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Review article

Regulation of LH secretion by RFRP-3 – From the hypothalamus to the pituitary

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ABSTRACT

RFamide-related peptides (RFRPs) have long been identified as inhibitors of the hypothalamus-pituitary-gonad axis in mammals. However, less progress has been made in the detailed roles of RFRPs in the control of LH secretion. Recent studies have suggested that RFRP-3 neurons in the hypothalamus can regulate the secretion of LH at different levels, including kisspeptin neurons, GnRH neurons, and the pituitary. Additionally, conflicting results regarding the effects of RFRP-3 on these levels exist. In this review, we collect the latest evidence related to the effects of RFRP-3 neurons in regulating LH secretion by acting on kisspeptin neurons, GnRH neurons, and the pituitary and discuss the potential role of the timely reduction of RFRP-3 signaling in the modulation of the preovulatory LH surge.

1. Introduction

It has long been established that the hypothalamic–pituitary–gonadal (HPG) axis (also known as the gonadotropic axis) plays an indispensable role in regulating mammalian reproduction (Harris, 1964). A small group of hypothalamic neurons terminating in the median eminence (ME) synthesize the gonadotropin-releasing hormone (GnRH), which in turn stimulates gonadotrope cells to synthesize and release pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Vadakkadath Meethal and Atwood, 2005). The release of FSH and LH to the circulation regulates gonadal development and promotes the formation of gametes and the production of sex steroids. The latter, in turn, forms a negative feed-back loop by inhibiting the production of GnRH and gonadotropins (Karsch and Evans, 1996; Knobil, 2005; Plant and Zeleznik, 2014; Vadakkadath Meethal and Atwood, 2005). However, many of the key mechanisms involved in the regulation of GnRH release, including the GnRH pulsatility and the preovulatory LH surge, remain poorly understood until recent times.

In the last two decades, several neuropeptides that control the synthesis and release of GnRH in the hypothalamus have been identified. Two different peptides, kisspeptins and RFamide-related peptides (RFRPs), are the most attractive neuropeptides because they act as the essential upstream regulators in the control of GnRH secretion, with a stimulatory and inhibitory effect, respectively. Considerable advances have been made concerning the stimulatory control of the HPG axis by kisspeptins in recent years (for reviews, see Javed et al. (2015), Piet et al. (2015), Ratnasabapathy and Dhillo (2013), Shruti and Prevot (2016), Skorupskaite et al. (2014)), while less progress has been made in the inhibitory modulation of the production of GnRH and LH. In this review, we collected the recent studies focused on how RFRP-3 regulates LH secretion across the different types of controls regarding levels of hypothalamic and pituitary. We also discussed the involvement of timely-reduction of RFRP-3 signaling in the modulation of preovulatory LH surge. Our discussion will be largely limited to data generated from mammals, primarily rodents, as these animal models have been widely used to study the functions of RFRP-3 and the underlying cellular and molecular mechanisms.

2. Discovery of RFRPs and related receptors

In 2000, a neuropeptide that has the sequence of Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH_2 (RFamide peptide) was isolated from avian brain (Tsutsui et al., 2000). This peptide was named GnIH, as it inhibited the release of gonadotropins from cultured anterior pituitaries of adult male quail in a dose-dependent manner (Tsutsui et al., 2000). Based on the molecular structures, mammalian GnIH peptides are termed as LPXRFamide peptides because these peptides possess a common LPXRFamide (X = L or Q) sequence at C terminal (for reviews,

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see Clarke and Parkington (2014), Kriegsfeld et al. (2015), Tsutsui et al. (2010), Tsutsui et al. (2012), Tsutsui et al. (2015)). Based on database searches of DNA sequences, Hinuma et al. reported a human gene *NPVF* that encodes at least three RF-amide-related peptides, RFRP-1, RFRP-2, and RFRP-3, among which RFRP-1 and RFRP-3 are functional peptides (Hinuma et al., 2000). Endogenous RFRP-1 and RFRP-3 peptides have been subsequently isolated as mature peptides in various mammalian species, including humans, cattle, and Siberian hamster. However, in rats and macaques, only RFRP-3 was biochemically characterized (Ubuka et al., 2012; Ubuka et al., 2009a; Ubuka et al., 2009b; Ukena et al., 2002; Yoshida et al., 2003) (for reviews, see Quillet et al. (2016), Tsutsui et al. (2015), Ubuka and Tsutsui (2014)). Despite that RFRP-1 displays closer structural homology to avian GnIH, functional studies have indicated that RFRP-3 is most likely the homolog of GnIH in the regulation of gonadotropin secretion in mammals (Ancel et al., 2012; Pineda et al., 2010a).

Similar to the discovery of RFamide neuropeptides, several orphan G protein-coupled receptors (GPCRs) were successively identified as the natural targets of endogenous mammalian RFamides in the early 2000 s. Bonini and collaborators cloned orthologs of two GPCRs for neuropeptide FF (NPFF) and designated them as NPFF1R (identical to GPR147) and NPFF2R (identical to GPR74) (Bonini et al., 2000). In the same year, Hinuma et al. reported a specific receptor for RFRP, OT7T022, which specifically responds to synthetic hRFRP-1 and hRFRP-3 (Hinuma et al., 2000). Independently, Elshourbagy and coworkers identified a receptor, HLWAR77, with which both neuropeptides AF (NPAF) and NPFF have high affinity (Elshourbagy et al., 2000). Additionally, Parker and collaborators cloned another putative receptor GPR74 (Parker et al., 2000). Studies using the systematic comparison of the pharmacological properties of these receptors clearly demonstrated the molecular and functional identity of OT7T022 with the NPFF1R/GPR147 receptor and HLWAR77 with the NPFF2R/GPR74 receptor (Yin et al., 2005; Yoshida et al., 2003) (for reviews, see Quillet et al. (2016)). Similarly, studies of the binding affinity and efficacy in the signal transduction pathways indicated that RFRPs have a higher affinity for GPR147, whereas NPFF has a potent agonistic activity for GPR74 (Bonini et al., 2000; Hinuma et al., 2000; Liu et al., 2001) (Table 1). Taken together, these studies suggested that GPR147 (NPFF1R, OT7T022) is the functional receptor for RFRPs. Therefore, in this review, we mainly focus on the functional role of RFRP-3 and its receptor GPR147 as well as on their implication in the regulation of LH secretion.

3. Distribution of RFRP-3 neurons

The distribution of RFRP-3 neurons in the hypothalamus has been characterized in various species, including rats (Johnson et al., 2007; Kriegsfeld et al., 2006; Legagneux et al., 2009; Peragine et al., 2017; Rizwan et al., 2009; Yano et al., 2003), hamsters (Gibson et al., 2008; Henningsen et al., 2016b; Kriegsfeld et al., 2006; Ubuka et al., 2012), mice (Kriegsfeld et al., 2006; Ukena and Tsutsui, 2001), sheep (Clarke et al., 2008; Dardente et al., 2008; Qi et al., 2009; Smith et al., 2008), rhesus macaques (Smith et al., 2010; Ubuka et al., 2009a), and humans (Ubuka et al., 2009b) (Table 2). Although there is a slight difference in the distribution of the RFRPs neuron cell bodies, nearly all of these studies suggested that the dorsomedial hypothalamic nucleus (DMN) is the key area where RFRP neuron cell bodies are clustered (Table 2). By contrast, the projections and fibers of RFRP neurons are detected throughout the brain. Interestingly, the fibers of RFRP neurons project to the preoptic area (POA) in primates and paraventricular regions of the hypothalamus in rodents (particularly the anteroventral periventricular nucleus (AVPV)), infundibulum (INF) area in humans and arcuate nucleus (ARC) in nonhuman animals, where many kisspeptin neurons are located (Table 2). Notably, kisspeptin neurons presented in these areas have recently been identified to be involved in steroid-mediated positive and negative feedback regulation of GnRH neurons (Dubois et al., 2015; Goodman et al., 2011; Merkley et al., 2015; Watanabe et al., 2014; Wintermantel et al., 2006; Yeo and Herbison, 2014) (for reviews, see Hrabovszky (2014), Lehman et al. (2010), Ronnekleiv et al. (2015), Shruti and Prevot (2016), Skorupskaite et al. (2014)).

Most GnRH cell bodies are located in the POA, and these cell bodies project neurosecretory axons to the ME, where the GnRH hormone is released by their nerve terminals in the external zone. GnRH further enters the pituitary portal blood system to regulate anterior pituitary function (Schwanzel-Fukuda et al., 1989; Suter et al., 2000). Although several studies have suggested that RFRP neuron fibers project to the ME area (Clarke et al., 2008; Gibson et al., 2008; Ubuka et al., 2009a; Ubuka et al., 2009b), some studies have reported that RFRP neuron fibers were not obviously detected in the ME area (Harbid et al., 2013; Johnson et al., 2007; Rizwan et al., 2009; Smith et al., 2010; Ukena and Tsutsui, 2001). This discrepancy may be partially attributed to the variability in species and sexual differences. For species differences, RFRP neurons scarcely project to the ME area in male or female rats (Johnson et al., 2007; Rizwan et al., 2009), although very low-density fibers were detected in male rats by Yano et al. (2003). For sexual differences, RFRP fibers are nearly absent in the ME area of female rhesus macaques (Smith et al., 2010) and male hamsters (Ubuka et al., 2012), whereas RFRP fibers are extensively appeared in male rhesus macaques (Ubuka et al., 2009a) and female hamsters (Gibson et al., 2008). This discrepancy could also be explained by the different antibodies used for immunostaining of RFRP fibers. The neuroanatomical location of RFRP fibers projecting into the ME area is of great importance as it indicates that RFRP neurons either directly inhibit the release of GnRH by decreasing the activity of GnRH nerve terminals or RFRP neurons release RFRP-3 to the ME area, which affects the function of the gonadotrope cells in the pituitary. In agreement with these studies, fibers of RFRP neurons were identified to project to GnRH neurons in POA as well as kisspeptin neurons in AVPV/RP3V and ARC. Additionally, the functional receptor of RFRP-3 GPR147 is expressed in these nuclei, although results from different studies displayed diverse proportions of neurons that express GPR147 (Poling et al., 2012; Poling et al., 2013; Rizwan et al., 2012) (Table 3). Furthermore, GPR147 receptors are extensively detected in the gonadotrope cells of humans (Ubuka et al., 2009b), hamsters (Gibson et al., 2008), pigs (Li et al., 2013), and sheep (Smith et al., 2012). Collectively, results from the immunochemical analyses strongly suggested that there exist at least three routes by which RFRP-3 control the secretion of LH, namely RFRP-3-kisspeptin-GnRH-LH, RFRP-3-GnRH-LH, and RFRP-3-LH.

At present, the *in vivo* effects of RFRP-3 on LH secretion have been investigated under different conditions (male or female, intact or gonadectomized, prepuberty or adult, and diestrus or proestrus, long

Table 1
Summarization of the names of the peptides and related receptors.

Peptides	Receptors	References
NPAF and NPFF	GPR74, (NPFF2R, HLWAR77)	Bonini et al. (2000), Elshourbagy et al. (2000), Hinuma et al. (2000), Liu et al. (2001), Parker et al. (2000), Yin et al. (2005), Yoshida et al. (2003)
RFRP-1 and RFRP-3	GPR147 (NPFF1R, OT7T022)	Bonini et al. (2000), Elshourbagy et al. (2000), Hinuma et al. (2000), Yin et al. (2005), Yoshida et al. (2003)

For more information about the sequence of these peptides and their binding affinities, the brilliant review from Quillet et al. (2016) is strongly recommended.

Table 2
Summarization of the locations and the projections of RFRP-3 cells in various species.

Distribution	Projections	Species	Reference
DMN	INF; ME; POA	Human	Ubuka et al. (2009b)
DMN, PVN	ME	Ewe	Clarke et al. (2008)
DMN, the lateral superior olive, and the nucleus of the solitary tract	Lateral parabrachial nucleus, the lateral reticular nucleus, and the superficial layer of spinal trigeminal nucleus and dorsal horn of the spinal cord. ME (−)	Mouse (male)	Ukena and Tsutsui (2001)
AH, DMN, premammillary nucleus	Lateral SEP, medial POA, Amg, ARC (+ + +); PVN, central gray (+ +); ME (+/−)	Siberian hamster (Male)	Ubuka et al. (2012)
DMN	ME (extensive); SCN (−)	Syrian hamster (female)	Gibson et al. (2008)
DMN; PVN	DMN; PVN; ARC (+ + +); lateral hypothalamic area, VMH (+)	Ewe	Qi et al. (2009)
DMN; PVN	No data	Ewe	Smith et al. (2008)
PVN, DMN; IPe	ME (−); POA and MBH	Rhesus macaque (female)	Smith et al. (2010)
IPe	POA, PVN, IPe, ARC, ME, dorsal hypothalamic area	Rhesus macaque(male)	Ubuka et al. (2009a)
PVN, DMN	No data	Soay sheep	Dardente et al. (2008)
Tuberal hypothalamus	No data	Rat (Male and female)	Legagneux et al. (2009)
DMN	Hippocampus, POA, PVN, AH, and rostral aspects of the lateral hypothalamus. ME (−)	Rat (Male and female)	Rizwan et al. (2009)
ARC, Lateral hypothalamic area (+ +); other areas of hypothalamus (+)	Mediodorsal thalamic nucleus, lateral posterior thalamic nucleus, ventral postmedial thalamic nucleus. ME (inner layer, lateral) (+)	Rat (male)	Yano et al. (2003)
DMN	ARC, AH, Amg, POA, PVN., SEP	Rat (female), mouse (female), LVG hamster (female)	Kriegsfeld et al. (2006)
Dorsal tuberomammillary nucleus, DMN	Diencephalon, Amg, MPOA, SEP, PVN. VMH (−), ME (−)	Rat (male)	Johnson et al. (2007)
DMH; VMH	POA/OVLT, MPN/AVPV, ARC, SCN, PVN, AH	Syrian hamster (Male and female)	Henningsen et al. (2016)
DMH, ARC	AVPV, BNST, MPOA, ME, NACs, SEP, PVN, Arc	Naked mole rats	Peragine et al. (2017)
DMH; VMH	Thalamus, POA, hypothalamus. ME (−)	Brushtail possum (female)	Harbid et al. (2013)
PVN, DMN	Lateral hypothalamus	Gilt	Thorson et al. (2017)
PVN, DMN	No data	Mare	Thorson et al. (2014)

Abbreviations: Amg, amygdala; INF, infundibulum; POA, preoptic area; MPOA, medial preoptic area; ME, Median eminence; DMH, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; MBH, medial basal hypothalamus; BNST, bed nucleus of the stria terminalis; NACs, nucleus accumbens shell; PVN, periventricular nucleus; SEP, lateral septal nucleus; IPe, intermediate periventricular nucleus; AH, anterior hypothalamic area; + + +, high density; + +, moderate density; +, low density; − not detected.

photoperiod or short photoperiod) in various species (summarized in Table 4). Different effects (negative, positive, or no effects) of RFRP-3 on LH secretion have been reported because of the varied study designs and the various animal models used to obtain these conflicting results. In this review, we have summarized literature from all studies that demonstrated the involvement of RFRP-3 in the control of LH secretion, mainly focus on the inhibitory role of this neuropeptide.

4. Action of RFRP-3 on kisspeptin neurons

Studies using immunohistochemical analyses revealed that in both male and female mice, about 12% of kisspeptin neurons in AVPV express Gpr147 and only a small number (5–6%) of kisspeptin neurons express Gpr74, whereas a relatively higher number (approximately 25%) of kisspeptin neurons in the arcuate nucleus co-express Gpr147 and Gpr74 (Poling et al., 2013). Furthermore, approximately 35% of kisspeptin cells in ARC receive RFRP-3 fiber contacts (Poling et al., 2013). In contrast, kisspeptin fibers did not project to RFRP-3 cells in either male or female mice (Poling et al., 2013), indicating that kisspeptin neurons do not directly signal to RFRP-3 neurons. Therefore, RFRP-3 may directly modulate a subset of kisspeptin neurons in the ARC area, and this action seems to be unilateral.

In female rats and mice, injection of RFRP-3 to the intracerebroventricular (ICV) area significantly decreased the mRNA levels of kisspeptin in the hypothalamus (Han et al., 2017; Xiang et al., 2015). An *in vitro* study confirmed that treatment with RFRP-3 decreased the expression of GnRH mRNA (but not kisspeptin mRNA) in the cultured hypothalamic cells of pigs (Li et al., 2013). In rats, a marked reduction in the activation of a subset of AVPV cells was observed after infusion of RFRP-3 in ICV (Anderson et al., 2009), however whether these AVPV cells contain kisspeptin cells has not been identified. We may speculate that these AVPV cells do not contain many kisspeptin cells, as a recent study indicated that RFRP-3 does not affect the expression of kisspeptin or activity of kisspeptin cells in the AVPV areas of female hamsters (Henningsen et al., 2017). Additionally, GPR147-null male mice displayed increased kisspeptin expression in

Table 3
Summarization of the proportion of GnRH and kisspeptin cells expressing GPR147 and receiving projections from RFRPs neurons.

Neurons	GPR147 expression	Projections receiving	Species	References
GnRH neurons in the POA area	86.1 ± 1.70%	No data	Siberian hamster	Ubuka et al. (2012)
	No data	> 80%	Ewe	Smith et al. (2008)
	No data	> 40%	LVG hamster	Kriegsfeld et al. (2006)
	No data	75 ± 3.2%	Rat	Johnson et al. (2007)
	33%	25–27%	Mouse	Rizwan et al. (2012)
	15%	No data	Mouse	Poling et al. (2012)
Kisspeptin neurons in the AVPV/ RP3V area	9–16%	19%	Mouse	Rizwan et al. (2012)
	12%	No data	Mouse	Poling et al. (2013)
Kisspeptin neurons in the ARC area	25%	35%	Mouse	Poling et al. (2013)

Table 4
Summarization of the *in vivo* effects of RFRP3 injection on LH secretion.

Species	Condition	Injection	Effects	Reference
Rat	Female, adult, OVX	IV	Serum LH levels decreased gradually for 2 h	Murakami et al. (2008)
Rat	Female, adult, OVX	Acute ICV	No change of mean LH concentration, pulse frequency, and pulse amplitude	Murakami et al. (2008)
Rat	Female, adult OVX, low-dose Estradiol	Acute ICV	No change of mean LH concentration, pulse frequency, and pulse amplitude	Anderson et al. (2009)
Rat	Female, adult OVX, high-dose Estradiol	Chronic ICV	LH concentration decreased but not very significant	Anderson et al. (2009)
Rat	Adult, male and female, intact and GNX	Acute ICV	LH concentration decreased	Pineda et al. (2010a,b)
Rat	Adult, male, GNX	IV	LH concentration decreased	Pineda et al. (2010a,b)
Rat	Adult, female OVX	IV	LH concentration not changed	Rizwan et al. (2009)
Rat	Adult, male, intact	Acute ICV	LH concentration decreased	Johnson et al. (2007)
Mouse	Prepubescent, female, intact; Prepubescent, female, OVX, E2 replacement; Adult, female, OVX; Adult, female, OVX, E2 replacement	Acute ICV	LH concentration decreased	Xiang et al. (2015)
Mouse	Prepubescent, female, OVX	Acute ICV	LH concentration not changed	Xiang et al. (2015)
Mouse	Adult, male, intact or castrated	Acute ICV	LH concentration increased	Ancel et al. (2017)
Mouse	Adult, female OVX, mimicking negative feedback	Acute ICV	LH concentration not changed	Ancel et al. (2017)
Mouse	Adult, female, intact, preovulatory LH surge; Adult, female, OVX mimicking preovulatory surge	Acute ICV	LH surge amplitude decreased	Ancel et al. (2017)
Mouse	Adult, intact, male or female, diestrus or proestrus	IP	LH concentration not changed	Ancel et al. (2017)
Human	Postmenopausal women	IV	LH concentration decreased	George et al. (2017)
Cattle	Male, 5 months old, castrated at 3 months of age	IV	LH pulse amplitude and average LH concentration unchanged, LH pulse frequency decreased	Kadokawa et al. (2009)
Syrian hamster	Adult, male, LP	Acute ICV	LH concentration increased	Elhabazi et al. (2017)
Syrian hamster	Adult, male, LP and SP	Acute ICV	LH concentration increased	Ancel et al. (2012)
Syrian hamster	Adult, female, OVX, LP	Acute ICV	LH concentration not changed	Ancel et al. (2012)
Syrian hamster	Adult, male, LP and SP	IP	Basal LH levels decreased but not very significant	Ancel et al. (2012)
Siberian hamster	Adult, male, LP	Acute ICV	LH concentration decreased	Ubuka et al. (2012)
Siberian hamster	Adult, male, SP	Acute ICV	LH concentration increased	Ubuka et al. (2012)
LVG hamster	Adult, female, OVX	Acute ICV and IP	LH concentration decreased	Kriegsfeld et al. (2006)
Syrian hamster	Adult female, intact, SP; Adult female, intact, LP, diestrus and the morning or midday or LH surge	Acute ICV	LH concentration not changed	Henningsen et al. (2017)
Syrian hamster	Adult, female, intact, LP, injected 30 min before LH surge	Acute ICV	LH surge amplitude decreased	Henningsen et al. (2017)
Syrian hamster	Adult, female, intact, SP	Chronic ICV	LH concentration increased	Henningsen et al. (2017)
Syrian hamster	Adult, female, intact, LP	Chronic ICV	LH concentration decreased	Henningsen et al. (2017)
Ewe	Adult, OVX	IV	LH pulse amplitude decreased	Clarke et al. (2008)
Ewe	OVX, anestrous	Acute ICV and IV	LH concentration not changed	Caraty et al. (2012)
Ewe	OVX	IV	LH pulse amplitude decreased; LH pulse frequency or mean LH concentration unchanged	George et al. (2017)
Ewe	Intact, acyclic, nonbreeding season	IV	LH concentration not changed	Decourt et al. (2016)
Ewe	OVX, estrogen-induced LH surge, breeding season	IV	LH concentration not changed	Decourt et al. (2016)
Ewe	Intact	IV	LH pulse amplitude and frequency decreased	Clarke et al. (2012)
Ewe	OVX, estrogen induced LH surge	IV	LH concentration decreased	Clarke et al. (2012)
Mare	Intact, mature, breeding season	IV	LH pulse amplitude, frequency and concentration unchanged	Thorson et al. (2014)
Gilt	OVX, prepuberty	ICV and IV	LH pulse amplitude, frequency and concentration unchanged	Thorson et al. (2017)

Abbreviations: LP, long photoperiod; SP, short photoperiod; OVX, ovariectomized; GNX, gonadectomized; IV, intravenous injection; ICV, Intracerebroventricular injection; IP, intraperitoneal injection.

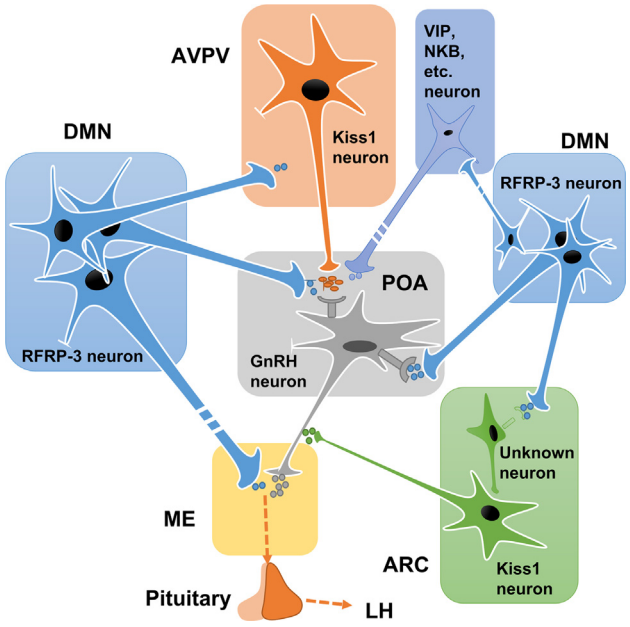

Fig. 1. Schematic diagram summarizing potential roles of RFRP-3 in regulating the secretion of LH. RFRP-3 cells in the DMN area project to various areas in the hypothalamus, including the AVPV, ARC, and POA areas. Contradictory results exist in the projection to the ME area. RFRP-3 indirectly regulates the c-Fos level of kisspeptin neurons in the ARC area but not in the AVPV area. Apart from directly inhibiting the activation of GnRH neuron, RFRP-3 may also affect the signaling to GnRH neurons from other neuronal fibers, possibly from kisspeptin neurons and VIP neurons. Abbreviations: ME, median eminence; POA, preoptic area; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; DMN, dorsomedial hypothalamic nucleus. RFRP-3, RFamide-related peptides; VIP, vasoactive intestinal polypeptide; NKB, neurokinin B.

the ARC area, higher serum FSH levels, and enhanced LH responses to GnRH (Leon et al., 2014), indicating that RFRP-3 may have an inhibitory effect on the expression and activity of kisspeptin. Indeed, local co-administration of RFRP-3 and kisspeptin in the POA area resulted in the suppression of GnRH release, while single administration of RFRP-3 failed to block kisspeptin-induced GnRH release in the ME (Glanowska and Moenter, 2015). These results suggested that RFRP-3 is effective at inhibiting the secretion of kisspeptin in the POA but not in the ME. Fibers of kisspeptin neurons in the ARC are in close contact with GnRH nerve terminals in the ME and have been proposed to stimulate GnRH release without involving the cell bodies of GnRH neurons (Shruti and Prevot, 2016; Yip et al., 2015). Therefore, RFRP-3 may inhibit kisspeptin-induced secretion of GnRH in the AVPV, which occurs mainly in the cell bodies of GnRH neurons (Fig. 1).

However, a recent study suggested that acute ICV injections of RFRP-3 modified neither the number of kisspeptin neurons nor the percentage of activated kisspeptin neurons in female Syrian hamsters (Henningsen et al., 2017). Interestingly, ICV administration of RFRP-3 in male Syrian hamster induces the activation of GnRH neurons and certain non-kisspeptinergic neurons (unidentified neurons) in the ARC (Ancel et al., 2012), suggesting that RFRP-3 stimulates gonadotrophin secretion in male Syrian hamster is not due to the activation of kisspeptin neurons in the ARC. Central injection of RFRP-3 in both intact and castrated male mice had a dose-dependent stimulatory effect on LH secretion, and this effect was diminished in kisspeptin receptor knockout mice, suggesting that the stimulating effects of RFRP-3 are partially dependent on the occurrence of kisspeptin/Kiss1R signaling, at least in male mice (Ancel et al., 2017). The discrepancy of RFRP-3 on the effect of kisspeptin/Kiss1R signaling could be attributed to the seasonal characteristics of the animal model, as RFRP-3 may have contrary effects on kisspeptin expression in both AVPV and ARC area during the short and long photoperiods (Henningsen et al., 2017). Similarly, RFRP-3 may have contrary effects on LH secretion in seasonal controlled animals (Ubuka et al., 2012) (reviewed by (Henningsen et al., 2016a; Kriegsfeld et al., 2015; Weems et al., 2015)). Taken together, the RFRP-3-induced actions on kisspeptin neurons are species- and sex-dependent. Future studies aimed at addressing the functional roles of RFRP-3 in regulating HPG by using animal models across a number of species in both male and female sexes to investigate the action of RFRP-3 on kisspeptin neurons will be of great interest. Notably, the fibers of RFRP neurons and expression of GPR47 are widely distributed in the central nervous system (Bonini et al., 2000; Henningsen et al., 2016b). Additionally, ICV administration of RFRPs induced the expression of c-Fos in many areas of the brain (Yano et al., 2003). To the best of our knowledge, whether RFRP-3 could affect the activation of GnRH neurons and GnRH secretion through other upstream factors, such as vasoactive intestinal polypeptide (VIP), neurokinin B (NKB), neuropeptide Y, dynorphin, and substance P (Fergani et al., 2016; Goodman et al., 2014; Maggi et al., 2016), has never been investigated. Therefore, further studies are needed to identify whether the upstream regulators of GnRH neurons, in addition to kisspeptin neurons, are involved in the modulation of GnRH neurons by RFRP-3.

5. Action of RFRP-3 on GnRH neurons

The percentage of GnRH neurons in the POA that express GPR147 or receive projections from RFRP neurons are dependent on animal models used for studies, with a relatively small percentage in mice compared to those in hamsters, rats, and sheep (Table 3). Like the various effects of RFRP-3 on kisspeptin neurons, RFRP-3 also exerts paradoxical effects on the activation of GnRH neurons or the secretion of GnRH in different types of animals. Several lines of evidence have suggested that RFRP-3 has inhibitory effect on the activity of GnRH neurons or GnRH secretion. Firstly, the GnRH expression and GnRH neuronal activation is significantly suppressed following the ICV injection of RFRP-3 in female rats (Anderson et al., 2009; Han et al., 2017) and female mice (Xiang et al., 2015). Secondly, an *in vitro* study in mice indicated that RFRP-3 might exhibit a rapid and repeatable inhibitory effect on the firing rate of 41% of GnRH neurons, with no difference detected in male, diestrus, or proestrus female mice (Ducret et al., 2009). Furthermore, the suppressive effect of RFRP-3 on the firing rate of GnRH neurons was maintained when the signal of neurotransmitters was blocked (Ducret et al., 2009), indicating that RFRP-3 directly acts on GnRH neurons. Thirdly, a brief 15-s application of RFRP-3 produced a strikingly non-desensitizing hyperpolarization in GnRH neurons. This action can block kisspeptin-induced activation of vesicular glutamate transporter 2 in the GnRH neurons (Wu et al., 2009), which is related to the release of GnRH at the nerve terminals of the ME (Kawakami et al., 1998a; Kawakami et al., 1998b). Fourthly, in a GnRH neuronal cell model mHypoA-GnRH/GFP, the mRNA expression of GnRH was attenuated by approximately 60% when cells were incubated with 100 nM of RFRP-3 (Gojska et al., 2014). In murine hypothalamic cells, mouse RFRPs (mRFRPs) significantly suppressed the stimulatory effect of kisspeptin on GnRH release and GnRH mRNA levels (Son et al., 2016). However, in a GnRH neuronal cell line, GT1-7 (Son et al., 2016), mRFRPs had no inhibitory effect on kisspeptin-induced signaling pathways, including the phosphorylation of extracellular signal-regulated kinase (ERK) and Ca^{2+} release that is indispensable for kisspeptin-induced secretion of GnRH (Glanowska and Moenter, 2015; Sukhbaatar et al., 2013). This *in vitro* functional study does not support the directly inhibitory effect of mRFRPs on kisspeptin signaling in GnRH neurons. However, it should be noted that the *in vitro* system using a GT1-7 cell line is not totally representative of the *in vivo* GnRH neurons. Interestingly, vasoactive intestinal polypeptide (VIP) has a stimulatory effect on the release of GnRH secretion in female but not in male mice, which can be significantly suppressed by cotreatment with mRFRP-3 (Son et al., 2016). Additionally, the same study also

indicated that mRFRP-3 may exert inhibitory effects on VIP-induced expression of c-Fos mRNA and release of GnRH *via* the AC/cAMP/PKA-specific pathway in GT1-7 cells (Son et al., 2016). In female rats, approximately 40% of all GnRH neurons, including those in the POA area, contain VIP2 receptor immunoreactivity. Additionally, VIP neuron fibers are in close apposition to a large number of VIP2 receptor-positive GnRH neurons (Smith et al., 2000). Furthermore, a recent study suggested that VIP directly induced the firing of GnRH neurons in both male and female mice (Piet et al., 2016). Taken together, these studies indicate that mRFRP-3 may directly inhibit the VIP-induced signaling pathway in GnRH neurons, which suppresses the stimulatory effect of VIP on GnRH secretion.

Intriguingly, some studies suggested that RFEP-3 may not always exert inhibitory effect on the GnRH neurons. In male Syrian hamsters, either long photoperiod or short photoperiod, acute central injection of RFRP-3 induced the expression of c-Fos in GnRH neurons and increased the mean LH concentration (Ancel et al., 2012; Elhabazi et al., 2017). Whereas, intraperitoneal injection of RFRP-3 had no significant effect on LH secretion, indicating that RFRP-3 regulates LH secretion by affecting the activity of GnRH neurons rather than that of gonadotrope cells, at least in male Syrian hamsters. In female Syrian hamsters of long photoperiod, acute ICV injection of RFRP-3 did not affect the concentration of LH except when RFRP-3 was injected 30 min before the time of LH surge, which significantly decreased the amplitude of LH surge (Henningsen et al., 2017). Additionally, both acute ICV injection and intraperitoneal injection of RFRP-3 decreased LH secretion in female LVG hamsters (Kriegsfeld et al., 2006), suggesting that peripheral RFRP-3 could affect gonadotrope cells directly in this type of hamsters. Interestingly, acute ICV injection of RFRP-3 had an opposite effect on LH secretion in male Siberian hamsters of both long photoperiod and short photoperiod (Ubuka et al., 2012) (Table 4). Obviously, photoperiod was an indispensable factor affecting the effect of RFRP-3 on GnRH/LH secretion in hamsters. Indeed, chronic ICV injection of RFRP-3 inhibited LH secretion in female Syrian hamster of long photoperiod, whereas the same injection reactivated the reproductive axis in female Syrian hamster of short photoperiod (Henningsen et al., 2017). In addition, an *in vitro* study in female pubertal pigs showed that RFRP-3 promoted the expression of GnRH and its release in hypothalamic cells (Li et al., 2013), indicating that the stimulatory effect of RFRP-3 on reproductive axis is not limited to seasonal breeders. However, another study in female prepubertal pigs suggested that neither ICV nor IV injection of RFRP-3 affected the secretion of LH (Thorson et al., 2017). The inconsistency of these two studies may be attributed to the different ages of pigs used, as the number and the activity of GnRH neurons are different between prepubertal and pubertal animals (Herbison, 2016). A study in female rats showed that the mean level of LH concentration and frequency of the pulsatile LH secretion were not affected after ICV injection of RFRP-3, suggesting that RFRP-3 does not affect LH secretion via the release of GnRH (Murakami et al., 2008). Obviously, finding of this study is in contrast to the inhibitory role of RFRP-3 in GnRH neurons identified in female rats (Anderson et al., 2009; Han et al., 2017). The discrepancy may be attributed to the ovariectomized mature rat model used by Murakami et al. because the ovariectomized mature rats in the study by Anderson et al. were subjected to an exogenous estrogen-induced GnRH/LH surge protocol, whereas the rats in the study by Han et al. were prepubescent without being ovariectomized. In agreement, the inhibitory effects of RFRP-3 on the HPG axis are closely related to the levels of estradiol and maturation of animals as shown in mice (Xiang et al., 2015).

In summary, the effect of RFRP-3 on GnRH neurons and the subsequent LH secretion is dependent on many factors, including animal species, gender, age, estrogen level, and photoperiod. Future studies aimed at comprehensively investigating the role of RFRP-3 in reproduction should take all these factors into consideration.

6. RFRP-3 suppresses GnRH signaling in the gonadotrope

The suppressive effect of RFRPs on the secretion of LH induced by GnRH has also been largely identified. The GPR147 receptor is highly expressed in pituitary glands, especially in gonadotrope cells of ewes (Smith et al., 2012), female hamsters (Gibson et al., 2008), and humans (Ubuka et al., 2009b). Additionally, RFRP neuron fibers have been shown to project to the ME area in several species (Clarke et al., 2008; Gibson et al., 2008; Ubuka et al., 2009a; Ubuka et al., 2009b), though no fiber projected to the ME area has been detected in mice and possums (Harbid et al., 2013; Ukena and Tsutsui, 2001). In ewes, the pulsatile secretion of RFRP-3 was identified in the hypophyseal portal blood, although the RFRP-3 peptide was virtually undetectable in peripheral blood plasma. Peripheral administration of RFRP-3 decreased GnRH-stimulated LH release in ovariectomized rats (Rizwan et al., 2009) and ewes (Smith et al., 2012), whereas it did not have any effect on the basal LH secretion (Rizwan et al., 2009). In pituitary cells isolated from female rats (Murakami et al., 2008), mature female cattle (Kadokawa et al., 2009), female pubertal pigs (Li et al., 2013) and ewes (Clarke et al., 2008; Sari et al., 2009), the suppressive effects of RFRP-3 on LH secretion or LHβ mRNA expression occurred only in the situation that GnRH coexists in pituitary cells. In adult GPR147-null male mice, the response of LH to GnRH was significantly enhanced (Leon et al., 2014). However, studies in OVX female rats (Anderson et al., 2009) and male hamsters (Ancel et al., 2012) showed that treatment with RFRP-3, either in the presence or the absence of GnRH, did not have any significant inhibitory effect on the secretion of LH in isolated anterior pituitary cells. Additionally, neither equine RFRP-3 nor ovine RFRP-3 could reduce adenohypophyseal responsiveness to GnRH in mares during the winter anovulatory period (Thorson et al., 2014). These inconsistent results may be attributed to the sexual differences and inter-species variations. We may speculate that the expression level of the receptor for RFRP-3 is relatively low, which attenuates the response and activity of RFRP-3 to inhibit GnRH-induced secretion of LH.

In gonadotrope cells, the low pulse frequency of GnRH (diestrus phase in rodents) seems to up-regulate the transcription of both LHβ and FSHβ by binding to the $G_{\alpha q}$/11. During the high pulse frequency of GnRH (proestrus phase in rodents), GnRHR engages $G_{\alpha s}$ apart from $G_{\alpha q}$/11 subfamily proteins (reviewed by Coss (2017), Maggi et al. (2016)). Using $G_{\alpha s}$ siRNA (small interfering RNA) approaches and stimulatory experiments in LβT2 gonadotrope cells and using a $G_{\alpha s}$ specific activator in mouse gonadotrope cells, studies have shown that the up-regulation of LHβ expression induced by GnRH occurred mainly via the $G_{\alpha s}$ subfamily proteins (Choi et al., 2012). The principle function of $G_{\alpha s}$ is to activate the adenylyl cyclase, which in turn, promotes the production of cAMP and activates the cAMP-dependent protein kinase. cAMP-dependent protein kinase subsequently activates ERK1/2 signaling and induces the mobilization of intracellular Ca^{2+}, both of which are indispensable for the up-regulation of LHβ expression induced by GnRH in pituitary cells (Bliss et al., 2009; Liu et al., 2002). Using overexpression technique, Hinuma and colleagues found that GPR147 can couple to G proteins $G_{\alpha i}$ but not to $G_{\alpha q}$ (Hinuma et al., 2000). Clarke et al. found that ovine RFRP-3 potently inhibited the generation of intracellular free Ca^{2+} elicited by GnRH in the gonadotrope cells (Clarke et al., 2008). Additionally, RFRP-3 significantly abolished the phosphorylation of ERK1/2 signaling stimulated by GnRH in pituitary cells isolated from gonadectomized ewes, rams, (Sari et al., 2009) and pigs (Li et al., 2013). Consistent with these results, in a mouse gonadotrope cell line, LβT2, mRFRPs effectively inhibited GnRH-induced LH secretion through the inhibition of cAMP signaling and ERK phosphorylation, which was mediated by the inhibition of the protein kinase A pathway (Son et al., 2012). The same study group demonstrated an inhibitory effect of mRFRP-3/GPR147 on the activation of the adenylate cyclase/cAMP/protein kinase A pathway in a mouse GnRH neuronal cell line (Son et al., 2016). Taken together, the results of these studies strongly suggested that RFRP-3/GPR147

functions via the inhibition of the adenylate cyclase/cAMP/protein kinase A pathway in both GnRH neurons and gonadotrope cell lines. However, whether such inhibitory effect on the adenylate cyclase/cAMP/protein kinase A pathway induced by RFRPs also exists in the *in vivo* GnRH neurons and gonadotropes remains to be determined. Using RF9 (initially considered an antagonist of GPR147), several studies have shown that RFRP-3 could not inhibit the secretion of gonadotropins independent of GnRH (Caraty et al., 2012; Pineda et al., 2010b; Rizwan et al., 2012). However, later studies have shown that RF9 is an agonist (rather than an antagonist) of kisspeptin receptor that in fact promotes the secretion of GnRH (Kim et al., 2015; Liu and Herbison, 2014; Min et al., 2015). Consequently, it is unsurprising that GnRH is involved in the stimulatory effect of RF9 on the secretion of gonadotrophins. Therefore, previous studies could hardly provide extra evidence towards the involvement of GnRH in RFRP-3-induced regulation of gonadotropin secretion. Recently, two novel antagonists of RFRP-3, RF313 and GJ14, were designed to exhibit a selectively antagonistic activity against GPR147 both *in vitro* and *in vivo* (Bihel et al., 2015; Elhabazi et al., 2017; Kim et al., 2015). Most likely, the specific antagonists of RFRP-3 will be more useful to investigate the role of RFRP-3 in regulating the secretion of LH and the underlying molecular mechanisms in future studies.

7. Direct effect of RFRP-3 on the secretion of LH in the pituitary

It is less likely that RFRP-3 directly affects the secretion of LH in the gonadotrope cells from the pituitary. In cultured pituitary cells isolated from female rats (Murakami et al., 2008), mature female cattle (Kadokawa et al., 2009), female pubertal pigs (Li et al., 2013) and ewes (Clarke et al., 2008; Sari et al., 2009), the suppressive effects of RFRP-3 on LH secretion or LHβ mRNA expression occurred only in the situation that GnRH coexists in the gonadotrope cells. However, RFRP-3 could inhibit LH secretion in the absence of GnRH in the pituitary tissues obtained from gonadectomized male rats (Pineda et al., 2010a). The concentration of RFRP-3 (10^{-8} M) that can inhibit LH secretion in the gonadectomized male rats was much higher (approximately 10,000 times) than its physiological concentration (10^{-12} M), as shown in rats (Murakami et al., 2008) and pig (Li et al., 2013). However, a lower concentration (less than 10^{-10} M) does not affect LH secretion (Pineda et al., 2010a), casting doubt on the physiological concentrations that induce such an inhibitory effect on LH secretion in the absence of GnRH. Additionally, sexual differences and the existence of gonads may contribute to the inconsistency. However, the concentration of RFRP-3 (10^{-8}M) could not inhibit LH secretion in other animals (Clarke et al., 2008; Kadokawa et al., 2009; Li et al., 2013) (Thorson et al., 2017), indicating that species variation was also related to the contradictory effect of RFRP-3 on LH secretion. Therefore, it is unsurprising that studies have not shown a negative association between secretory pulses of GnIH-3 (portal) and peripheral LH levels (Smith et al., 2012), because the suppressive effect of RFRP-3 on LH secretion is dependent on the existence of GnRH.

8. Timely reduction of RFRP-3 signaling may be involved in the modulation of the preovulatory LH surge

In mammals, estrogen triggers the preovulatory LH surge at the end of the follicular phase. During proestrus phase in female rats and hamsters, the expression of RFRP-3 in the hypothalamus or activation of RFRP cells was lower than that in the diestrus phase (Gibson et al., 2008; Jorgensen et al., 2014; Salehi et al., 2013). In agreement, the number of RFRP-3 cells in the DMH area was reduced in the late follicular phase compared with that in the luteal phase of ewes (Clarke et al., 2012; Jafarzadeh Shirazi et al., 2014). Additionally, during the LH surge in female hamsters and mice, the number of c-Fos-positive RFRP-3 neurons was significantly reduced (Gibson et al., 2008; Henningsen et al., 2017; Poling et al., 2017). In addition, activation of RFRP cells was reinstated shortly after the end of the LH surge, and this process is estradiol-dependent (Gibson et al., 2008). These studies showed that the onset and end of the LH surge are accompanied by the synchronous changes in RFRP neuronal activation. Probably, RFRP-3 neurons play an indispensable role in modulating the amplitude of the LH surge or overall duration of the surge. Indeed, acute ICV administration of RFRP-3 significantly decreased the amplitude of LH surge when given before the LH surge in female Syrian hamsters (Henningsen et al., 2017) and mice (Ancel et al., 2017). Additionally, the estrogen-induced surge in LH secretion was suppressed by the intravenous injection of RFRP-3 in ewes (Clarke et al., 2012). However, kisspeptin-induced LH responses in humans and ewes were not affected by IV injection of RFRP-3 (Decourt et al., 2016; George et al., 2017). Notably, whether RFRP-3 could affect LH secretion without kisspeptin administration in humans has not been identified, although it has been identified in postmenopausal women (George et al., 2017). Additionally, the sexual differences in the study should not be ruled out because studies in hamsters have shown that acute central injection of RFRP-3 had a stimulatory effect on the reproductive axis in males (Ancel et al., 2012; Simonneaux et al., 2013) and an inhibitory effect on the reproductive axis in females (Henningsen et al., 2017). However, it is difficult to explain the phenomenon in ewes. Interestingly, in rhesus macaques, the expression of RFRP-3 was higher in the late follicular phase than in the luteal phase (Smith et al., 2010). To the best of our knowledge, this is the only study that investigated the expression of RFRP-3 across the menstrual cycle in primates. Future studies are necessary to investigate the reason for the difference in the expression of RFRP-3 across the menstrual cycle between primates and other mammals.

At present, the molecular mechanisms underlying the transient inhibition of RFRP-3 neuronal activation, changes in the number of the RFRP-3 neurons, and differential gene expression across the menstrual cycle have not been clearly elucidated. It seems that the estrogen levels may partially contribute to the difference because estrogen receptor α (ERα) is expressed in RFRP neurons in mice and hamsters (Kriegsfeld et al., 2006; Molnar et al., 2011; Poling et al., 2012). Additionally, treatment with E2 significantly decreased the total amount of RFRP mRNA in the DMN and overall relative amount of RFRP mRNA per cell (Molnar et al., 2011; Poling et al., 2012). However, the coexpression of ERα does not significantly differ between male and female mice (Molnar et al., 2011; Poling et al., 2012). Furthermore, some studies have suggested that E2 treatment did not alter the number of RFRP neuronal cells, the cellular content of RFRP mRNA (Gojska and Belsham, 2014; Smith et al., 2008), or activation of RFRP neurons and their RFRP-3 expression (Iwasa et al., 2012; Kriegsfeld et al., 2006). In addition to the effects of estrogen, the influence of the circadian clock signal from the hypothalamic suprachiasmatic nuclei (SCN) should be taken into account. Unlike male hamsters, which show neither circadian nor day-to-night variations in the mRNA expression of RFRP and neuron activation of RFRP-3 (Revel et al., 2008; Saenz de Miera et al., 2014), female hamsters and mice show a significantly reduced number of c-Fos-positive RFRP-3 neurons around the time of the LH surge, although the mRNA level was not significantly changed (Gibson et al., 2008; Henningsen et al., 2017; Poling et al., 2017). Therefore, the sexual differences in the circadian activation of RFRP neurons may contribute to the sexually dimorphic basis of the LH surge. In agreement, marked fiber projections from the SCN to the RFRP cells (around 20.4–48.3%) have been identified in female hamsters (Gibson et al., 2008). Another study in female hamsters also showed that RFRP-3 cells receive close appositions from SCN-derived vasopressin (AVP) and VIP terminal fibers, and the injection of VIP, but not AVP, markedly reduced the percentage of RFRP-3/c-Fos-labeled cells in the afternoon but not in the morning (Russo et al., 2015). These results indicated that SCN-derived VIP may be related to the temporally reduced activation of RFRP neurons, and may reduce the release of RFRP-3 around the time of the LH surge. It should also be noted that the activation of RFRP neurons is also regulated by stress and photoperiod (for reviews, see

Henningsen et al. (2016a), Iwasa et al. (2017)). Whether these factors are involved in the temporal RFRP-3 mRNA expression and RFRP neuronal activation during the time of the LH surge remains to be further investigated.

9. Conclusion

The work of the last decade has greatly expanded our knowledge concerning the roles of RFRP-3 in regulating the secretion of LH. This review comprehensively discussed the effects of RFRP-3 on LH secretion at different levels, including kisspeptin neurons, GnRH neurons, and the pituitary. However, the conflicting results regarding the effects (no effect, inhibition, or promotion) of RFRP-3 on LH secretion and the activation of RFRP-3 neuronal cells across the menstrual cycle need to be further elucidated. Timely reduction of RFRP-3 signaling may be involved in the modulation of the preovulatory LH surge. Notably, the avenues opened by the antagonists, RF313 and GJ14, will be helpful in providing more evidence on the multifaceted role of RFRP-3 in the control of LH secretion.

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