salmo salar	Atlantic salmon	Actinopterygii	Salmoniformes	Salmonidae
Ictalurus punctatus	Channel catfish	Actinopterygii	Siluriformes	Ictaluridae
Pygocentrus nattereri	Red-bellied piranha	Actinopterygii	Characiformes	Serrasalmidae
Danio rerio	Zebrafish	Actinopterygii	Cypriniformes	Cyprinidae
Carassius auratus	Goldfish	Actinopterygii	Cypriniformes	Cyprinidae
Cyprinus carpio	Common carp	Actinopterygii	Cypriniformes	Cyprinidae
Sinocyclocheilus rhinocerous	horned golden-line barbel	Actinopterygii	Cypriniformes	Cyprinidae
Sinocyclocheilus grahami	golden-line barbel	Actinopterygii	Cypriniformes	Cyprinidae

Scientific name is indicated in the common name section if there is no common name. Classification is based on NCBI Taxonomy Browser besides Dicentrarchus labrax and Stegastes partitus, which are based on World Register of Marine Species.

that align to human RFRP-3 include all LPQRFamide in teleost fish precursor polypeptides that encode the third LPXRFamide-like or LPXRFamide peptide. The mature teleost fish GnIH peptide structure was only identified in goldfish by immunoaffinity chromatography and mass spectrometry (8), which aligns to human RFRP-3 (**Figure 1**). The possible N-terminal sequences of teleost fish LPXRFamide or LPXRFamide-like peptides are also well conserved within fish if the peptides were cleaved after the first basic amino acid (Arg or Lys). These results suggest that three or two LPXRFamide or LPXRFamide-like peptides exist in teleost fish. The elucidation of the physiological roles of different endogenous LPXRFamide or LPXRFamide-like peptides should require further investigation in future studies.

DISTRIBUTION OF GnIH AND GnIH RECEPTORS (GnIH-Rs) IN FISH

The presence and distribution of GnIH orthologs and their receptors have been explored mainly in the brain but also in peripheral tissues of different vertebrate species by using PCR, immunoassays, immunohistochemistry, and/or *in situ* hybridization. Although the location of GnIH varies among species and the method used for the detection, some features, such as the presence of a periventricular preoptic/hypothalamic GnIH cell population and a profuse brain GnIH innervation, are recurring among studies (25). In mammals, several studies have been performed in rodents, ovine, and primates (including humans), with GnIH cells being identified mainly in the hypothalamus (paraventricular nucleus, dorsomedial nucleus, mediobasal, and ventromedial hypothalamus), as well as in the olfactory bulbs, hippocampus, medulla oblongata, and/or spinal cord, but GnIH is also produced in the eye, testis, and ovary (3, 6, 22, 26, 27). The paraventricular nucleus of the avian and reptile hypothalamus also contains GnIH cells (1, 28–34), in addition the nucleus accumbens and the upper medulla of the Japanese grass lizard was also identified as containing GnIH cells (35). In amphibians, studies carried out in the bullfrog, the European green frog and newt have identified GnIH neurons in the mediobasal telencephalon (medial septum, nucleus of the diagonal band of Broca,

and the medial and dorsal pallium) and the diencephalon (anterior preoptic area, suprachiasmatic nucleus, ventral, and dorsal hypothalamic nuclei) (36–39). The pattern of GnIH innervation in the brain of tetrapods is highly consistent with the presence of GnIH-Rs (25, 40). In most tetrapod species studied to date, GnIH neurons project to the median eminence to control anterior pituitary function, and GnIH-Rs are present in the gonadotropes (10, 23, 25, 40). GnIH neurons also project to GnRH-1 and GnRH-2 neurons, which also express GnIH-Rs, at least in birds [GnRH-1 and GnRH-2 neurons; (10, 33, 40)] and hamsters [only GnRH-1 neurons; (7)]. The presence of a profuse GnIH innervation and/or GnIH-Rs outside the hypothalamus and pituitary, from the telencephalon to the rhombencephalon, suggest that GnIH could be involved in functions other than reproduction and could exert pleiotropic actions in vertebrates.

Despite teleost fishes constituting the most abundant group of vertebrates, studies reporting the distribution of GnIH cells and their projections are scarce and have been addressed in only a few species (**Figure 2**). In addition, most of these studies have used heterologous antibodies and, only recently, specific antibodies to fish GnIH orthologous peptides have been developed in sea bass and tilapia (41, 42). In goldfish, developing Indian major carp *Labeo rohita*, cichlid fish *Cichlasoma dimerus*, and sea bass, immunohistochemistry has revealed the presence of GnIH-immunoreactive (ir) cells in the terminal nerve/olfactory bulbs (8, 41, 43, 44). However, no labeled cells were detected in the terminal nerve of goldfish by using *in situ* hybridization, which could reflect that GnIH transcripts exhibit low levels or that the antibody used is cross-reacting with another unknown peptide(s) present in this region (8). There are reports showing that NPFF, a sister gene of GnIH, is expressed in the terminal nerve cells of dwarf gourami, *Colisa lalia*, and medaka, *Oryzias latipes* (45, 46). In this sense, the use of a more sensitive technique as laser capture microdissection followed by quantitative real time PCR evidenced that GnIH transcripts can also be detected in terminal nerve GnIH-ir cells of sea bass (41), indicating that immunostained cells in this area represent true GnIH-synthesizing cells, at least in this species. Recently, Corchuelo et al. (47) characterized GnIH in the olfacto-retinal system of zebrafish (*Danio rerio*) by using RT-PCR and qPCR, detecting *gnih* expression in the olfactory epithelium, olfactory bulbs and retina of zebrafish during different stages of oocyte maturation. Moreover, these authors showed inverse expression of *gnih* and *gnrh3* in the olfactory bulbs (47). The presence of GnIH-ir cells in the ventral telencenphalon (Vl) was also reported in the sea bass brain (41). In agreement with these observations, conventional PCR showed important LPXRFamide mRNA levels in the olfactory bulbs/telencephalon of sea bass (41). A conspicuous population of GnIH-ir neurons was consistently detected in the periventricular region of the preoptic area/hypothalamus of all vertebrate groups analyzed, including fish. In the lamprey (*Petromyzon marinus*), the presence of a GnIH cell population has been reported in the hypothalamic bed nucleus of the tract of the postoptic commissure (12). GnIH cells were located in the posterior periventricular nucleus (NPPv) of the caudal preoptic area of goldfish, sockeye salmon, Indian major carp, *Cichlasoma dimerus*, sea bass, and tilapia (8, 15, 41–44), in the ventral zone of the periventricular hypothalamus of zebrafish (48), as well as in the periventricular preoptic nucleus (NPP) and magnocellular preoptic nucleus (NPOm) of Indian major carp (43). Moreover, GnIH-ir perikarya were found in the dorsal mesencephalic tegmentum (close to the nucleus of the medial longitudinal fascicle), as well as in the rhombencephalon of sea bass (secondary gustatory nucleus) and adult Indian major carp (nucleus reticularis, octaval nucleus, and motor nucleus of the vagal nerve) (41, 43). These results are consistent with the important GnIH mRNA levels detected in the midbrain-hindbrain of sea bass by conventional PCR, and laser capture microdissection of caudal GnIH-ir cells followed by qPCR (41), but similar mesencephalic and rhombencephalic cell masses have not been reported in other teleost species.

A common feature between species analyzed is the profuse innervation of GnIH cells within the fish brain, which is particularly evident in the preoptic area, hypothalamus, optic tectum, semicircular torus, and caudal midbrain tegmentum but also has been reported in the olfactory bulbs, ventral/dorsal telencephalon, habenula, pineal, ventral thalamus, vascular sac, pretectum, rostral midbrain tegmentum, posterior tuberculum, reticular formation, and facial-vagal sensory lobe (8, 15, 41–44). The ventral telencephalon, the preoptic area, and the mediobasal hypothalamus of fish have a known presence of GnRH-1 cells, the latter also containing kisspeptin (Kiss2) neurons (49, 50). The presence of GnIH-ir projections and GnIH-Rs have been reported in GnRH-1 cells of mammals (6, 7, 23, 51) and birds (33, 34, 52), as well as on kisspeptin neurons of mammals (53). In zebrafish, LPXRFa fibers interact with GnRH-3 soma from the preoptic area (48) but neither LPXRFa-ir fibers nor LPXRFa-R were found closely associated or coexpressed with GnRH-1, GnRH-3, or Kiss2 neurons in tilapia (42). Whether the association of GnIH with GnRH and kisspeptin neurons exhibits seasonal plasticity in fish or is dependent on the physiological conditions of the animals, as reported in birds (54) should be clarified in future research. But at least in sea bass, diencephalic GnIH expression exhibits marked seasonal variations, with higher transcript levels in the resting season related to the reproductive season (55).

Gonadotropin-inhibitory hormone-ir fibers coursing through the ventral hypothalamus also innervate the fish pituitary, as has been observed in goldfish, sockeye salmon, developing Indian major carp, sea bass, and tilapia (8, 15, 41–43). However, no GnIH-ir fibers or GnIH-ir cells were detected in the pituitary of the cichlid fish *Cichlasoma dimerus* or adult Indian major carp (43). The NPPv of goldfish and sea bass contains hypophysiotropic neurons (41, 56) that could represent the source of GnIH projections reaching the pituitary. In sea bass, DiI tract-tracing and immunohistochemical studies revealed that GnIH cells of the ventral telencephalon could also be the origin of the pituitary GnIH innervation (41). GnIH-ir fibers present in the pituitary of sea bass and tilapia were observed in close proximity of FSH and LH cells of the proximal pars distalis, supporting the neuroendocrine effects of GnIH on gonadotropin synthesis and/or secretion reported in both species (41, 42). Furthermore, most LH cells from the ventral part of the proximal pars distalis were immunolabeled with the tilapia LPXRFa antibody, suggesting that GnIH can also exert autocrine/paracrine effects in the pituitary of this species (42). The sea bass also exhibited GnIH-ir terminals

adjacent to GH cells (41), whereas tilapia displayed positive GnIH fibers innervating POMC cells from the rostral pars distalis and α-MSH and somatolactin cells of the pars intermedia (42).

The actions of GnIH are elicited through its binding with GnIH-Rs belonging to the seven transmembrane G protein-coupled receptor family. From the two potential GnIH-Rs identified in vertebrates (GPR147 and GPR74), GPR147 appears to represent the functional receptor for GnIH (25). Less abundant are the studies concerning the identification and distribution of GnIH-Rs in fish. These studies have identified one GnIH-R

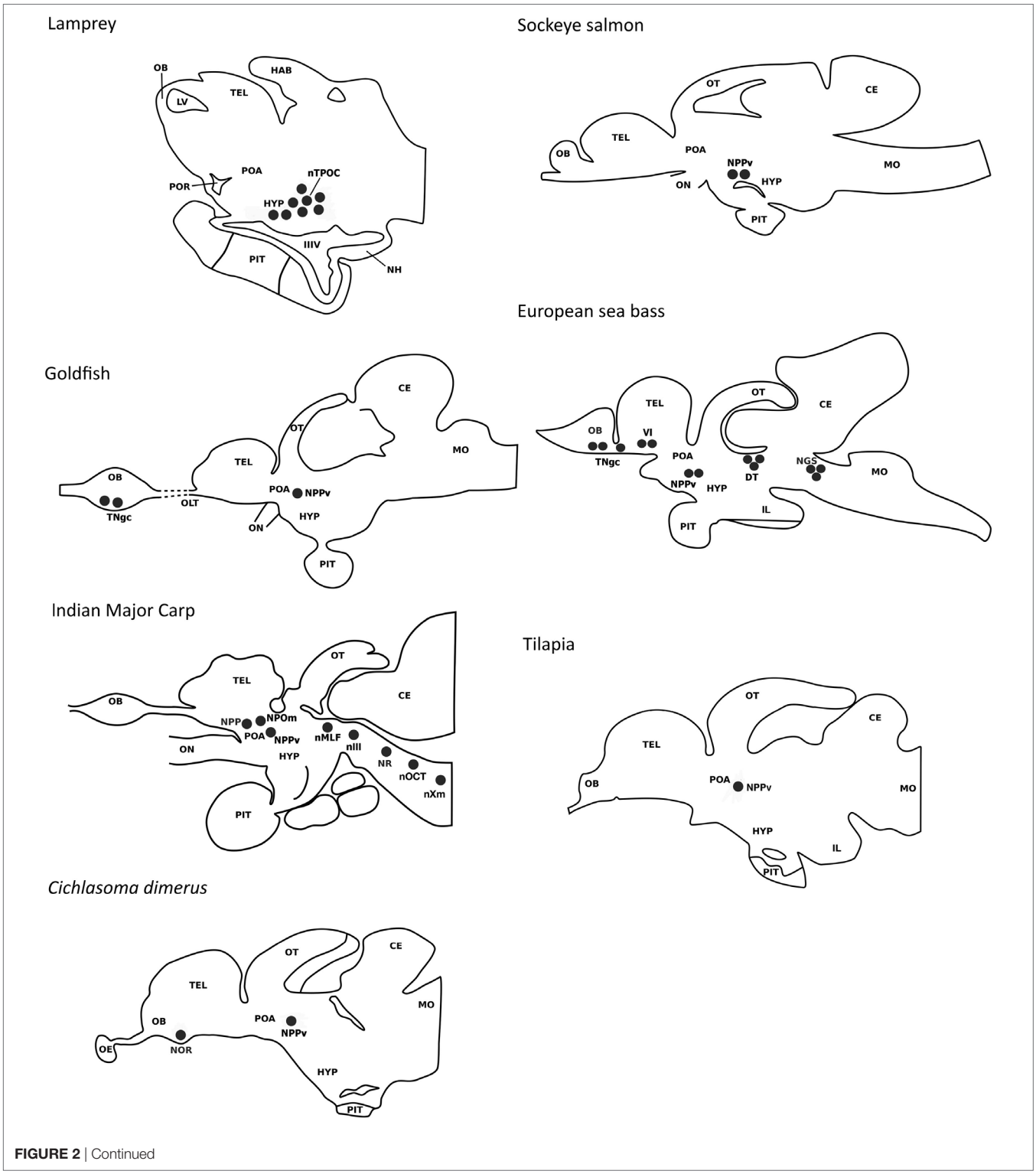

FIGURE 2 | Continued

FIGURE 2 | Continued
Comparison of the localization of gonadotropin-inhibitory hormone (GnIH) cells in a jawless fish (lamprey) and various teleost species (European sea bass, sockeye salmon, Indian major carp, goldfish, cichlid *Cichlasoma dimerus*, tilapia). GnIH cell populations are represented by black circles. In the lamprey, cells have been reported in the hypothalamic bed nucleus of the tract of the postoptic commissure. In teleosts, the presence of a prominent population of GnIH cells in the posterior periventricular nucleus of the caudal preoptic area is a common feature. GnIH cells have also been reported in the terminal nerve/olfactory bulbs of some teleost species (European sea bass, *Cichlasoma dimerus*, and developing Indian major carp). GnIH cells were also detected in the terminal nerve of the olfactory bulb of goldfish by immunohistochemistry, but not by *in situ* hybridization. Additional GnIH cell masses were detected in the ventral telencephalon of the European sea bass, as well as in the dorsal tegmentum and rhombencephalon of the European sea bass and adult Indian major carp. Abbreviations: CE, cerebellum; DT, dorsal tegmentum; HAB, habenula; HYP, hypothalamus; IIIV, third ventricle; IL, inferior lobe of the hypothalamus; LV, lateral ventricle; MO, medulla oblongata; NGS, secondary gustatory nucleus; NH, neurohypophysis; nIII, oculomotor nucleus; nMLF, nucleus of the median longitudinal fascicle; nOCT, octaval nucleus; NOR, nucleus olfacto-retinalis; NPOm, magnocellular preoptic nucleus; NPP, periventricular preoptic nucleus; NPPv, posterior periventricular nucleus; NR, nucleus reticularis; nTPOC, nucleus of the tract of the postoptic commissure; nXm, motor nucleus of the vagal nerve; OB, olfactory bulb; OE, olfactory epithelium; OLT, olfactory tract; ON, optic nerve; OT, optic tectum; PIT, pituitary; POA, preoptic area; POR, preoptic recess; TEL, telencephalon; TNgc, terminal nerve ganglion cells; Vl, lateral nucleus of the ventral telencephalon.

(GPR147 type) in several species such as the torafugu (31), grass puffer, *Takifugu niphobles* (25), tilapia (17), and sea bass (41), and one GPR147/GnIH receptor was also predicted from gene databases of the coelacanth *L. chalumnae*, spotted gar, Mexican tetra, *Astyanax mexicanus*, rainbow trout, *Oncorhynchus mykiss* and bicolor damselfish, *Stegastes partitus* (25). In tilapia, the binding of tiGnIH to GPR147/GnIH-R activates cAMP/PKA (CRE) and Ca^{2+}/PKC (SRE) pathways (17). Interestingly, three different GPR147/GnIH-Rs have been isolated and characterized in two cypriniform species including the zebrafish (16) and the goldfish (57). In zebrafish, receptor activation studies using a heterologous cell-based system, revealed that all three zebrafish LPXRFa peptides activate GPR147/GnIH-R2 and GPR147/GnIH-R3 *via* the PKA/cAMP pathway (48). However, no dose response of the SRE pathway (PKC/Ca^{2+}) was detected by any of the three LPXRFa peptides with any of the three GPR147/GnIH-Rs in this species (48). In addition, two different NPFFR receptors from GPR74 subtype were identified in the torafugu, zebrafish and tilapia (16, 17, 31). The effect of medaka GnIH and NPFF on the intracellular cAMP/PKA pathway was investigated in HEK293-T cells transfected with GPR147 or GPR74 subtypes by CRE-luciferase assays (46). GnIH dose-dependently inhibited CRE-luciferase activity stimulated by forskolin in HEK-293-T cells expressing GPR147 at low concentrations from 10^{-13} to 10^{-9} M, but this inhibitory effect diminished dose-dependently at higher concentrations from 10^{-9} to 10^{-5} M. A similar effect of NPFF was observed in HEK293-T cells expressing GPR74-1 or GPR74-2. When GnIH or NPFF was applied without forskolin, a dose-dependent increase in CRE-luciferase activity was observed at concentrations of 10^{-8} M and higher. It is likely that switch of coupling of GPR147/GPR74 from G_i to G_s proteins from lower to higher concentrations of GnIH or NPFF may happen in fish (46).

The presence of GnIH-Rs has been identified in central and peripheral tissues of zebrafish, grass puffer, goldfish, and tilapia by RT-PCR (16, 17, 58–60). In zebrafish, the three GnIH receptor genes are expressed in the brain, eye, testis, kidney, spleen, heart, and gill, two of them (*gnih-r1* and *gnih-r3*) are also expressed in the pituitary and one GnIH receptor gene (*gnih-r3*) is present in the ovary (16). In the grass puffer and the tilapia, the GnIH receptor is expressed in the brain and pituitary, the latter species also exhibiting GnIH-R transcripts in the ovary, liver, anterior, and posterior intestine, fat, muscle, and heart (17, 58). GnIH-R mRNA was primarily detected in the brain, pituitary, retina, and gonad of cinnamon clownfish, *Amphiprion melanopus* (60). To date, the precise cellular localization of GnIH-Rs has only been elucidated in the gonads of goldfish by using *in situ* hybridization (59), and in the brain and pituitary of tilapia by using a combination of *in situ* hybridization and immunohistochemistry (17, 42). In goldfish, GnIH-R1 and 2 were localized exclusively to the oocytes before the cortical alveolus stage and to the interstitial tissue to the testis (59). Tilapia GnIH-R-ir cells were distributed widely in the brain, being evident in the olfactory bulbs, dorsal and ventral telencephalic areas, preoptic area, ventral and dorsal thalamus, pretectum, pregromerular area, tuberal hypothalamus, lateral recess, dorsal tegmentum, periventricular gray zone of optic tectum, semicircular torus, posterior tuberal region, granular, and molecular layers of the corpus of the cerebellum, reticular area, superior raphe, and central gray (42). In the tilapia pituitary, immunoreactive GnIH-R cells lie in the dorsal and ventral parts of the rostral pars distalis, and the pars intermedia. In the ventral part of the proximal pars distalis, LH cells were labeled with the GnIH-R antiserum, whereas only a few FSH cells from the dorsal part of the proximal pars distalis appeared immunostained. ACTH cells from the rostral pars distalis and α-MSH cells from the pars intermedia also exhibited GnIH-R immunoreactivity (42). The distribution of GnIH-R-ir cells and/or GnIH-ir fibers in the pituitary of tilapia and sea bass could suggest a role of GnIH not only in reproduction but also in the stress response and growth/metabolism of fish.

PHYSIOLOGICAL ACTIONS OF GnIH IN FISH

The physiological actions of GnIH and related peptides in fish are summarized in **Table 2**. As it has been described in many studies and reviews, the reproductive inhibitory effects of GnIH were first established in birds and mammals through its reported actions on GnRH and gonadotropin synthesis and secretion (1–3, 61). However, the role of GnIH orthologs in reproduction remains controversial in fish and, as indicated above, the observed inhibitory or stimulatory effects of GnIH could be dependent on the species, sex, reproductive strategies and stages, the peptides used, dose, their route of administration and/or the elapsed time after treatment (**Figure 3**).

TABLE 2 | Molecular structure and physiological actions of GnIH in teleost fish.

Common name of species	Common classification	Putative peptide sequence	Physiological action	Reference
Goldfish	*gfGnIH-1*	PTHLHAN**LPLRFa**	Stimulation of pituitary FSH, LH and GH release (gfGnIH-1,-2,-3, *in vitro*; sockeye salmon)	Amano et al. (15)
			Stimulation of pituitary *lhβ, fshβ, prl*, and *gh* synthesis (gfGnIH-1, *in vitro*; grass puffer)	Shahjahan et al. (58, 62)
			Inhibition of *gnrh-3 and fshβ* synthesis (gfGnIH-2,-3, *in vivo* ip; female goldfish)	Qi et al. (57)
			Inhibition of *lhβ* synthesis (gfGnIH-2, *in vivo* ip; female goldfish)	Qi et al. (57)
			Inhibition of GnRH-stimulated pituitary *lhβ* and *fshβ* synthesis (gfGnIH-3, *in vitro*; mixed sex goldfish)	Qi et al. (57)
	gfGnIH-2	AKSNIN**LPQRFa**	Increase of plasma testosterone levels (gfGnIH-2,-3, *in vivo* ip; male goldfish)	Qi et al. (59)
			Stimulation of *star* and *3βhsd* synthesis (gfGnIH-2,-3, *in vivo* ip; male goldfish)	Qi et al. (59)
			Inhibition of *cyp19* synthesis (gfGnIH-2,-3, *in vivo* ip; male goldfish)	Qi et al. (59)
			Stimulation of *fsh-r*, *lh-r*, *star*, and *3βhsd* synthesis (gfGnIH-2,-3, *in vitro*; male goldfish)	Qi et al. (59)
			Stimulation or inhibition of LH release, *lhβ*, *fshβ*, and/or *gnih-r* expression depending on the maturational status and administration route (gfGnIH-3, *in vivo* ip and *in vitro*; mixed sex goldfish)	Moussavi et al. (19, 20, 63)
	gfGnIH-3	SGTGLSAT**LPQRFa**	Attenuation of GnRH-2 and GnRH-3 effects on LH secretion and gonadotropin subunit mRNA levels in particular reproductive stages (gfGnIH-3, *in vivo* ip and *in vitro*; mixed sex goldfish)	Moussavi et al. (20)
			Inhibition of GH release and stimulation of *gh* expression (gfGnIH-3, *in vivo* ip; mixed sex goldfish)	Moussavi et al. (63)
			Attenuation of GnRH-2 and GnRH-3 effects on GH release and *gh* expression in a reproductive-dependent manner (gfGnIH-3, *in vivo* ip and *in vitro*; mixed sex goldfish)	Moussavi et al. (63)
			Inhibition of plasma levels of GnRH, FSH and LH, and *sbgnrh*, *lhβ, fshβ*, and *gthα* expression (gfGnIH-3, *in vivo* ip; immature, male and female cinnamon clownfish).	Choi et al. (60)
			Stimulation of *gnih*, *gnih-r* and melatonin receptor expression, and of plasma levels of melatonin (gfGnIH-3, *in vivo* ip; immature, male and female cinnamon clownfish)	Choi et al. (60)
Zebrafish	*zfGnIH-3*	SGTGPSAT**LPQRFa**	Decreases in plasma LH level (*in vivo* ip; female goldfish).	Zhang et al. (16)
			Reduction of *gnrh-3* expression (*in vitro*, adult male brain slices of zebrafish).	Spicer et al. (48)
			Downregulation of *lhβ* and common α subunit expression (*in vitro*, adult male pituitary explants of zebrafish)	Spicer et al. (48)
cichlid fish *Cichlasoma dimerus*	*cdGnIH-1*	TPNSSPN**LPQRFa**	Inhibition of LHβ and FSHβ release and stimulation of GH release (cdGnIH-1, *in vitro*; mixed sex cichlid)	Di Yorio et al. (44)
	cdGnIH-2	APNQV**LPQRFa**	Stimulation of FSHβ release (cdGnIH-2 *in vitro*; mixed sex cichlid)	Di Yorio et al. (44)
Tilapia	*tiGnIH-2*	QSDERTPNSSPN**LPQRFa**	Stimulation of LH and FSH release (tiGnIH-2, *in vivo* ip and *in vitro*; female tilapia).	Biran et al. (17)
Orange-spotted grouper	*grGnIH-1*	LFPPTAKPFQLHAN**MPMRFa**	Inhibition of *gnrh-1* synthesis (grGnIH-1,-2,-3, *in vivo* ip; female grouper)	Wang et al. (64)
	grGnIH-2	ESVPGDDSAPNSTPN**MPQRFa**	Inhibition of *lhβ* synthesis (grGnIH-2, *in vivo* ip; female grouper)	Wang et al. (64)
	grGnIH-3	EAQNPI**LPQRL**	Stimulation of *gnrh-3* synthesis (grGnIH-3, *in vivo* ip; female grouper)	Wang et al. (64)
			Stimulation of *lh-r* synthesis (grGnIH-2, *in vitro*; female grouper)	Wang et al. (65)
			Stimulation of *star* and *3βhsd1* synthesis (grGnIH-1, *in vivo* ip and *in vitro*; female grouper)	Wang et al. (65)
Sea bass	*sbGnIH-1*	PLHLHAN**MPMRFa**	Inhibition of brain *gnrh2, kiss1, kiss2*, *kiss1-r*, *gnih*, and *gnih-r* expression (sbGnIH-2 *in vivo* icv; male sea bass)	Paullada-Salmerón et al. (18)

(*Continued*)

TABLE 2 | Continued

Common name of species	Common classification	Putative peptide sequence	Physiological action	Reference
	sbGnIH-2	SPNSTPN**MPQRFa**	Inhibition of brain *gnrh-1* expression (sbGnIH-1 *in vivo* icv; male sea bass)	Paullada-Salmerón et al. (18)
			Inhibition of pituitary *fshβ, lhβ, gh,* and *gnrh-r-II-1a* expression (sbGnIH-1,-2 *in vivo* icv, im; male sea bass)	Paullada-Salmerón et al. (18, 66)
			Decreases in plasma LH levels (sbGnIH-1,-2 *in vivo* icv, im; male sea bass)	Paullada-Salmerón et al. (18, 66)
			Decreases in plasma FSH levels (sbGnIH-1 *in vivo* im; male sea bass)	Paullada-Salmerón et al. (66)
			Decreases in plasma T and 11-KT levels (sbGnIH-1, 2 *in vivo* im; male sea bass)	Paullada-Salmerón et al. (66)
			Increases in diurnal activity (sbGnIH-1,-2 *in vivo* im; male sea bass)	Paullada-Salmerón et al. (66)

Characteristic C-terminal five amino acid sequences are highlighted in bold. icv, intracerebroventricular injection; im, intramuscular injection; ip, intraperitoneal injection.

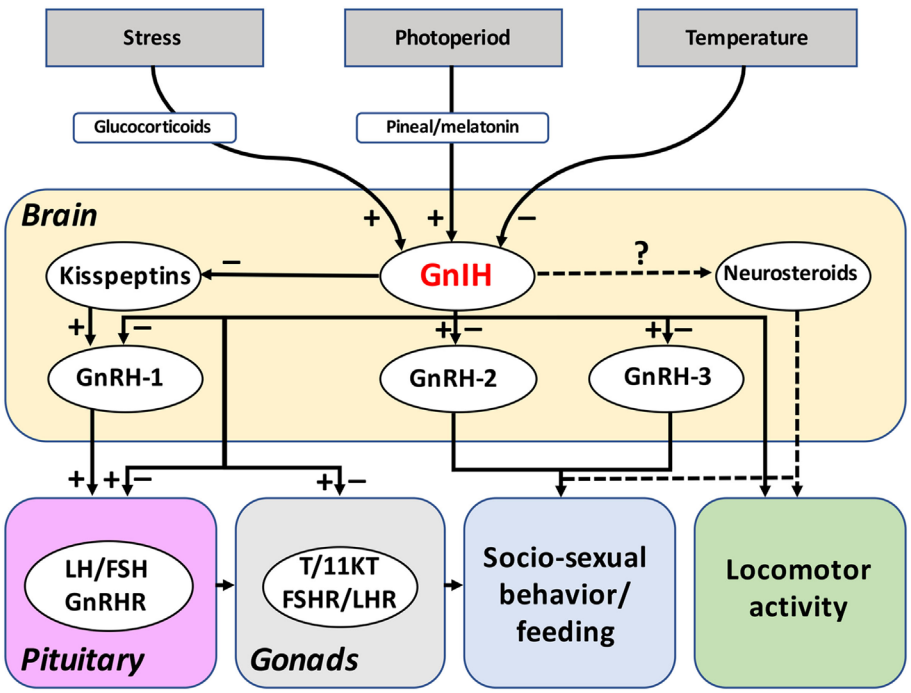

FIGURE 3 | Reported functions and regulation of GnIH system in fish. The reported actions of GnIH on the brain-pituitary-gonad axis of fish are summarized in this figure. Most of the studies have been concentrated on GnIH actions on gonadotropin synthesis and release, evidencing both inhibitory and stimulatory effects. In contrast, only a few studies have addressed the effects of GnIH on neuroendocrine systems and in gonadal physiology in fish. The putative actions of GnIH in fish behavior (socio-sexual behavior, locomotor activity) and feeding could be mediated by its effects on GnRH-2, GnRH-3, kisspeptins, and/or neurosteroids synthesis. Evidences obtained suggest that GnIH could also be mediating the effects of photoperiod, temperature, and stress on the reproductive and other axes of fish, as it has been reported in birds and mammals. Dotted lines refer to suspected actions. Abbreviations: FSH, follicle-stimulating hormone; FSHR, FSH receptor; GnIH, gonadotropin-inhibitory hormone; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; LH, luteinizing hormone; LHR, LH receptor; T, testosterone; 11KT, 11-ketotestosterone.

GnIH Actions on Neuroendocrine Systems in Fish

The presence of profuse GnIH fiber projections and/or their receptors in the ventral telencephalon, the preoptic area, and the hypothalamus of several fish species (8, 15, 41–43, 57) suggests that GnIH might be acting at the central level to modulate the main neuroendocrine systems controlling the reproductive process. To date, most of the reported effects of different GnIH orthologs on brain neuroendocrine systems are inhibitory (**Table 2**). For example, icv injection of sbGnIH-1 in mature male sea bass, and intraperitoneal (ip) injections of grouper GnIH (grGnIH)-1, -2, and -3 in female grouper (*Epinephelus*

coioides), and of goldfish GnIH (gfGnIH)-3 in inmature, male and female cinnamon clownfish decreased *gnrh-1* mRNA levels, the latter species also exhibiting reduced plasma GnRH levels after GnIH treatment (41, 60, 64). In mature male sea bass, the effects of GnIH on GnRH-2 expression appear dependent of the route of administration because centrally (icv)-administered sbGnIH-2 reduced *gnrh-2* mRNA levels, whereas the same GnIH form injected peripherally (intramuscular) increased the *gnrh-2* expression (18, 66). In turn, gfGnIH-2 and gfGnIH-3 peptides inhibited the expression of *gnrh-3* gene, having no effects on *gnrh-2* transcript levels in female goldfish (57), and zebrafish GnIH (zfGnIH)-3 reduced *gnrh-3* expression in brain slices of zebrafish (48), but stimulatory effects of grGnIH-3 on *gnrh-3* expression have been reported in ip-injected female groupers (64). The kisspeptin system of sea bass is also modulated by GnIH where sbGnIH-2 icv-injected animals exhibited decreased *kiss1*, *kiss2*, and *kiss1r* transcript levels (18). These results are consistent with the presence of abundant GnIH-ir fibers in the habenula, preoptic area, rostral mediobasal hypothalamus and around the lateral recess of sea bass, where Kiss1 and Kiss2 neurons are present (41, 50). Results obtained in cinnamon clownfish and sea bass showed that GnIH can also modulate brain *gnih* and *gnih-r* mRNA levels, indicating the existence of an autocrine regulation on the brain GnIH system (18, 41, 60). However, the nature of these autoregulatory actions seems to be dependent on the route of administration because centrally (icv)-injected sbGnIH-2 decreased *gnih* and *gnih-r* expression in sea bass (18), whereas peripherally (intramuscular or intraperitoneal)-administrated sbGnIH-2 and gfGnIH-3 increased *gnih* and *gnih-r* mRNA levels in cinnamon clownfish and sea bass, respectively (41, 60).

Role of GnIH in Fish Pituitary

The distribution pattern of GnIH fibers and its receptors described above reinforces the involvement of this neuropeptide in the regulation of pituitary hormone synthesis and secretion in fish (**Figure 3**). The first physiological study developed in teleost fish demonstrated that *in vitro* treatment of cultured pituitary cells with exogenous GnIH (goldfish GnIH-1, -2, and -3 peptides) stimulated the release of LH and FSH in sockeye salmon (15). Likewise, *in vitro* treatment with gfGnIH-1 enhanced *fshβ* and *lhβ* gene expression in grass puffer pituitary (58) and *Cichlasoma dimerus* GnIH (cdGnIH)-2 peptide also provoked an increase of *in vitro* FSH-β release in intact pituitary cultures of this cichlid fish (44). In female tilapia, both *in vivo* and *in vitro* studies revealed that tiGnIH-2 induced a significant increase in FSH and LH secretion (17).

On the other hand, *in vivo* and *in vitro* studies performed in goldfish examining the effects of gfGnIH-3 on pituitary *lhβ*, *fshβ*, and *gnih-r* mRNA levels, as well as on LH secretion showed mixed results (19, 20). Intraperitoneal (ip) injection of gfGnIH-3 peptide reduced LH plasma levels at early- and mid-gonadal recrudescence, as well as the expression of *gnih-r* mRNA levels in the pituitary at mid-late and late recrudescence stages, but increased the mRNA levels of both *lhβ* and *fshβ* at early to late recrudescence. Furthermore, the incubation of cultured pituitary cells with gfGnIH-3 provoked a decrease of *lhβ* and *fshβ* transcript levels at early and late recrudescence, but elevated the gene expression of *lhβ* at mid-recrudescence and stimulated the LH secretion at late-gonadal recrudescence. Moussavi and colleagues have further investigated the interaction of gfGnIH-3 with two forms of GnRH, GnRH-2 and GnRH-3, both *in vivo* and *in vitro* (20). Administration of gfGnIH-3 or GnRH alone evoked an increase of *lhβ* and *fshβ* mRNA levels at early-, mid-, and late-gonadal recrudescence, but gfGnIH-3 treatment showed no effect on transcription of *lhβ* gene at late recrudescence. Conversely, co-injection of gfGnIH-3 with GnRH-3 reduced the expression of *lhβ* mRNA levels at early recrudescence, and *lhβ* and *fshβ* mRNA levels during mid- and late-gonadal recrudescence, whereas the co-treatment of gfGnIH-3 with GnRH-2 decreased the transcript levels of *lhβ* at mid and late recrudescence. Additionally, during early- and mid-gonadal recrudescence, treatment with gfGnIH-3 caused an inhibition of plasma LH levels, and this reduction was also observed in fish co-injected with GnRH-2 or GnRH-3 in early gonadal recrudescence. Treatments with GnRH-2 and GnRH-3 stimulated *gnih-r* expression at mid and late recrudescence, while opposite effects, i.e., reduced *gnih-r* mRNA levels, were observed after gfGnIH-3 treatment during late recrudescence. *In vitro* experiments showed that exposure to gfGnIH-3 suppressed GnRH-2-induced LH response at mid recrudescence. Taken together, these results suggest that GnIH in goldfish can exert complex stimulatory or inhibitory effects on gonadotropin synthesis and secretion, as well as on GnRH regulation of this pituitary function, depending on the reproductive stage of the animals and administration route (19, 20).

In contrast, inhibitory effects of GnIH on gonadotropins have been reported in other studies performed in species such as goldfish, cinnamon clownfish, cichlid *C. dimerus*, orange-spotted grouper, sea bass, and zebrafish (16, 18, 44, 48, 57, 60, 64, 66). The ip administration of zfGnIH-3 decreased LH plasma levels in female goldfish at 1 and 3 h post injection at the highest doses tested (16). Another *in vivo* study performed in female goldfish showed that animals treated with gfGnIH-2 and gfGnIH-3 peptides had reduced *fshβ* mRNA levels in the pituitary gland, while only the gfGnIH-2 form was able to decrease *lhβ* expression significantly (57). Nevertheless, *in vitro* administration of gfGnIH-2 and gfGnIH-3 in cultured pituitary cells, although it had no effect on either *fshβ* or *lhβ* mRNA levels, decreased GnRH-stimulated *fshβ* and *lhβ* expression (57). In the pituitary gland of the female grouper, injections of grGnIH-2 peptide decreased *lh*β mRNA levels (64). In 2016, Choi and collaborators also evaluated the effects of GnIH on transcript and plasma levels of gonadotropins in immature, and mature male and female cinnamon clownfish showing that ip injection with heterologous gfGnIH-3 reduced *gthα, fshβ*, and *lhβ* expression, as well as plasma FSH and LH (60). In the cichlid fish *Cichlasoma dimerus*, cdGnIH-1 peptide also inhibited LHβ and FSHβ release in intact pituitary cultures (44). Recent studies performed in the male sea bass showed that treatment with both sbGnIH-1 and sbGnIH-2 peptides exerted inhibitory actions on the synthesis and release of gonadotropins (18, 66). Findings revealed that the central (18) and peripheral (66) administration of sbGnIH-1 or sbGnIH-2 peptides reduced LH plasma levels, as well as pituitary *lhβ* mRNA levels. Furthermore, only the icv injection

with sbGnIH-2 determined a reduction of pituitary *fshβ* and *gnrh-r-II-1a* receptor mRNA levels (18), whereas intramuscular administration of sbGnIH-1 elicited a decrease in FSH plasma levels (66). In pituitary explants, zfGnIH-3 downregulated *lhβ* and common α subunit expression (48).

In addition to its effects on gonadotropins, GnIH has also been shown to be involved in the regulation of the synthesis and/or release of other adenohypophyseal hormones in fish. A pioneer study performed in sockeye salmon showed that *in vitro* treatment of cultured pituitary cells with the three goldfish GnIH peptides (gfGnIH-1, -2, and -3) stimulated GH release (15). Accordingly, *in vitro* gfGnIH-1 increased *gh* transcript levels, as well as those of prolactin, in grass puffer (62) and cdGnIH-1 augmented the release of GH in pituitary cultures of a cichlid species (44). However, ip injection of tiGnIH-2 did not show any change in GH release in female tilapia (17). Another study in goldfish showed that ip injection of gfGnIH-3 reduced GH plasma levels at early, mid, and late recrudescence, but elevated *gh* gene expression in the pituitary (63). These authors also showed that gfGnIH-3 attenuates the effects of GnRH-2 and GnRH-3 on GH release and *gh* expression in a reproductive-related manner (63). Icv injections of sbGnIH-2 inhibited *gh* mRNA levels in a dose dependent manner in male sea bass (18). The presence of GnIH fibers in the proximal pars distalis of the sea bass pituitary, innervating GH-ir cells, and in the pars intermedia (41), as well as coursing close to somatolactin, α-MSH and ACTH cells of tilapia, the two latter cell types also exhibiting GnIH-receptor immunoreactivity (42), reinforces the assumption that GnIH can modulate the synthesis and secretion of some pituitary hormones other than gonadotropins.

Role of GnIH on Gonadal Physiology

It has been demonstrated that not only the brain, but also the gonads may be a source of GnIH. Based on different studies in birds and mammals (67–71), GnIH has also been considered as a peripheral regulator of gonadal functions. The fish gonads also synthesize GnIH, as reflected in different studies showing *gnih* expression in the ovary and/or the testis of zebrafish, tilapia and sea bass (16, 17, 41, 47). Recently, *gnih* transcripts were identified in the cortical vesicles of previtellogenic oocytes of zebrafish, as well as in the follicular cells and in the zona radiata of the vitellogenic oocytes (47). Although the expression of GnIH and GnIH-R has been reported in the gonads of different teleost species (16, 17, 41, 47, 59, 65), most of the research has been focused on GnIH actions in gonadotropin synthesis and secretion, and only a few studies have addressed the role of GnIH in gonadal gametogenesis and/or steroidogenesis of fish (59, 65, 66). Qi and collaborators (59) studied the effects of GnIH on steroidogenesis in male and female goldfish gonads. Implantation of gfGnIH-2 or gfGnIH-3 did not induce any change in estradiol plasma levels in females, but both GnIH peptides increased serum testosterone levels in male goldfish. Additionally, ip injection with both GnIH forms enhanced the gene expression of *steroidogenic acute regulatory protein* (*star*) and *3β-hydroxysteroid dehydrogenase* (*3βhsd*) genes and decreased *cyp19* mRNA levels in the testes of the goldfish. *In vitro* analysis using gfGnIH-2 and gfGnIH-3 in cultured testicular cells also significantly increased *fshr*, *lhr*, *star*, and *3βhsd* transcript levels, but reduced *cyp19* expression. However, ovarian mRNA levels of gonadotropin receptors and steroid-synthesizing enzymes were unaffected after both *in vivo* and *in vitro* GnIH treatments (59). In contrast, in a recent study performed in female grouper, treatment with grGnIH-2 increased *lhr* mRNA levels in cultured ovary fragments, and grGnIH-1 peptide stimulated the ovarian expression of *star* and *3βhsd* both *in vitro* and *in vivo* (65). On the other hand, the peripheral sbGnIH-1 and sbGnIH-2 implants in male European sea bass caused an inhibition of testosterone and 11-ketotestosterone plasma levels at particular reproductive stages (early- and mid-spermatogenesis), without affecting plasma levels of the progestin 17,20β-dihydroxy-4-pregnen-3-one (66). Moreover, intramuscular implant of both GnIH peptides determined a delay in the development of testis, which exhibited abundant type A spermatogonia (SgA) and only scattered and isolated clusters of spermatozoids at the spermiation phase (66). Taken together, these findings suggest that GnIH may regulate the reproductive axis of teleost fish by acting not only at the brain and pituitary levels but also on gonadal physiology, the nature of its actions depending on the species, the sex and the reproductive stage (**Figure 3**).

Role of GnIH in Fish Behavior

First reports of GnIH effects on socio-sexual behavior and/or sexual motivation were obtained in birds (34, 72, 73) and mammals (51, 74). Despite many studies that have explored the interactions between GnIH and the reproductive axis, how GnIH is involved in the regulation of reproductive, social and other behaviors in fish is still almost unknown. To date, the only study addressing the role of GnIH in fish behavior has been accomplished in the European sea bass (66), showing that both sbGnIH-1 and sbGnIH-2 affected the diurnal to nocturnal ratio of locomotor activity along the reproductive cycle. In this study, whereas control animals progressively decreased their diurnal habits as spermatogenesis progressed and they reached the spermiation phase, GnIH treatment induced a significant elevation in their diurnal pattern of activity along the same gametogenic stages, and this diurnal activity only decreased when the GnIH treatment ceased at the spawning season (66). It is interesting to note that the European sea bass is a rhythmic species that exhibits diurnal feeding and locomotor activity patterns during most of the year, but switches to nocturnal during the reproductive season at winter (75, 76). Moreover, diencephalic expression of *gnih* in sea bass was higher during the resting season and lower during the reproductive season (55). Therefore, it is plausible to consider that this seasonal pattern of GnIH expression could be on the basis of this natural diurnal to nocturnal shift observed in the reproductive season of this species.

As indicated above, the midbrain of Indian major carp and European sea bass contains a GnIH cell population located in the dorsal mesencephalic tegmentum (41, 43). Based on their location and the profuse GnIH innervation in sensory-motor areas, a role in regulating behavior (locomotor activity, mating, feeding) may be considered for this GnIH cell population in sea bass (41, 77). GnIH has been found to be involved in the modulation of socio-sexual behavior in birds by acting on tegmental GnRH-2 cells, and promoting the conversion of testosterone into neuroestrogens

via the stimulation of brain cytochrome P450 aromatase activity (10, 34, 73). Furthermore, kisspeptin and GnIH seem also to interact in the regulation of social behavior in mammals and teleosts (78). Interestingly, both sbGnIH-1 and sbGnIH-2 forms decrease plasma testosterone levels, and sbGnIH-2 modulates brain *gnrh2* and *kisspeptin* gene expression in sea bass (18, 66). Several studies performed in fish have reported the potential involvement of neurosteroids in socio-sexual behaviors. For instance, in *Lythrypnus dalli*, a socially induced decrease in brain aromatase levels resulted in increased aggression (79). In turn, treatments with aromatase inhibitors decreased aggressive behavior in African male cichlid fish *Astatotilapia burtoni* (80) and reduced courtship activities in male Endler guppy *Poecilia reticulata* (81). Altogether, these results suggest that GnIH might also be involved in the regulation of reproductive, social and/or locomotor behaviors in fish through its actions on GnRH-2, kisspeptins and/or neurosteroid synthesis and release (**Figure 3**).

REGULATION OF THE GnIH SYSTEM IN FISH

A range of evidence indicates that the GnIH system is mediating the effects of photoperiod on different physiological processes in Tetrapods, with results in photoperiodic mammals, birds and amphibians suggesting that its expression is modulated through a melatonin-dependent process (7, 32, 38, 82, 83). Unfortunately, much less is known regarding the regulatory mechanisms of GnIH in fish, although scarce data available support the idea that environmental cues such as photoperiod and temperature are also regulating daily and seasonal profiles of GnIH in this group of vertebrates (**Figure 3**).

In general, photoperiodic regulation of reproduction in fish is mediated by plasma melatonin release from the pineal gland, acting at all levels of the reproductive axis (84, 85). Data collected in fish also suggest that melatonin might exert its action, at least in part, through GnIH neurons (**Figure 3**). A recently published study analyzed the relationship between melatonin and GnIH in the cinnamon clownfish, and reported that GnIH was co-localized with the melatonin receptor MT-R1 in diencephalic cells (60). Likewise, GnIH cells in sea bass are located in regions known to exhibit melatonin-binding sites (41, 86). Bidirectional connections between the pineal organ and GnIH cells appear to exist in fish, because the pineal organ projects to the NPPv and the dorsal tegmental area, where GnIH cells have been identified in teleosts (87–89), and GnIH-ir fibers have been identified in the fish pineal organ (41). In addition, in grass puffer, the expression of *gnih* and its receptor showed diurnal and circadian rhythmicity at the spawning stage, in association with melatonin receptor expression, suggesting that the action of GnIH is cyclic possibly due to regulation by melatonin and the functional role of the GnIH system is in the regulation of lunar-synchronized spawning (58, 90). Interestingly, Cowan and co-workers reported, for the first time in fish, that GnIH gene expression is regulated by the pineal organ. Their findings in sea bass revealed that pinealectomy (Px) reduced the expression of *gnih* in a regional- (in mid-hindbrain, but not in the telencephalon or diencephalon) and reproductive- (in reproductive season but not in resting) dependent manner (55). Moreover, a seasonal difference in *gnih* and *gnih-r* expression was observed in the diencephalon, where both Px and control groups exhibited higher transcript levels of these genes at resting than in the reproductive season (55). All these evidences support the hypothesis that melatonin could play an important role in the regulation of the GnIH system in fish, which could mediate, in turn, in the transduction of environmental information to other reproductive-related systems.

In fish, increasing evidence supports the decisive role of photoperiod and/or temperature on larval development, sex determination/differentiation and puberty (91–94). However, ontogenetic studies of the GnIH system and its developmental regulation by environmental factors have not been adequately addressed in this vertebrate group. In a recent study, Paullada-Salmerón and colleagues performed the first analysis of *gnih* and *gnih* receptor expression pattern throughout the first year of life in the European sea bass (95). This study revealed that both *gnih* and *gnih-r* showed significantly increased expression from hatching to the second/third week of life, a subsequent decrease in mRNA levels until 120 days post-fertilization (dpf) and then a further increase at the onset of sex differentiation (150 dpf). Afterward, *gnih* transcript levels dropped significantly during the sex differentiation period (150–240 dpf) and continued at that level for the remainder of that year. The results also revealed daily variations in developmental expression of *gnih* and *gnih-r*, with higher diurnal mRNA levels at early stages (until 25 dpf), and a shift to higher nocturnal expression at 300–360 dpf coinciding with the winter (reproductive season) (95). In the same study, Paullada-Salmerón and co-workers further investigated the effects of rearing temperature during the thermosensitive period on the expression of the *gnih* and its receptor in sea bass. Early exposure to high temperatures (21°C), which is known to provoke a masculinization of the progeny (96), decreased the levels of *gnih* and *gnih-r* transcripts during early development and these effects were also evident at the end of the sex differentiation period (240–300 dpf), indicating that temperature can exert remarkable effects on the transcription of both genes (95). Altogether, these results could indicate that the GnIH system might not only be involved in the modulation of the reproductive cycle but could also be a mediator in sex differentiation and puberty in fish. Involvement of GnIH in the regulation of reproductive development and puberty has been also studied in birds and mammals (97, 98).

Several findings suggest that stress may act through GnIH neurons to inhibit reproductive function in birds and mammals (10, 99). Both acute and chronic stress upregulate hypothalamic GnIH gene expression in rats, and this stress-induced increase of GnIH is blocked by adrenalectomy (100). Glucocorticoid receptors are present in GnIH neurons, as revealed by immunohistochemistry, and could sustain these effects (101). In quail and mice, corticosterone induces GnIH expression *via* the glucocorticoid receptor present in GnIH neurons and these actions appear to be mediated by the glucocorticoid-response element (GRE) present in the promoter of the GnIH gene (101). Although there is no report indicating the involvement of GnIH in stress response in fish, evidence suggests that a similar mechanism to that reported in Tetrapods may be operating in fish (**Figure 3**). Promoter

prediction searching has revealed the presence of several putative GRE in the zebrafish *gnih* and *gnih-r* gene promoter sequences (25). Stimulatory effects of cortisol on *gnih* transcript levels have been reported in the cinnamon clownfish (102). The presence of GnIH-ir fibers in close proximity to α-MSH and ACTH cells, which also exhibit GnIH-R immunoreactivity, was identified recently in the pituitary of tilapia (42). These results indicate that the role of GnIH in the mediation of the stress response could be evolutionarily conserved in vertebrates.

CONCLUDING REMARKS

Increasing research is showing that GnIH is present throughout the vertebrate lineage from fish to mammals. Most of the teleost GnIH precursor polypeptides identified to date encode three LPXRFamide or LPXRFamide-like peptides, although some species exhibit only two LPXRFamide-like peptide sequences. The presence of a diencephalic GnIH cell population in the preoptic area/hypothalamus, the profuse GnIH innervation in the brain and the hypophysiotropic character of GnIH seem also to represent a common feature for all vertebrate groups, including fish. However, recent studies in teleosts are revealing that GnIH cells can also be found in cells from the olfactory bulbs, ventral telencephalon, dorsal midbrain tegmentum, and rostral rhombencephalon, likely to be coexisting with other RFamide neuropeptides. Functional studies of GnIH in fish have mainly focused on its effects on gonadotropin synthesis and secretion, but increasing evidence is showing that it can also regulate reproduction by modulating brain GnRH and kisspeptin systems, as well as gonadal gametogenesis and steroidogenesis. Contrary to that referenced in birds and mammals, in which inhibitory actions of GnIH represent the main feature, both inhibitory and stimulatory actions of GnIH have been reported in the reproductive axis of fish (**Figure 3**). This diversity in actions could be related to the species, the sex of the animals, the physiological status, the route, and the time elapsed after administration of the GnIH peptide. Nevertheless, the results obtained in a recent dose-response study (18), as well as in work analyzing the ligand dose-dependent activation of GPR147/GPR74 receptors by GnIH and NPFF (46), suggest that the GnIH actions on the reproductive axis of fish could be inhibitory at low (physiological) concentrations, and stimulatory at higher (pharmacological) concentrations, which could also explain the diversity of GnIH actions reported in fish studies. Whether centrally- and peripherally- (e.g., gonadal) synthesized GnIH exert different effects on reproduction and other physiological processes remains an open question. Reinforcing and building on the knowledge acquired in the last decade on GnIH in fish will require further efforts to clarify the role of this RFamide neuropeptide in functions other than reproduction, such as feeding, stress response, and behavior, as well as in elucidating the intracellular pathways and regulatory mechanisms involved in GnIH actions.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Dual Actions of Mammalian and Piscine Gonadotropin-Inhibitory Hormones, RFamide-Related Peptides and LPXRFamide Peptides, in the Hypothalamic–Pituitary–Gonadal Axis

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Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide that decreases gonadotropin synthesis and release by directly acting on the gonadotrope or by decreasing the activity of gonadotropin-releasing hormone (GnRH) neurons. GnIH is also called RFamide-related peptide in mammals or LPXRFamide peptide in fishes due to its characteristic C-terminal structure. The primary receptor for GnIH is GPR147 that inhibits cAMP production in target cells. Although most of the studies in mammals, birds, and fish have shown the inhibitory action of GnIH in the hypothalamic–pituitary–gonadal (HPG) axis, several *in vivo* studies in mammals and many *in vivo* and *in vitro* studies in fish have shown its stimulatory action. In mouse, although the firing rate of the majority of GnRH neurons is decreased, a small population of GnRH neurons is stimulated by GnIH. In hamsters, GnIH inhibits luteinizing hormone (LH) release in the breeding season when their endogenous LH level is high but stimulates LH release in non-breeding season when their LH level is basal. Besides different effects of GnIH on the HPG axis depending on the reproductive stages in fish, higher concentration or longer duration of GnIH administration can stimulate their HPG axis. These results suggest that GnIH action in the HPG axis is modulated by sex-steroid concentration, the action of neuroestrogen synthesized by the activity of aromatase stimulated by GnIH, estrogen membrane receptor, heteromerization and internalization of GnIH, GnRH, and estrogen membrane receptors. The inhibitory and stimulatory action of GnIH in the HPG axis may have a physiological role to maintain reproductive homeostasis according to developmental and reproductive stages.

Keywords: gonadotropin-releasing hormone, GPR147, aromatase, neuroestrogen, GPR30, receptor heteromerization, receptor internalization, sex steroids

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INTRODUCTION

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide that was initially isolated from the brain of Japanese quail, which decreases luteinizing hormone (LH) concentration in the culture medium of the anterior pituitary gland (1). *In vivo* administration of quail GnIH also decreases gonadotropin synthesis as well as gonadal development and maintenance in quail (2).

The C-terminal of GnIH peptides has an LPXRFamide (LPXRFa, X = L or Q) motif. Therefore, peptides orthologous to GnIH are also called RFamide-related peptide (RFRP) in mammals and LPXRFa peptides in non-mammalian and non-avian vertebrates (3). Most of the studies in mammals, birds, and fish have shown inhibitory effects of GnIH on the hypothalamic–pituitary–gonadal (HPG) axis; however, several *in vivo* and *in vitro* studies in mammals and fish show its stimulatory effects (3, 4). Here, we highlight studies that show stimulatory effects of GnIH on the HPG axis and investigate their physiological or pharmacological mechanisms.

ENDOGENOUS MATURE GnIH PEPTIDES

Human RFRP-1 and -3 (5), macaque RFRP-3 (6), Siberian hamster RFRP-1 and -3 (7), rat RFRP-3 (8), bovine RFRP-1 (9) and -3 (10), European starling GnIH (11), zebra finch GnIH (12), chicken GnIH (13), quail GnIH (1), quail GnIH-related peptide (RP) 2 (14), red-eared slider LPXRFamide-1, 2, 3 (15), frog growth hormone-releasing hormone (fGRP), fGRP-RP-1, fGRP-RP-2, and fGRP-RP-3 (16, 17), Japanese red-bellied newt LPXRFa-1, -2, -3, -4 (18), and goldfish LPXRFa-3 (19) are identified as endogenous mature LPXRFa peptides by cDNA sequencing, immunoaffinity chromatography, and mass spectrometry in gnathostomes (3). Lamprey is a jawless fish that is one of the most primitive among vertebrates. Lamprey LPXRFamide peptide precursor gene encompasses C-terminal QPQRFamide (LPXRFa-1a, 1b) and RPQRFamide peptides (LPXRFa-2) that have been identified by mass spectrometry (20). LPXRFamide peptide precursor gene is also found in amphioxus, one of the most primitive chordates (protochordates), which encompasses three mature C-terminal RPQRFamide peptides (PQRFa-1, PQRFa-2, and PQRFa-3) (21). Identified and putative amino-acid sequences of GnIH peptides are summarized in **Table 1**. Although the C-terminal LPXRFa structure is key for binding of GnIH to its receptor (22), the N-terminal structure may modify the action of GnIH. Studies are needed to investigate the function of the N-terminal of GnIH and the differential effect of orthologous LPXRFa peptides encoded in the precursor polypeptide (**Table 1**).

GnIH RECEPTOR

Yin et al. characterized the binding activity of quail GnIH and GnIH-RPs to a G-protein-coupled receptor (GPCR) GPR147. The membrane fraction of COS-7 cells transfected with quail GPR147 cDNA specifically bound GnIH and GnIH-RPs that have a C-terminal LPXRFa motif with similar affinities (22). Hinuma et al. identified a specific receptor for GnIH (RFRP) in mammals, which was identical to GPR147 and named it OT7T022 (28). In the same year, Bonini et al. reported two GPCRs for neuropeptide FF (NPFF), a neuropeptide that has a PQRFamide (PQRFa) motif at its C-terminal that modulates pain, and designated as NPFF1 (identical to GPR147) and NPFF2 (identical to GPR74) (29). LPXRFa peptide precursor gene and PQRFa peptide precursor gene are thought to have diverged from a common ancestral gene through gene duplication (20, 21). GPR147 and GPR74 genes are also paralogous (30). The binding affinities of RFRPs to GPR147 and GPR74 and their signal transduction pathways show their higher affinity to GPR147 than NPFF that has a potent agonistic activity on GPR74 (10, 29, 31), suggesting that GPR147 (NPFF1, OT7T022) is the primary receptor for GnIH (3). However, this may not apply to teleost fishes as they generally have several subtypes of GPR147 and/or GPR74 (32).

INTRACELLULAR SIGNALING OF GnIH RECEPTOR

Gonadotropin-inhibitory hormone peptides suppress the production of cAMP by binding to GPR147 on the cells, suggesting that GPR147 couples to $G_{\alpha i}$ protein that inhibits adenylate cyclase (AC) (28, 33). Son et al. investigated the precise mechanism of GnIH cell-signaling pathway in a mouse gonadotrope cell line, LβT2 (34). Mouse RFRPs (mRFRPs) suppress GnRH-induced cAMP signaling. mRFRPs also inhibit GnRH-stimulated extracellular signal-regulated kinase (ERK) phosphorylation and gonadotropin subunit gene transcription by inhibiting the protein kinase A (PKA) pathway. Therefore, mRFRPs function as GnIH to inhibit GnRH-induced gonadotropin subunit gene transcription by inhibiting AC/cAMP/PKA-dependent ERK activation in gonadotropes (34) (**Table 2**).

Son et al. further investigated the signal transduction pathway that conveys the inhibitory action of GnIH in GnRH neurons by using a mouse GnRH neuronal cell line, GT1–7 (46). Although GnIH significantly suppressed the stimulatory effect of kisspeptin on GnRH release in hypothalamic culture, GnIH had no inhibitory effect on the protein kinase C (PKC) pathway stimulated by kisspeptin in GnRH neurons. On the other hand, GnIH eliminated the stimulatory effect of vasoactive intestinal polypeptide (VIP) on AC activity, p38 and ERK phosphorylation, and c-Fos mRNA expression in GT1–7. This shows the specific inhibitory mechanism of GnIH action on AC/cAMP/PKA pathway, and demonstrates a common mechanism of GnIH action in gonadotropes and GnRH neurons (34, 46) (**Table 2**).

EXISTENCE OF GnIH AND GnIH RECEPTOR IN THE HPG AXIS

Gonadotropin-inhibitory hormone precursor mRNA is expressed in the hypothalamus of all vertebrates investigated (3). GnIH neuronal axons terminate on GnRH1 neurons in the preoptic area (POA) that terminate at the median eminence and stimulate gonadotropin secretion from the anterior pituitary gland in birds (11, 12, 52–55) (**Figure 1**). *In situ* hybridization of GPR147 mRNA combined with GnRH immunocytochemistry shows expression of GPR147 mRNA in GnRH1 neurons in birds (11). GnIH (RFRP) axons also terminate on the hypophysiotropic type of GnRH neurons in humans (5), monkey (6), sheep (56), hamsters (7, 45), rats

TABLE 1 | Amino-acid sequences of RFRPs, GnIHs, and LPXRFa peptides in chordates.

	Animal	Name	Sequence	Reference
Mammals	Human	RFRP-1	MPHSFANLPLRFa	(5)
		RFRP-3	VPNLPQRFa	(5)
	Macaque	RFRP-1[a]	MPHSVTNLPLRFa	(6)
		RFRP-3	SGRNMEVSLVRQVLNLPQRFa	(6)
	Bovine	RFRP-1	SLTFEEVKDWAPKIKMNKPVVNKMPPSAANLPLRFa	(9)
		RFRP-3	AMAHLPLRLGKNREDSLSRWVPNLPQRFa	(10)
	Horse	RFRP-3[a]	IPNLPQRFa	(23)
	Rat	RFRP-1[a]	SVTFQELKDWGAKKDIKMSPAPANKVPHSAANLPLRFa	(8)
		RFRP-3	ANMEAGTMSHFPSLPQRFa	(8)
	Siberian hamster	RFRP-1	SPAPANKVPHSAANLPLRFa	(7)
		RFRP-3	TLSRVPSLPQRFa	(7)
	Syrian hamster	RFRP-1[a]	VPHSAANLPLRFa	(45)
		RFRP-3[a]	VPSLPQRFa	(45)
Birds	Quail	GnIH	SIKPSAYLPLRFa	(1)
		GnIH-RP-1[a]	SLNFEEMKDWGSKNFMKVNTPTVNKVPNSVANLPLRFa	(14)
		GnIH-RP-2	SSIQSLLNLPQRFa	(14)
	Chicken	GnIH	SIRPSAYLPLRFa	(13)
		GnIH-RP-1[a]	SLNFEEMKDWGSKNFLKVNTPTVNKVPNSVANLPLRFa	(24)
		GnIH-RP-2[a]	SSIQSLLNLPQRFa	(24)
	White-crowned sparrow	GnIH[a]	SIKPFSNLPLRFa	(62)
		GnIH-RP-1[a]	SLNFEEMEDWGSKDIIKMNPFTASKMPNSVANLPLRFa	(62)
		GnIH-RP-2[a]	SPLVKGSSQSLLNLPQRFa	(62)
	European starling	GnIH	SIKPFANLPLRFa	(11)
		GnIH-RP-1[a]	SLNFDEMEDWGSKDIIKMNPFTVSKMPNSVANLPLRFa	(11)
		GnIH-RP-2[a]	GSSQSLLNLPQRFa	(11)
	Zebra finch	GnIH	SIKPFSNLPLRFa	(12)
		GnIH-RP-1[a]	SLNFEEMEDWRSKDIIKMNPFAASKMPNSVANLPLRFa	(12)
		GnIH-RP-2[a]	SPLVKGSSQSLLNLPQRFa	(12)
Reptiles	Anole lizard	GnIH[a]	SIKPAANLPLRFa	ENSACAG00000013069
		GnIH-RP-1[a]	SMDLESMNDWELNKIIRRTTPEMKKMAHAAVNLPLRFa	ENSACAG00000013069
		GnIH-RP-2[a]	APDVQSLSRSLANLPQRFa	ENSACAG00000013069
	Red-eared slider turtle	GnIH	SIKPVANLPLRFa	15
		GnIH-RP-1	STPTVNKMPNSLANLPLRFa	15
		GnIH-RP-2	SSIQSLANLPQRFa	15
	Chinese softshell turtle	GnIH[a]	IIKPVANLPLRFa	ENSPSIG00000017952
		GnIH-RP-1[a]	SLNFEELKDWGSKNIIKMSTPTVNKMPNSVANLPLRFa	ENSPSIG00000017952
		GnIH-RP-2[a]	TPFVKTSSQLFPNLPQRFa	ENSPSIG00000017952
Amphibians	Bullfrog	fGRP/R-RFa	SLKPAANLPLRFa	(16, 26)
		fGRP-RP-1	SIPNLPQRFa	(17)
		fGRP-RP-2	YLSGKTKVQSMANLPQRFa	(17)
		fGRP-RP-3	AQYTNHFVHSLDTLPLRFa	(17)
	Red-bellied newt	nLPXRFa-1	SVPNLPQRFa	(18)
		nLPXRFa-2	MPHASANLPLRFa	(18)
		nLPXRFa-3	SIQPLANLPQRFa	(18)
		nLPXRFa-4	APSAGQFIQTLANLPQRFa	(18)
Teleost fish	Goldfish	gfLPXRFa-1[a]	PTHLHANLPLRFa	(19)
		gfLPXRFa-2[a]	AKSNINLPQRFa	(19)
		gfLPXRFa-3	SGTGLSATLPQRFa	(19)
	Medaka	mdLPXRFa-1[a]	PLHMHANMPLRFa	XM_004073848
		mdLPXRFa-2[a]	VSNSSPNMPQRFa	XM_004073848
		mdLPXRFa-3[a]	EAPSPVLPQRFa	XM_004073848
	Grass puffer	LPXRFa-1[a]	SLDMERINIQVSPTSGKVSLPTIVRLYPPTLQPHHQHVNMPMRFa	(79)
		LPXRFa-2[a]	DGVQGGDHVPNLNPNMPQRFa	(79)
		RYa[a]	SWKVIRLCEDCSKVQGVLKHQVRYa	(79)
	Tiger puffer	LPXRFa-1[a]	SLDMERINIQVSPTSGKVSLPTIVRLYPPTLQPRHQHVNMPMRFa	(79)
		LPXRFa-2[a]	DGVQGGDHVPNLNPKMPQRFa	(79)
		RYa[a]	SWKVIRLCEDCSKVQGVLKHQVRYa	(79)
Agnathans	Sea lamprey	lLPXRFa-1a	SGVGQGRSSKTLFQPQRFa	(20)
		lLPXRFa-1b	AALRSGVGQGRSSKTLFQPQRFa	(20)
		lLPXRFa-2	SEPFWHRTRPQRFa	(20)
Protochordates	Amphioxus	PQRFa-1	WDEAWRPQRFa	(21)
		PQRFa-2	GDHTKDGWRPQRFa	(21)
		PQRFa-3	GRDQGWRPQRFa	(21)

Ensembl or Genbank accession numbers are cited for some reptile GnIHs or medaka LPXRFa peptides. C-terminal LPXRFa (X = L or Q) sequences are underlined.
[a]*Putative peptides hypothesized from mRNA and deduced amino-acid sequences.*

TABLE 2 | Effect of GnIH on the HPG axis of mammals.

In vivo (animal) or *in vitro* (pituitary or cell line)	Concentration or dose of peptides	Rout of administration, culture medium	Administration time, sample collection, measurement	Effect	Reference
In vivo					
Postmenopausal women	50-μg/kg/h human RFRP-3	iv	Continuous administration for 3 h	LH secretion was decreased during RFRP-3 administration	George et al. (35)
Estrous ewes	1-mg/h human RFRP-3	iv	2-h infusion	LH secretion was decreased during and after RFRP-3 administration	Clarke et al. (36)
Ovariectomized ewes treated with EB to induce LH surge	1-mg bolus + 0.5 mg/h human RFRP-3	iv	8-h infusion	EB-induced LH surge was blocked by RFRP-3	Clarke et al. (36)
Hypothalamo-pituitary disconnected ovariectomized ewes	50, 100, 200 ng GnRH during 400-μg/h human RFRP-3	iv	Blood was collected −5, 5, 10, 15, 20, 30 min after GnRH administration	RFRP-3 decreased 100-ng GnRH-induced LH secretion	Smith et al. (37)
Castrated male calves	90-μg bovine RFRP-3	iv	6 injections at 10-min intervals	LH pulse frequency was decreased during 1-h injection period	Kadokawa et al. (38)
Male rats	10, 100, 500 ng rat RFRP-3	icv	Blood was collected 20 min after administration	LH concentration was decreased by administration of 10-, 100-, or 500-ng RFRP-3	Johnson et al. (39)
Male rats	0.1, 0.5, 1, 5 nmol rat RFRP-3	icv	Blood was collected 15–120 min after administration	Total LH secretion until 120 min after administration was decreased by 5-nmol RFRP-3. FSH concentration was decreased at 15 min by 5-nmol RFRP-3. Total FSH secretion until 120 min after administration was decreased by 5-nmol RFRP-3	Pineda et al. (40)
Gonadectomized male rats	0.1, 0.5, 1, 5 nmol rat RFRP-3	icv	Blood was collected 15–120 min after administration	LH concentration was decreased at 15 min by 5-nmol RFRP-3. Total LH secretion until 120 min after administration was decreased by 1- and 5-nmol RFRP-3. Total FSH secretion until 120 min after administration was decreased by 5-nmol RFRP-3	Pineda et al. (40)
Gonadectomized male rats	10-nmol rat RFRP-3	iv	Blood was collected 15–120 min after administration	LH concentration was decreased at 60 min. Total LH secretion until 75 min after administration was decreased. FSH concentration was decreased at 60 and 75 min after administration	Pineda et al. (40)
Ovariectomized rats	1, 5 nmol rat RFRP-3	icv	Blood was collected 15–120 min after administration	LH concentration was decreased at 15 min by 1-nmol RFRP-3. Total LH secretion until 120 min after administration was decreased by 5-nmol RFRP-3	Pineda et al. (40)
Ovariectomized rats	1-μg rat RFRP-3	iv	Blood was collected 30, 60, 120 min after administration	LH concentration was decreased 120 min after administration	Murakami et al. (41)
Ovariectomized rats with E2 + P4 to induce LH surge	2.5, 25 ng/h rat RFRP-3	icv using osmotic pump	Brains were collected 2 days later at the surge peak	25-ng/h 25-ng/h RFRP-3-reduced c-Fos expression in GnRH neurons and anteroventral periventricular region that provides stimulatory input to GnRH neurons	Anderson et al. (42)
Prepubertal female mice	100, 500, 1,000 ng RFRP-3	icv	Hypothalamus and blood was collected 4 h after administration	GnRH mRNA, Kiss1 mRNA, and LH concentration was decreased by 500- and 1,000-ng RFRP-3	Xiang et al. (43)
Ovariectomized or E2-treated ovariectomized prepubertal or adult female mice	20-nmol RFRP-3	icv	Blood was collected 4 h after administration	RFRP-3 decreased LH concentration in only E2-treated ovariectomized prepubertal female mice but both E2-treated or not treated ovariectomized adult female mice	Xiang et al. (43)

(*Continued*)

TABLE 2 | Continued

In vivo (animal) or *in vitro* (pituitary or cell line)	Concentration or dose of peptides	Rout of administration, culture medium	Administration time, sample collection, measurement	Effect	Reference
Male Syrian hamsters	150, 500, 1,500, 5,000-ng Syrian hamster RFRP-3	icv	Blood was collected 30 and 120 min after administration	LH concentration was increased 30 min after administration of 500-, 15,00-ng RFRR-3. FSH concentration was increased 30 min after administration of 1,500-ng RFRR-3. Testosterone concentration was increased 120 min after administration of 1,500-ng RFRR-3	Ancel et al. (44)
Male Syrian hamsters acclimatized to SD	12-μg/day Syrian hamster RFRP-3	icv using osmotic pump	Blood was collected after 5 weeks of continuous administration	Testosterone concentration and paired testicular weight were increased to LD levels	Ancel et al. (44)
Ovariectomized Syrian hamsters	100, 300, 500 ng GnIH (icv), 600-ng GnIH (ip)	icv, ip	Blood was collected 5 (icv), 30 (icv and ip) min after administration	LH concentration was decreased 5 and 30 min after icv administration of 500-ng GnIH, and 30 min after ip administration of 600-ng GnIH.	Kriegsfeld et al. (45)
Male Siberian hamsters acclimatized to LD or SD	100- and 500-pmol Siberian hamster RFRP-1 or RFRP-3	icv	Blood was collected 5 and 30 min after administration	LH concentration was decreased 5 and 30 min after administration of 500-pmol RFRP-1, 100- and 500-pmol RFRP-3, 30 min after administration of 100-pmol RFRP-1 in LD. LH concentration was increased 30 min after administration of 500-pmol RFRP-1 or 500-pmol RFRP-3 in SD	Ubuka et al. (7)
In vitro					
Hypothalamic tissue of male mice	10^{-7}, 10^{-6} M RFRP-3 with 10^{-6} M kisspeptin	Medium 199	After 1-h incubation medium was collected.	10^{-6} M RFRP-3 suppressed 10^{-6} M kisspeptin-induced GnRH release	Son et al. (46)
Hypothalamic tissue of female mice	10^{-6} M RFRP-3 with 10^{-6} M VIP	Medium 199	After 1-h incubation medium was collected.	10^{-6} M RFRP-3 suppressed 10^{-6} M VIP-induced GnRH release	Son et al. (46)
GFP labeled GnRH neurons of transgenic mice	0.01–1-μM GnIH or RFRP-3	aCSF	15-s application	GnIH and RFRP-3 produced a non-desensitizing hyperpolarization [IC_{50}: 34 nM (GnIH), 37 nM (RFRP-3)] *via* a direct postsynaptic Ba^{2+}-sensitive K^+ current mechanism	Wu et al. (47)
GFP labeled GnRH neurons of transgenic mice	1-μM RFRP-3	aCSF	5-min application	RFRP-3 exhibited rapid and repeatable inhibitory effects on the firing rate of 41% of GnRH neurons. RFRP-3 increased the firing rate of 12% of GnRH neurons	Ducret et al. (48)
Mouse GnRH neuronal cell line (GT1–7)	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M RFRP-1 and -3 with 10^{-6} M VIP	DMEM	6 (CRE assay) or 1 (p38, ERK assay) h application	10^{-6} M VIP-induced CRE activity was suppressed by 10^{-8}, 10^{-7}, 10^{-6} M RFRP-1, 3. 10^{-6} M VIP-induced p38 and ERK phosphorylation was suppressed by 10^{-7}, 10^{-6} M RFRP-3	Son et al. (46)
Mouse GnRH neuronal cell line (mHypoA-GnRH/GFP)	10-, 100-nM human RFRP-3	DMEM	1-, 2-, 4-h application	GnRH mRNA expression was decreased by 100-nM RFRP-3 at 1-, 2-, 4-h application	Gojska et al. (49)
Ewe dispersed pituitary cells	10^{-14}, 10^{-12}, 10^{-10}, 10^{-8} M human RFRP-3 with 10^{-9} M GnRH	DMEM	Medium was collected after 2-h incubation	GnRH-induced LH release was decreased by 10^{-12}, 10^{-10}, 10^{-8} M RFRP-3. GnRH-induced FSH release was decreased by 10^{-10}, 10^{-8} M RFRP-3	Clarke et al. (50)
Gonadectomized ewe and ram dispersed pituitary cells	10^{-12}, 10^{-9} M human RFRP-3 with 10^{-9} M GnRH	DMEM with 10% fetal calf serum	Medium was collected 8, 16, 24 h during incubation and finally pituitary cells were collected	GnRH-induced LH release was decreased by 10^{-12}, 10^{-9} M RFRP-3 at 8-, 16-, 24-h in ewe pituitary cells. GnRH-induced LH release was decreased by 10^{-12}, 10^{-9} M RFRP-3 at 8-, 16-h in ram pituitary cells. GnRH-induced FSH release was decreased by 10^{-12}, 10^{-9} M RFRP-3 at 16-, 24-h in ewe pituitary cells. GnRH-induced FSH release was decreased by 10^{-12}, 10^{-9} M RFRP-3 at 8-, 16-h in ram pituitary cells. GnRH-induced LHβ, FSHβ expression, ERK phosphorylation were decreased by 10^{-12}, 10^{-9} M RFRP-3 in ewe and ram pituitary cells	Sari et al. (51)

(*Continued*)

TABLE 2 | Continued

In vivo (animal) or *in vitro* (pituitary or cell line)	Concentration or dose of peptides	Rout of administration, culture medium	Administration time, sample collection, measurement	Effect	Reference
Cattle dispersed pituitary cells	10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} M bovine RFRP-3 with 10^{-9} M GnRH	DMEM	Medium was collected after 2-h incubation	10^{-10}, 10^{-8}, 10^{-6} M RFRP-3 decreased GnRH-induced LH release	Kadokawa et al. (38)
Gonadectomized male rat pituitaries	10^{-10}, 10^{-8}, 10^{-6} M rat RFRP-3 with or without 10^{-9} M GnRH	DMEM	After 2-h incubation medium was collected	Basal LH concentration was decreased by 10^{-8}, 10^{-6} M RFRP-3. LH concentration stimulated by GnRH was decreased by 10^{-10}, 10^{-8} M RFRP-3.	Pineda et al. (40)
Female rat dispersed pituitary cells	10^{-16}, 10^{-14}, 10^{-12} M rat RFRP-3 with 10^{-9} M GnRH	DMEM with 10% fetal bovine serum	After 24-h incubation medium was collected	LH concentration stimulated by GnRH was decreased by 10^{-12} M RFRP-3	Murakami et al. (41)
Mouse gonadotrope cell line (LβT2)	10^{-7}, 10^{-6} M RFRP-3 with 10^{-7} M GnRH	DMEM	1 h (gonadotropin subunit gene expression), 2 h (LH release) application	10^{-7} M GnRH-induced gonadotropin subunit gene expression was suppressed by 10^{-6} M RFRP-1, 3. 10^{-8} M GnRH-induced LH release was suppressed by 10^{-7}, 10^{-6} M RFRP-1, 3	Son et al. (34)
Mouse gonadotrope cell line (LβT2)	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M RFRP-3 with 10^{-7} M GnRH	DMEM	75-min (cAMP assay), 6-h (CRE assay) or 15-min (ERK assay) application	10^{-7} M GnRH-induced cAMP production was suppressed by 10^{-7}, 10^{-6} M RFRP-1, 3. 10^{-7} M GnRH-induced CRE activity was suppressed by 10^{-8}, 10^{-7}, 10^{-6} M RFRP-1, 3. 10^{-7} M GnRH-induced ERK phosphorylation was suppressed by 10^{-6} M RFRP-1, 3	Son et al. (34)

aCSF, artificial cerebrospinal fluid; CRE, cAMP response element; DMEM, Dulbecco's modified Eagle's medium; E2, 17β-estradiol; EB, estradiol benzoate; ERK, extracellular signal-regulated kinase; FSH, follicle-stimulating hormone; GFP, green fluorescent protein; icv, intracerebroventricular administration; ip, intraperitoneal administration; iv, intravenous administration; LD, long day; LH, luteinizing hormone; P4, progesterone; SD, short day; VIP, vasoactive intestinal polypeptide.

Stimulatory effects on the HPG axis are underlined.

(39, 57), mice (58), frog (59), zebrafish (60), and lamprey (20). Double-immunohistochemistry using GPR147 and GnRH antibodies shows GPR147 on GnRH neurons in hamsters (7) (**Figure 1**).

Abundant GnIH-immunoreactive (ir) fibers exist in the median eminence of humans (5), monkey (6), sheep (50), quail (1, 25, 61), sparrow (52, 62), and turtle (15). It has been clearly shown that GPR147 mRNA is expressed in the gonadotropes of human pituitary (5). GPR147-ir cells are located in the cephalic and caudal lobes of the chicken pituitary gland and they are colocalized with LHβ or FSHβ mRNA-containing cells (63). Therefore, it is likely that GnIH can directly act on the pituitary to inhibit gonadotropin synthesis and/or release from the pituitary in most birds and relatively large mammalian species (3) (**Figure 1**). On the other hand, GnIH may not act directly on the pituitary in some birds and rodents, as there are few or no GnIH-ir fibers in the median eminence of Rufous-winged sparrows (64), hamsters (7, 45), and rats (65). In teleost fishes, GnIH-ir fibers directly innervate the pituitary (4), which have been observed in goldfish (19), sockeye salmon (66), Indian major carp (67), sea bass (68), and tilapia (69). In the tilapia pituitary, LH cells were labeled by GnIH receptor antibody (69) (**Figure 1**).

STIMULATORY EFFECTS OF GnIH ON THE HPG AXIS

An electrophysiological study has shown that RFRP-3 exhibits rapid and repeatable inhibitory effects on the firing of 41% of GnRH neurons in adult mice (48). However, stimulatory effect of RFRP-3 was observed in 12% of GnRH neurons (**Table 2**). No stimulatory effect of RFRP-3 on the firing of GnRH neurons was observed in diestrus mice but 18% of GnRH neurons were stimulated by RFRP-3 in proestrus female mice (48).

To understand the physiological roles of GnIH in mammalian reproduction, GnIH precursor cDNA and endogenous mature peptides have been identified in the Siberian hamster brain (7). GnIH mRNA expression and number of GnIH-ir perikarya, fibers that innervate GnRH neurons are higher in long days (LD), breeding season, compared with short days (SD), non-breeding season. Intracerebroventricular (icv) administration of hamster RFRP-1 or RFRP-3 to male Siberian hamster inhibits plasma LH concentration 5 and 30 min after administration in LD but stimulates plasma LH concentration 30 min after administration in SD (7) (**Table 2**). It has been also shown that central chronic administration of RFRP-3 to male Syrian hamsters adapted to SD fully restores testicular weight and plasma testosterone concentration (44, 70) (**Table 2**).

Moussavi et al. investigated the effect of intraperitoneal (ip) administration of goldfish LPXRFa-3 on LHβ and FSHβ subunit mRNA levels in the pituitary and serum LH concentration during gonadal cycle in goldfish (71). Circulating 17β-estradiol (E2) level is very low at early gonadal recrudescence (gr), increasing at mid-gr, very high at mid-late gr, and decreasing at late gr stages. LPXRFa-3 increased LHβ and FSHβ mRNA levels at early to mid-late and late gr, respectively. However, serum LH

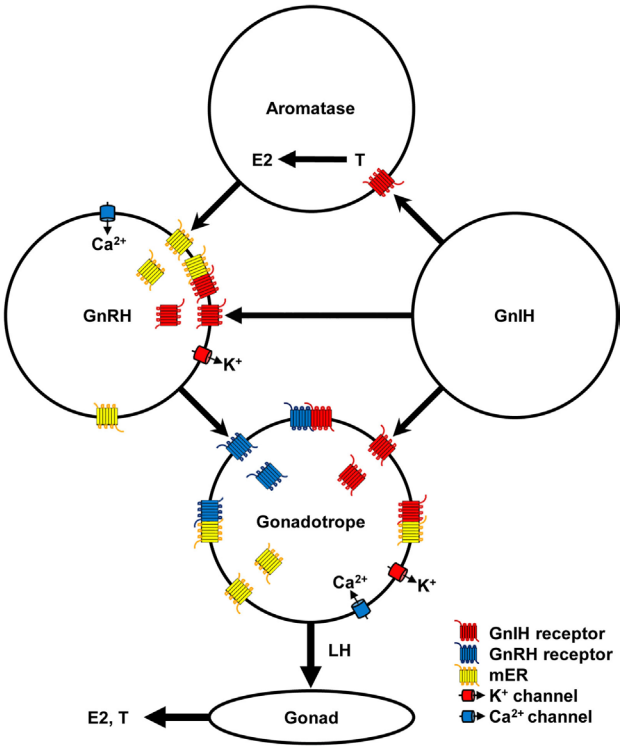

FIGURE 1 | Schematic diagram of the mechanism of gonadotropin-inhibitory hormone (GnIH) action in the hypothalamic–pituitary–gonadal axis. GnIH neurons act on aromatase and gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus and gonadotrope in the pituitary *via* GnIH receptor. Aromatase neurons synthesize estradiol-17β (E2) from testosterone (T) in the hypothalamus and E2 can act on GnRH neurons *via* membrane estrogen receptor (mER). GnIH stimulates K^+ channel to hyperpolarize GnRH neurons and gonadotrope, and decrease GnRH and luteinizing hormone (LH) release, respectively. E2 stimulates Ca^{2+} channel to depolarize GnRH neurons and stimulates GnRH release. GnRH stimulates GnRH receptor and Ca^{2+} channel to depolarize gonadotrope and stimulates LH release. Low concentration of E2 inhibits Ca^{2+} channel on the gonadotrope and LH release stimulated by GnRH. LH stimulates synthesis and release of E2 and T from ovary and testis, respectively. GnIH and GnRH receptors and GPR30 (mER) belong to Class A G-protein coupled receptor family and may form heteromers to modulate ligand binding affinity and signal transduction. Binding of GnIH, GnRH, and E2 with their receptors can downregulate their cognate receptors by internalization. These complex stimulatory and inhibitory mechanisms may regulate reproductive homeostasis according to developmental and reproductive stages.

level is decreased by LPXRFa-3 administration at early to mid gr (**Table 3**). Moussavi et al. further examined the effect of ip administration of LPXRFa-3 with two native goldfish GnRHs, salmon GnRH (sGnRH) and chicken GnRH (cGnRH)-II (72). Ip administration of gfLPXRF-3 alone elevated pituitary LHβ and FSHβ mRNA levels at early and mid-gr, and only FSHβ mRNA at late gr. Coadministration of LPXRFa-3 attenuated the stimulatory effect of sGnRH on LHβ in early recrudescence, and LHβ and FSHβ mRNA levels in mid and late gr, as well as cGnRH-II-elicited increase in LHβ mRNA expression at mid and late gr. Ip administration of gfLPXRF-3 reduced serum LH levels in early and mid gr (**Table 3**).

Ip administration of grouper GnIH-I, II, and III decreased GnRH1 mRNA level in the hypothalamus (77). However, GnRH3 mRNA level in the hypothalamus was increased by ip administration of GnIH-III. On the other hand, LHβ mRNA level in the pituitary was decreased by GnIH-II (**Table 3**). Ip administration of lamprey LPXRFa-2 increased GnRH-I and III content in the brain, gonadotropin β mRNA level in the pituitary [(20), **Table 3**]. A study in European sea bass has shown that intramuscular administration of sea bass GnIH-2 increased GnRH2 and kiss1 receptor mRNA levels in the brain (27). On the other hand, GnIH-1, 2 decreased pituitary LHβ mRNA level and plasma LH level. Plasma FSH level was only decreased by GnIH-1 (**Table 3**).

In addition, 48-h incubation of grass puffer pituitary with LPXRFa-1 (10^{-7} M) increased LHβ and FSHβ mRNA levels [(79), **Table 3**]. Although LH and FSH release from *Cichlasoma dimerus* pituitary was decreased by 24-h incubation with LPQRFa-1 (10^{-6} M), FSH release was increased by LPQRFa-2 (10^{-6} M) [(80), **Table 3**]. Also, 6-h incubation of Nile tilapia pituitary with pyroglutamic-LPXRFa-2 (10^{-7} and 10^{-6} M) increased LH release and pyroglutamic-LPXRFa-2 (only 10^{-6} M) increased FSH release [(81), **Table 3**].

Effect of goldfish LPXRFa-3 on gonadotropin synthesis and release was tested in dispersed goldfish pituitary cells collected at different gr stages (71). LHβ mRNA level was decreased by LPXRFa-3 (10^{-8} and 10^{-7} M) at early gr, but increased by LPXRFa-3 (10^{-9} M) at mid-gr, and decreased by LPXRFa-3 (10^{-8} and 10^{-7} M) at late gr. FSHβ mRNA levels was decreased by LPXRFa-3 (10^{-8} and 10^{-7} M) at early gr, by LPXRFa-3 (10^{-9}, 10^{-8}, 10^{-7} M) at mid-gr, and by LPXRFa-3 (10^{-7} M) at late gr. On the other hand, LH concentration in the media was increased by LPXRFa-3 (10^{-8} M) at late gr (**Table 3**). In dispersed pituitary cells of male sockeye salmon, LH release was increased by goldfish LPXRFa-1, 2 (10^{-7} and 10^{-5} M), and LPXRFa-3 (10^{-9} and 10^{-5} M). FSH release was increased by goldfish LPXRFa-1 (10^{-9} and 10^{-5} M), LPXRFa-2 (10^{-7}, 10^{-5} M), and LPXRFa-3 (10^{-7} M) (66, **Table 3**).

POSSIBLE MACHNISM OF THE STIMULATORY EFFECTS OF GnIH ON THE HPG AXIS

The mechanism of GnIH (RFRP-3) effect on the electrophysiological activity of GnRH neurons was studied in transgenic mice having vesicular glutamate transporter 2 (vGluT2)-GnRH neurons (47). GnIH and RFRP-3 produced a non-desensitizing hyperpolarization with IC_{50} values of 34 and 37 nM, respectively, in vGluT2-GnRH neurons *via* a direct postsynaptic Ba^{2+}-sensitive K^+ current mechanism (**Figure 1**, **Table 2**).

It is known that E2 secreted from the ovary negatively and positively act on the hypothalamus and pituitary to regulate the HPG axis in females. However, it is also known that E2 is synthesized from androgen by aromatase neurons in the hypothalamus (82). Recent studies have shown that E2 synthesized in the brain (neuroestrogen) directly and rapidly act on GnRH neurons *via* membrane estrogen receptor (mER) to regulate GnRH release (83, 84). GPR30 (85, 86), ERβ (87, 88) or other membrane receptors are thought to transduce the rapid effect of E2 on GnRH

TABLE 3 | Effect of GnIH on the HPG axis of amphioxus, lamprey, and teleost fishes.

In vitro (cell line or pituitary) or *in vivo* (animal)	Concentration or dose of peptides	Culture medium, rout of administration	Administration time, sample collection, measurement	Effect	Reference
In vivo					
European sea bass	1, 2, 4 μg sea bass GnIH-1, 2	icv	6 h after administration brain, pituitary, and blood were collected	GnRH1 mRNA level in the brain was decreased by 1, 2, 4 μg GnIH-1. GnRH2 mRNA level in the brain was decreased by 1, 2, 4 μg GnIH-2. Kiss1 mRNA level in the brain was decreased by 2-μg GnIH-2. Kiss2 mRNA level in the brain was decreased by 2, 4 μg GnIH-2. Kiss1 receptor mRNA level in the brain was decreased by 2-μg GnIH-2. GnIH mRNA level in the brain was decreased by 1, 2 μg GnIH-2. GnIH receptor mRNA level in the brain was decreased by 1, 2 μg GnIH-2. LHβ mRNA level in the pituitary was decreased by 1, 2, 4 μg GnIH-2. FSHβ mRNA level in the pituitary was decreased by 2, 4 μg GnIH-2. GnRH receptor II1a mRNA level in the pituitary was decreased by 2, 4 μg GnIH-2. Plasma LH level was decreased by 4-μg GnIH-1 and 1-μg GnIH-2	Paullada-Salmerón et al. (73)
Goldfish	2-μg goldfish LPXRFa-3	ip	Injected twice with 12-h interval and pituitaries and blood were collected 12 h after the second injection	LHβ mRNA level was increased at early to mid-late gr. FSHβ mRNA levels was increased at early to late gr. Serum LH concentration was decreased at early to mid-gr	Moussavi et al. (71)
Goldfish	2-μg goldfish LPXRFa-3	ip	Injected twice with 12-h interval with or without 4-μg sGnRH or cGnRH-II and pituitaries and blood were collected 2 h after the second injection	LHβ level was increased by LPXRFa-3 at early to mid-gr. FSHβ mRNA levels was increased LPXRFa-3 at early to late gr. Serum LH concentration was decreased by LPXRFa-3 at early to mid-gr. LHβ mRNA level increased by sGnRH was decreased by LPXRFa-3 at early to late gr. LHβ level increased by cGnRH-II was decreased by LPXRFa-3 at mid to late gr. FSHβ mRNA level increased by sGnRH was decreased by LPXRFa-3 at mid to late gr	Moussavi et al. (72)
Sexually mature female goldfish	1-μg/g bw zebrafish LPXRFa-3	ip	Injected twice with 3-h interval and blood was collected 1 and 3 h after the second injection	Serum LH concentration was decreased by LPXRFa-3 either at 1 and 3 h after the second injection	Zhang et al. (74)
Female goldfish at late vitellogenic stage	100-ng/g bw goldfish LPXRFa-2, 3	ip	After 12-h administration hypothalamus and pituitary were collected	sGnRH mRNA level in the hypothalamus was decreased by LPXRFa-2, 3. LHβ mRNA level in the pituitary was decreased by LPXRFa-2. FSHβ mRNA level in the pituitary was decreased by LPXRFa-2, 3	Qi et al. (75)
Immature, mature male and female cinnamon clownfish	100-ng/g bw goldfish LPXRFa-3	ip	After 0, 6, 12, and 24-h administration with or without 100-ng/g bw sbGnRH brain, pituitary and blood were collected	GnIH and GnIH receptor mRNA levels in the brain were increased at 6, 12 and 24 h.[a] GnIH and GnIH receptor mRNA levels in the brain decreased by sbGnRH were increased at 6, 12 and 24 h.[a] sbGnRH mRNA level in the brain, plasma GnRH, FSH, LH levels, pituitary GTHα, FSHβ, LHβ mRNA levels were decreased at 6, 12 and 24 h.[a] sbGnRH mRNA level in the brain, plasma GnRH, FSH, LH levels, pituitary GTHα, FSHβ, LHβ mRNA levels increased by sbGnRH were decreased at 6, 12 and 24 h[a]	Choi et al. (76)
Female orange-spotted grouper	100-ng/g bw grouper GnIH-I, II, III	ip	Injected twice with 6-h interval and hypothalamus and pituitary were collected 6 h after the second injection	GnRH1 mRNA level in the hypothalamus was decreased by grouper GnIH-I, II, III. GnRH3 mRNA level in the hypothalamus was increased by grouper GnIH-III. LHβ mRNA level in the pituitary was decreased by grouper GnIH-II	Wang et al. (77)
Lamprey	50, 100 μg/kg bw lamprey LPXRFa-1a, 1b, 2	ip	Injected twice with 24-h interval and brain and pituitary were collected 48 h after the second injection	Lamprey GnRH-I, III content in the brain, gonadotropin β mRNA level in the pituitary were increased by 100-μg/kg bw LPXRFa-2	Osugi et al. (20)

(*Continued*)

TABLE 3 | Continued

In vitro (cell line or pituitary) or *in vivo* (animal)	Concentration or dose of peptides	Culture medium, rout of administration	Administration time, sample collection, measurement	Effect	Reference
European sea bass	1-μg sea bass GnIH-1, 2/g bw in coconut oil	im	Injected on day 17 from October to January and blood was collected on day 22 from October to January. Brain and pituitary were collected on day 17 of February (spermiation stage)	Plasma testosterone and 11-ketotestosterone levels were decreased by sbGnIH-1, 2 in November and December (early and mid-spermatogenesis). GnRH2, sbGnIH, sbGnIH receptor, kiss1 receptor mRNA levels in the brain were increased by sbGnIH-2. LHβ mRNA level in the pituitary was decreased by sbGnIH-1 and -2. Plasma FSH level was decreased by sbGnIH-1. Plasma LH level was decreased by sbGnIH-1 and -2	Paullada-Salmerón et al. (27)
Flatfish	0.1, 1 μg/g bw flatfish GnIH-2, 3	im	Injected twice with 12-h interval and brain and pituitary were collected 4 and 8 h after the second injection	GnRH3 mRNA level in the brain was decreased by 1-μg/g bw GnIH-3 at 4 h after administration. LHβ mRNA level in the pituitary was decreased by 0.1, 1 μg/g bw GnIH-3 at 4 h after administration	Aliaga-Guerrero et al. (78)
In vitro					
Primary culture of male zebrafish pituitary	10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} M zebrafish LPXRFa-3	Culture media	After 18-h incubation pituitary was collected	Common α mRNA level was decreased by 10^{-12}, 10^{-11}, 10^{-10} M LPXRFa-3. LHβ mRNA level was decreased by 10^{-11}, 10^{-10} M LPXRFa-3	Spicer et al. (60)
Primary culture of grass puffer pituitary	10^{-9}, 10^{-7} M goldfish LPXRFa-1	RPMI medium	After 48-h administration pituitaries were collected	LHβ, FSHβ mRNA levels were increased by 10^{-7} M LPXRFa-1	Shahjahan et al. (79)
Primary culture of *Cichlasoma dimerus* pituitary	10^{-8}, 10^{-6} M *Cichlasoma dimerus* LPQRFa-1, -2	Leibovitz L-15 medium with 10% fetal bovine serum	After 24-h incubation medium was collected	LH and FSH concentration was decreased by 10^{-6} M LPQRFa-1. FSH concentration was increased by 10^{-6} M LPQRFa-2	Di Yorio et al. (80)
Primary culture of male Nile tilapia pituitary	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M Pyroglutamic-tilapia LPXRFa-2	Culture medium	After 6-h incubation medium was collected	LH concentration was increased by 10^{-7}, 10^{-6} M pyroglutamic-LPXRFa-2. FSH concentration was increased by 10^{-6} M pyroglutamic-LPXRFa-2	Biran et al. (81)
Dispersed goldfish pituitary cells	10^{-9}, 10^{-8}, 10^{-7} M goldfish LPXRFa-3	Medium 199 with 1% horse serum	After 12-h administration medium and cells were collected	LHβ mRNA level was decreased by 10^{-8} and 10^{-7} M LPXRFa-3 at early gr, increased by 10^{-9} M LPXRFa-3 at mid-gr, decreased by 10^{-8} and 10^{-7} M LPXRFa-3 at late gr. FSHβ mRNA levels was decreased by 10^{-8} and 10^{-7} M LPXRFa-3 at early gr, by 10^{-9}, 10^{-8}, 10^{-7} M LPXRFa-3 at mid-gr, by 10^{-7} M LPXRFa-3 at late gr. LH concentration in the media was increased by 10^{-8} M LPXRFa-3 at late gr	Moussavi et al. (71)
Dispersed female goldfish pituitary cells	10^{-7} M goldfish LPXRFa-2, 3	Medium 199 with 10% fetal bovine serum	After 12-h administration with 10^{-7} M LHRH-A cells were collected	FSHβ mRNA level increased by LHRH-A was decreased by 10^{-7} M LPXRFa-3.	Qi et al. (75)
Dispersed male sockeye salmon pituitary cells	10^{-9}, 10^{-7}, 10^{-5} M goldfish LPXRFa-1, 2, 3	MEM	After 2-h administration medium was collected	LH concentration in the media was increased by 10^{-7} and 10^{-5} M LPXRFa-1, 2, and 10^{-9}, 10^{-5} M LPXRFa-3. FSH concentration in the media was increased by 10^{-9} and 10^{-5} M LPXRFa-1, 10^{-7}, 10^{-5} M LPXRFa-2, and 10^{-7} M LPXRFa-3	Amano et al. (66)

(*Continued*)

TABLE 3 | Continued

In vitro (cell line or pituitary) or *in vivo* (animal)	Concentration or dose of peptides	Culture medium, rout of administration	Administration time, sample collection, measurement	Effect	Reference
COS-7 cells transfected with orange-spotted grouper GnIH receptor	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M grouper GnIH-I, -II, -III	DMEM with 10% fetal bovine serum	After 24-h incubation CRE or SRC-luciferase activity was measured	Forskolin-induced CRE-luciferase activity was decreased by 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M grouper GnIH-I, II and 10^{-6} M grouper GnIH-III. SRE-luciferase activity was decreased by 10^{-9}, 10^{-7}, 10^{-6} M grouper GnIH-I	Wang et al. (77)
COS-7 cells transfected with amphioxus PQRFa receptor 1	10^{-7}, 10^{-6} M amphioxus PQRFa-1, 2, 3	DMEM	After 6-h administration CRE-luciferase activity was measured	Forskolin-induced CRE-luciferase activity was decreased by 10^{-6} M PQRFa-1, 2, and 1 0^{-7}, 10^{-6} M PQRFa-3	Osugi et al. (21)

bw, body weight; cGnRH-II, chicken GnRH-II; CRE, cAMP response elements; DMEM, Dulbecco's modified Eagle's medium; FSH, follicle-stimulating hormone; gr, gonadal recrudescence; icv, intracerebroventricular administration; im, intramuscular administration; ip, intraperitoneal administration; LH, luteinizing hormone; LHRH-A, [D-Ala^6, Pro^9 NEt]-LHRH; MEM, minimum essential medium; sGnRH, salmon GnRH; sbGnRH, sea bream GnRH.

[a]*Only changed at 12 and 24 h in some groups.*

Stimulatory effects on the HPG axis are underlined.

release (83, 89). E2 stimulates GnRH release by increasing intracellular Ca^{2+} concentration (90) and electrophysiological activity of GnRH neurons (91, 92). More recently, it has been shown that GnIH neurons terminal on aromatase neurons that express GnIH receptor and increase neuroestrogen concentration in the hypothalamus by stimulating aromatase activity in quail (93, 94). Therefore, it is possible that GnIH stimulates the electrophysiological activity of some GnRH neurones (48) by increasing neuroestrogen concentration in the hypothalamus. GnIH may further stimulate LH release that was shown in hamsters (7) by stimulating the activity of aromatase neurons and increasing neuroestrogen concentration in the hypothalamus and stimulating the electrophysiological activity of GnRH neurons and GnRH release (**Figure 1**).

Binding of GnRH with GnRH receptor on gonadotropes results in the activation of intracellular $G_{\alpha q/11}$ and phospholipases and generation of the second messengers, inositol 1-, 4-, 5-tris-phosphate, diacylglycerol, and arachidonic acid, which stimulate Ca^{2+} mobilization and PKC activity. Ca^{2+} mobilization initiates gonadotropin release (**Figure 1**). PKC activates mitogen-activated protein kinases (MAPKs) such as ERK, jun-N-terminal kinase, and p38 MAPK, which initiate the transcriptional activity of gonadotropin subunit genes (95). GnRH receptor also couples with $G_{\alpha s}$ to stimulate AC/cAMP/PKA pathway, which was shown in LβT2 cells (96) and rat gonadotropes (97). Because GnIH signaling pathway triggered by $G_{\alpha i}$ does not interfere with $G_{\alpha q/11}$ triggered pathway, GnIH may suppress gonadotropin subunit gene transcription by inhibiting AC/cAMP/PKA pathway stimulated by GnRH receptor and $G_{\alpha s}$ (34). GnIH may also suppress gonadotropin release by hyperpolarizing gonadotropes by activating K^+ channel *via* GnIH receptor [(47), **Figure 1**].

However, recent studies of GPCR have shown that GPCR not only functions as a monomer or homodimer but also as a heterodimer with different GPCR resulting in modulation of ligand binding affinity, signal transduction, and internalization of the receptors (98, 99). It has been shown that Class A GPCRs form homo- and heteromers (100). As GnRH and GnIH receptors, and GPR30 all belong to Class A GPCR family (101), it is possible that they form heteromers in GnRH neurons and/or gonadotropes to modify the action of their ligands. Some of the stimulatory effect of GnIH on the HPG axis may be due to heteromerization of GnIH and GnRH receptor and GPR30 (**Figure 1**).

A recent study has shown that centrally administered GnIH can decrease plasma LH concentration in ovariectomized (OVX) prepubertal female mice that were treated with E2 but not in OVX mice that were not treated with E2 (43) (**Table 2**). E2 can abolish intracellular free Ca^{2+} concentration and LH release in ovine pituitary culture induced by GnRH (102). The inhibitory effect of low concentration of E2 on LH release was shown in bovine anterior pituitary mediated by GPR30 expressed on the gonadotrope (103, 104). These results suggest the modification of GnIH action by E2 in the hypothalamus and pituitary (**Figure 1**).

Finally, it is known for a long time that binding of GnRH with GnRH receptors is followed by aggregation, complex formation and internalization (105). Chronic administration of GnRH or antagonist administration can desensitize pituitary gonadotropes, downregulate GnRH receptor and suppress serum LH,

FSH and sex-steroid levels (106–108). It is therefore possible that chronic central administration of GnIH (RFRP-3) to male Syrian hamsters adapted to SD restores testicular weight and plasma testosterone concentration by downregulation of GnIH receptor in the hypothalamus and pituitary (44, 70) (**Table 2**). It is also possible that stimulatory effect of GnIH on the pituitary of fish is due to downregulation of GnIH receptor by chronic administration (79, 80), high concentration of GnIH (66, 80, 81) or antagonistic effect of LPXRFa peptides of different species (66, 79) (**Table 3**). Inhibitory effects of GnIH on the HPG axis are shown when GnIH peptides are tested with relatively low concentrations in a shorter time frame (73–76) (**Tables 2** and **3**; **Figure 1**).

Complex mechanism may be involved in *in vivo* studies that show stimulatory and inhibitory effects of GnIH on the HPG axis in addition to downregulation of receptors and changes in the number of receptors depending on reproductive and developmental stages and endogenous sex-steroid levels (**Tables 2** and **3**; **Figure 1**). It is also important to note that GnIH peptides are produced in gonads (3, 109) and it has been shown that they have direct effects on gonadal activates in mammals (110–114), birds (115–117) and fishes (118). Most of these studies showed inhibitory effects of GnIH peptides on gonadal activities, but stimulatory activity of GnIH peptides was also shown in mouse ovary (114) and goldfish testis (118). Therefore, *in vivo* studies that showed effects of GnIH peptides on gonadal activates (**Tables 2** and **3**) may include direct effects of GnIH peptides on the gonads.

CONCLUSION

Gonadotropin-inhibitory hormone orthologous peptides have a characteristic LPXRFamide C-terminal motif in most vertebrate species, which is critical for receptor binding. The primary receptor for GnIH is GPR147 that inhibits cAMP production in target cells. GnIH generally decreases gonadotropin synthesis and release by directly acting on the gonadotrope or by decreasing the activity of GnRH neurons. However, one study shows stimulatory effects of GnIH on the electrophysiological activity of some GnRH neurons in mice (48). Stimulatory effect of GnIH on GnRH neurons in the hypothalamus may be explained by the action of neuroestrogen synthesized in the hypothalamus by the stimulatory action of GnIH on aromatase neurons that terminate on GnRH neurons that express estrogen membrane receptor. GnIH may further stimulate LH release that was shown in hamsters by stimulating the electrophysiological activity of GnRH neurons and GnRH release (7, 44). Peripheral sex-steroid levels may also modify the action of GnIH (7, 44, 71, 72). Some of the stimulatory effects of GnIH on the HPG axis may be due to heteromerization of GnIH and GnRH receptors and GPR30 in GnRH neurons and/or gonadotropes, which modifies ligand binding and signaling transduction mechanism. Stimulatory effect of GnIH on the HPG axis may also be due to internalization of GnIH receptor by high concentration or chronic administration of GnIH or antagonistic effect of the peptides administered (20, 66, 77, 79–81). Besides pharmacological effect of administered peptides, the general inhibitory action of GnIH by decreasing cAMP concentration and inducing hyperpolarization in target cells and the additional stimulatory action of GnIH by neuroestrogen synthesis, receptor heteromerization, and internalization may have a physiological role to maintain reproductive homeostasis according to developmental and reproductive stages.

AUTHOR CONTRIBUTIONS

TU wrote the manuscript and IP edited the manuscript.

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