Effects and Therapeutic Potentials of Kisspeptin Analogs: Regulation of the Hypothalamic-Pituitary-Gonadal Axis

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Key Words

Kisspeptin · KISS1R · Kisspeptin analog · Testosterone · Desensitization

Abstract

The hypothalamic peptide kisspeptin (metastin), the endogenous ligand of the G protein-coupled receptor KISS1R, plays a critical role in controlling GnRH release from hypothalamic GnRH neurons and thereby regulates hypothalamic-pituitary-gonadal functions. Although the therapeutic potential of kisspeptin is attractive, its susceptibility to proteolytic degradation limits its utility. To overcome this, KISS1R agonists or antagonists as peptide analogs or small molecules have been investigated. Kisspeptin analogs have been most extensively studied by reducing the length of the peptide from the original 54 amino acids to 10 amino acids or less and by substituting key amino acid residues. Also, 2 investigational kisspeptin agonist analogs have been evaluated in clinical studies in men; in agreement with animal studies, abrupt elevations in gonadotropin and testosterone levels were observed as an acute effect, followed by rapid reductions in these hormones as a chronic effect. Some studies of small-molecule KISS1R antagonists have also been pub-

lished. In this review, we present a brief overview on kisspeptin/KISS1R physiology in reproductive functions and summarize the available knowledge of both agonists and antagonists. We also focus on the kisspeptin agonist analogs by summarizing key pharmacological findings from both clinical and preclinical studies, and discuss their potential therapeutic utility.

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Introduction

Kisspeptin (Kp) is encoded by the *KISS1* gene and is a peptide ligand of the G protein-coupled receptor KISS1R (GPR54) [1–3]. The physiological roles of the Kp/KISS1R system in mammalian reproductive functions were first demonstrated in 2003–2004. Defective onset of puberty was observed to be associated with mutation of *KISS1R* in humans or deletion of *Kiss1r* in mice [4–6]. Moreover, central or peripheral Kp administration to several mammalian species, including humans, induced robust release of GnRH and gonadotropin [7–12]. Subsequent studies have revealed that GnRH neurons express *Kiss1r* mRNA and are depolarized by Kp exposure in vitro [13–15], sug-

gesting that Kp directly regulates GnRH neurons. Anatomical investigations, most extensively in rodents, have revealed two populations of Kp neurons: one is within the arcuate nucleus (ARC) close to the median eminence and the other is around the anterior hypothalamic region, especially in the anteroventral periventricular nucleus (AVPV) [16–18]. In rodents, ARC Kp neurons are common to both males and females, and *Kiss1* mRNA expression is negatively regulated by steroid hormones [16–19]. In contrast, AVPV Kp neurons clearly show sexual dimorphism and are abundant in females, and *Kiss1* mRNA expression is positively regulated by estradiol [16–18]. Recent findings strongly suggest that ARC Kp neurons are critical for GnRH pulses [20–24], whereas AVPV Kp neurons regulate preovulatory GnRH/LH surges [18, 19, 25]. Although anatomical differences have been observed across species (detailed discussion of the neuroanatomy of kisspeptin system in human is present in this special issue by Hrabovszky [26]), it is now widely accepted that Kp plays a pivotal role in regulating both GnRH pulses and surges in mammals [27]. Although Kp acts within the hypothalamus, several studies showed that peripheral Kp administration can stimulate GnRH neurons [7, 9–12], presumably via circumventricular organs such as median eminence or the organum vasculosum of the lamina terminalis [28–30], thus offering attractive therapeutic targets.

The *KISS1* gene produces a 145-amino-acid polypeptide, which is then enzymatically processed into Kp-54, a C-terminally amidated 54-amino-acid peptide [1–3]. Ohtaki et al. [1] evaluated agonistic and receptor-binding activities of several N-terminal truncated peptides in vitro by $[Ca^{2+}]_i$ mobilization assays using human KISS1R-expressing CHO cells and competitive binding analyses using membrane fractions prepared from the CHO cells, respectively, and revealed that the C-terminal 10-amino acid peptide Kp-10, Tyr⁴⁵-Asn⁴⁶-Trp⁴⁷-Asn⁴⁸-Ser⁴⁹-Phe⁵⁰-Gly⁵¹-Leu⁵²-Arg⁵³-Phe⁵⁴-NH₂ (the superscripted numbers indicate the position of the amino acid residues based on Kp-54), has 3- to 10-fold more potent in vitro agonistic and receptor-binding activity than the Kp-54 against human KISS1R [1]. This 10-amino-acid sequence is well conserved among several species, including nonhuman primates, rodents, domestic animals, frogs and fish; in most mammals, except for primates, the RY-amide structure rather than the RF-amide structure has been identified (table 1). Although Kp-10 administration induces gonadotropin release in vivo, its susceptibility to enzyme cleavage potentially limits its utility [31].

KISS1R Agonists

Both peptide and small-molecule approaches have been studied. Kuohung et al. [32] established a high-throughput screening system to evaluate both small-molecule agonists and antagonists and identified a potential agonist for KISS1R out of 110,000 compounds screened. However, its in vitro EC₅₀ value is more than 0.1 mM and the GnRH-releasing activity in vivo remains to be confirmed. On the other hand, short-form peptide agonist analogs of Kp-10 have been reported by us and other investigators [31, 33–35].

Kp Agonist Analogs with Improved Biological Stability and Potency

We initially found that more than 50% of Kp-10 was rapidly metabolized in mouse serum within 1 min, and using high-performance liquid chromatography/electrospray ionization mass spectrometry coupling experiments, we found two major peptide fragments predominating because of cleavage of Tyr⁴⁵-Asn⁴⁶ and Arg⁵³-Phe⁵⁴ by serum proteases, respectively [31].

Arg⁵³-Phe⁵⁴

Arg-containing peptides are well-known substrates for trypsin-like proteases [36–38], although the substitution of Arg⁵³ with natural amino acids such as L-Ala or L-Leu or the corresponding D-amino acid D-Arg decreased in vitro agonistic activity [31]. Therefore, guanidino-*N*-alkyl (*N*^ω-alkyl) arginine derivatives, which retain the positive charge of Arg and exhibit various lipophilic and electrostatic characteristics, were then evaluated by substituting Arg⁵³. These experiments indicated that the *N*^ω-methylarginine analog showed 3-fold more potent in vitro agonistic activity in $[Ca^{2+}]_i$ mobilization assays than Kp-10, with resistance to trypsin cleavage between positions 53 and 54 [31].

Tyr⁴⁵-Asn⁴⁶

It is widely recognized that incorporation of D-amino acids improves the biological potencies of peptides by altering conformational properties and increasing the resistance to enzymatic degradation [37, 38]. Indeed, Kp-10 derivatives with D-Tyr⁴⁵ showed acceptable activity and evaded N-terminal degradation, and the residual ratio of an analog with D-Tyr⁴⁵ and Arg(Me)⁵³ was highly improved to 18.1% after 1 h of incubation in mouse serum. However, several metabolites were still observed in the

Table 1. Alignment of putative Kp-10 amino acid sequences of *KISS1* and *KISS2* gene products

Species name	C-terminal 10 amino acid residues										GenBank No.
KISS1 gene product											
<i>Homo sapiens</i> (human)	Y	N	W	N	S	F	G	L	R	F	AAC79512.1
<i>Macaca mulatta</i> (rhesus monkey)	Y	N	W	N	S	F	G	L	R	F	XP_001098284.2
<i>Pan troglodytes</i> (chimpanzee)	Y	N	W	N	S	F	G	L	R	F	AA05977.1
<i>Gorilla gorilla gorilla</i> (western lowland gorilla)	Y	N	W	N	S	F	G	L	R	F	XP_004028270.1
<i>Sus scrofa</i> (pig)	Y	N	W	N	S	F	G	L	R	Y	BAH47553.1
<i>Bos taurus</i> (cattle)	Y	N	W	N	S	F	G	L	R	Y	BAM28696.1
<i>Capra hircus</i> (goat)	Y	N	W	N	S	F	G	L	R	Y	BAH89066.1
<i>Ovis aries</i> (sheep)	Y	N	W	N	S	F	G	L	R	Y	AFW03832.1
<i>Felis catus</i> (domestic cat)	Y	N	W	N	S	F	G	L	R	Y	XP_003999526.1
<i>Mus musculus</i> (house mouse)	Y	N	W	N	S	F	G	L	R	Y	BAM11250
<i>Rattus norvegicus</i> (Norway rat)	Y	N	W	N	S	F	G	L	R	Y	EDM09773.1
<i>Suncus murinus</i> (house shrew)	Y	N	R	N	S	F	G	L	R	Y	BAL02985.1
<i>Xenopus laevis</i> (African clawed frog)	Y	N	W	N	S	F	G	L	R	Y	BAI50705.1
<i>Danio rerio</i> (zebrafish)	Y	N	L	N	S	F	G	L	R	Y	ABV03802.1
<i>Carassius auratus</i> (goldfish)	Y	N	L	N	S	F	G	L	R	Y	ACI96030.1
<i>Oryzias latipes</i> (Japanese medaka)	Y	N	L	N	S	F	G	L	R	Y	BAF98208
<i>Cyprinus carpio</i> (common carp)	Y	N	L	N	S	F	G	L	R	Y	AFM08413.1
<i>Dicentrarchus labrax</i> (European seabass)	Y	N	L	N	S	F	G	L	R	Y	ACM07422.1
KISS2 gene product											
<i>Danio rerio</i> (zebrafish)	F	N	Y	N	P	F	G	L	R	F	ACJ50541.1
<i>Carassius auratus</i> (goldfish)	F	N	Y	N	P	F	G	L	R	F	ACS34769.1
<i>Oryzias latipes</i> (Japanese medaka)	F	N	Y	N	P	F	G	L	R	F	BAG86623.1
<i>Cyprinus carpio</i> (common carp)	F	N	Y	N	P	F	G	L	R	F	AFM08414.1
<i>Dicentrarchus labrax</i> (European seabass)	F	N	F	N	P	F	G	L	R	F	ACM07423.1

Conserved amino acid residues are in bold.

electrospray ionization mass spectrometry spectra, including products of cleavage between Trp⁴⁷-Asn⁴⁸, Phe⁵⁰-Gly⁵¹, and Gly⁵¹-Leu⁵² [33].

Phe⁵⁰-Gly⁵¹ and Gly⁵¹-Leu⁵²

Kp analogs with D-amino acid substitutions at residues Phe⁵⁰-Gly⁵¹ showed improved metabolic stability but reduced in vitro agonistic activity. Pseudopeptides have become important for medicinal chemistry. For example, Kp analogs with 5-amino-acid residues containing (*E*)-alkene and hydroxyethylene-type isosteres showed high stability in mouse serum [34]. In our study, other simple pseudopeptides including azapeptides at residues Phe⁵⁰-Gly⁵¹ without altering the side chain moieties were then tested. The replacement of Gly⁵¹ with an azaGly residue, which involved a simple nitrogen atom replacement of the α -carbon on Gly⁵¹, improved KISS1R agonistic activity, presumably because of the change in the overall conformation of peptide analogs, thus avoiding enzymatic degradation [33]. This indicated that some β -turn conformations,

which are reportedly observed in azapeptides [39–41], may contribute to interactions with KISS1R. Moreover, incorporation of the azaGly⁵¹ residue improved metabolic stability of Phe⁵⁰-Gly⁵¹ and Gly⁵¹-Leu⁵² bonds, which were resistant to serum proteases such as chymotrypsin, neutral endopeptidases and matrix metalloproteinase-9 [33].

Trp⁴⁷-Asn⁴⁸

Substitution of Trp⁴⁷ with other amino acids such as serine, threonine, β -(3-pyridyl)alanine or D-tryptophan (D-Trp), produced several azaGly⁵¹ analogs that were resistant to metabolic degradation after 1 h of incubation in mouse serum. The replacement of key amino acids with azaGly⁵¹ resulted in metabolically stable analogs that could be easily synthesized using solid-phase methods without preparing dipeptide isostere units. Among these, the D-Trp⁴⁷ analog showed not only high metabolic stability but also excellent KISS1R agonistic activity (fig. 1), although other analogs showed decreased agonistic activities compared with Kp-10 [31, 33].

Fig. 1. Summary of optimization results: a biological stable lead peptide KISS1-305 with high agonistic activity was obtained from 5 amino acid substitutions of Kp-10.

Table 2. Representative Kp agonist analogs

Peptide	Position											
	45	46	47	48	49	50	51	52	53	54		
Kp-10	Tyr	Asn	Trp	Asn	Ser	Phe	Gly	Leu	Arg	Phe	NH ₂	
KISS1-305	D-Tyr		D-Pya(4)	Asn	Ser	Phe	azaGly	Leu	Arg(Me)	Phe	NH ₂	
TAK-448	Ac	D-Tyr	Hyp	Asn	Thr	Phe	azaGly	Leu	Arg(Me)	Trp	NH ₂	
TAK-683	Ac	D-Tyr	D-Trp	Asn	Thr	Phe	azaGly	Leu	Arg(Me)	Trp	NH ₂	

KISS1-305, TAK-448 and TAK-683

In order to identify Kp agonist analogs with improved metabolic stability and enhanced potency in vivo, a series of Kp-10 analogs containing 3 key replacements, N-terminal D-Tyr, azaGly⁵¹ and Arg(Me)⁵³, were synthesized and studied in vitro and in vivo. In addition to the substitutions mentioned above, deletion of Asn⁴⁶ to avoid inactivation of the peptide through chemical isomerization and substitution of Trp⁴⁷ produced highly potent and long-acting analogs suitable for in vivo study. Positions 45–47 were considered to be flexible for receptor recognition. This was supported by data showing that modifications at positions Tyr⁴⁵–Trp⁴⁷ of nonapeptide analogs resulted in small decreases in biological activity [35]. These experiments demonstrated that both agonistic activity and metabolic stability were important

for the performance of these peptides. Thus, the biologically stable agonist analog KISS1-305, D-Tyr⁴⁶-D-Pya(4)⁴⁷-Asn⁴⁸-Ser⁴⁹-Phe⁵⁰-azaGly⁵¹-Leu⁵²-Arg(Me)⁵³-Phe⁵⁴-NH₂ was identified [35] (fig. 1), and subsequent studies eventually led to the identification of the 2 investigational Kp agonist analogs TAK-448 and TAK-683 [35] (table 2).

Acute Effects of Kp or Its Agonist Analogs on Reproductive Functions

Because Kp is critical for GnRH physiology, two possible approaches were considered as therapeutic targets. The first was the improvement or stimulation of the reproductive functions by inducing GnRH release from

the hypothalamus, which can potentially be used to treat conditions such as hypogonadism, infertility and anovulation. The second was the suppression of the reproductive functions, allowing treatment of hormone-dependent diseases such as prostate cancer, benign prostate hyperplasia, endometriosis and breast cancer. As mentioned above, abrupt elevations of FSH/LH levels have been observed in several species as an acute effect. The first human study with Kp was conducted by Dhillon et al. [10], who administered 90-min intravenous infusions of Kp-54 (0.125–40 pmol/kg/min) in men. This treatment resulted in dose-dependent elevations in plasma FSH/LH levels and caused a slight elevation in testosterone levels. George et al. [12] demonstrated that intravenous bolus administration of Kp-10 in healthy men dose-dependently elevated plasma LH and FSH levels up to 1 µg/kg. However, the administration of 3 µg/kg of Kp-10 failed to elicit a statistically significant increase in LH and FSH levels; the underlying mechanisms remain unclear. Chan et al. [11] investigated the effects of intravenous injections of Kp-10 (0.24 nmol/kg) in men and observed not only elevated gonadotropin levels but also a possibility of resetting the GnRH/LH pulse clock. The acute effects of TAK-683 (0.01–2 mg/man) were also investigated in healthy male volunteers [42]. In this study, plasma gonadotropin levels peaked at 8 h, followed by an increase in plasma testosterone levels at 16–48 h after dosing.

The metabolic state is known to affect hypothalamic functions, and obesity and type 2 diabetes mellitus (T2DM) are frequently associated with hypogonadism [43, 44]. George et al. [45] demonstrated that the administration of a single bolus of Kp-10 in hypotestosteronemic men with T2DM induced LH elevations comparable with those in normal healthy men, suggesting that GnRH neurons maintain responsiveness to Kp stimuli in hypotestosteronemic patients with T2DM. This finding is in accordance with the results of previous animal studies in diabetic models [46–48]. In women, responsiveness to Kp varied across menstrual cycles; the most apparent LH elevations may be associated with increased LH pulsatility that occurred during the late follicular phase, which is the pre-ovulatory phase, whereas attenuated responsiveness was observed during the early follicular phase [49–52]. Similar estrous cycle dependence has also been reported in female rats [53]. One potential explanation for these variable responses, including sexual dimorphism, is the differences in the sex steroid hormone milieu as recently suggested by George et al. [54]; however, the precise mechanisms remain unknown.

Chronic Effects of Kp or Its Agonist Analogs on Reproductive Functions

It is widely known that continuous exposure to hormones or drugs leads to desensitization or tachyphylaxis, and one of the most famous examples is the suppression of gonadal functions by the continuous administration of GnRH or its agonist analogs, demonstrating that pulsatile GnRH release is critical in order to maintain pituitary function [55–57]. Pulsatile Kp release has been also proposed [24], and several studies have shown that continuous Kp administration also results in desensitization/tachyphylaxis of hypothalamic-pituitary-gonadal (HPG) functions in rats, monkeys and women with hypothalamic amenorrhea [58–62]. We have also evaluated the effects of continuous administration of Kp agonist analogs in male rats, monkeys and dogs and demonstrated rapid and profound testosterone suppression [63, 64]. These studies essentially showed similar outcomes: initiation of chronic dosing primarily induces abrupt elevation in gonadotropin/testosterone levels, followed by a rapid decline in gonadotropin/testosterone release. In contrast, differences have been observed between natural Kp and Kp agonist analogs. Specifically, Kp agonist analogs more profoundly suppressed testosterone levels in rats and monkeys than natural Kp [58, 61–64]. Moreover, Seminara et al. [59] showed that NMDA induced gonadotropin release in monkeys after continuous Kp-10 treatments, suggesting the preservation of GnRH-releasing ability of the GnRH neurons, whereas in our study LH was not elevated by NMDA after continuous KISS1-305 administration to male rats [63]. Although species differences should be considerable, these results may reflect differences in biological potency between Kp-10 and Kp agonist analogs. Accordingly, phase I clinical studies have been completed showing that subcutaneous infusion of TAK-448 (0.01–1 mg/man/day) and TAK-683 (0.01–2 mg/man/day) for 2 weeks in healthy male volunteers rapidly but reversibly reduced testosterone levels in a dose-dependent manner [42, 65].

In contrast, George et al. [12] showed that continuous subcutaneous infusion of Kp-10 (4 µg/kg/h) for 22.5 h in healthy men increased plasma LH levels throughout the infusion period, without apparent desensitization. Moreover, 11-hour infusions of Kp-10 at the same dose in hypotestosteronemic men with T2DM led to successful elevations in LH and testosterone levels [45]. The lack of apparent desensitization in these studies may be attributed to shorter dosing durations. Jayasena et al. [61, 62] investigated the effects of repeated subcutaneous dosing

	ORX	TAK-448
GnRH neural response	Normal*	Attenuated (desensitization)
Gnrh mRNA	Unchanged	Unchanged
GnRH peptide	Gradual decrease	Rapid decrease
Pituitary response	Responsive*	Responsive
Fshb/Lhb mRNA	Increase	Decrease
FSH/LH protein	Increase	Gradual decrease
Plasma FSH/LH	Increase	Decrease
Plasma T	Decrease	Decrease

Fig. 2. Summary of the status of the HPG axis under continuous administration of Kp analogs in comparison with ORX in male rats. Each description represents the changes from the vehicle control based on the results described in [42]. For example, *Gnrh* mRNA expression levels were unchanged regardless of the treatment, whereas hypothalamic GnRH contents were gradually (ORX) or rapidly reduced (Kp analog treatment). Items with asterisks are presumable (and not confirmed in [42]).

of Kp-54 (6.4 nmol/kg) in normal healthy women and women with hypothalamic amenorrhea. In the latter, twice-daily injections of Kp-54 eventually resulted in desensitization to exogenous Kp-54, while twice-weekly dosing of Kp-54 avoided the desensitization. In contrast, in healthy women, twice-daily administration of Kp-54 for a week did not cause desensitization, and their menstrual cycles were preserved [66]. As mentioned above, differences in the sex steroid hormone milieu may explain the differences, although more detailed pathophysiological studies are required for more precise explanations.

Continuous Administration of Kp or Kp Agonist Analogs and Suppression of HPG Function: Mechanism of Action

Suppression of GnRH Release by Chronic Administration of Kp or Kp Agonist Analogs

Under continuous administration of Kp or Kp agonist analogs associated with lowering of LH/testosterone lev-

els, bolus Kp/Kp agonist analogs cannot induce LH release [58–60, 63]. However, our studies and others showed that single bolus administration of GnRH or its agonist analogs induces LH release [59, 63] (fig. 2), suggesting that the continuous administration of Kp/Kp agonist analogs does not desensitize the pituitary but suppresses GnRH release. In fact, Ohkura et al. [67] showed suppression of GnRH pulses in goats treated continuously with TAK-683. We also observed marked suppression of pituitary *Fshb/Lhb* mRNA and protein expression after continuous administration of Kp agonist analogs [63] (fig. 2), supporting a mechanism involving the suppression of GnRH pulses. This may be associated with attenuated but not desensitized responsiveness of the pituitary to GnRH, particularly after long-term treatment with Kp agonist analogs.

Desensitization of GnRH Neurons

In goats, continuous treatment with TAK-683 suppressed GnRH pulses as stated above, although the animals maintained multiple unit activity (MUA) volleys [68], which are thought to be a consequence of GnRH pulse generator activities within the mediobasal hypothalamus, and recent studies have suggested that ARC Kp neurons are possible sources of this electrical activity [21, 22]. Thus, under the continuous administration of TAK-683 it is plausible that GnRH neurons become insensitive to endogenous Kp pulses. Investigations in mouse GnRH neurons and KISS1R-expressing CHO cells suggest that KISS1R desensitization occurs within 12 h. A potential mechanism of KISS1R desensitization may involve receptor internalization and subsequent reduction in cell surface KISS1R, uncoupling to G proteins, altered downstream signaling, or ubiquitination [69, 70]. In in vivo experiments, continuous Kp-10 infusion in monkeys induced abrupt elevations in LH levels for a few hours [59]. In male rats, continuously administered Kp-54 elevated plasma LH levels both at 6 and 12 h after initiation of continuous dosing, although the LH levels almost returned to the baseline level at 24 h [58]. We observed similar LH-releasing profiles after continuous subcutaneous administration of Kp agonist analogs to adult male rats, which led to marked LH release and apparent c-Fos expression within GnRH neurons at 4 and 8 h. However, LH release declined and c-Fos expression disappeared within 24 h [63]. Taken together, these data suggest that continuous administration of Kp or its agonist analogs rapidly desensitizes KISS1R both in vitro and in vivo.

Fig. 3. Schematic illustration of hypothalamic GnRH/KNDy neurons and effects of KISS1R agonists/antagonists on pulsatile GnRH/LH release. Dyn = Dynorphin; POA = preoptic area; ME = median eminence; (+) = tonic effect; (–) = inhibitory effect. **a** Normal state of hypothalamic GnRH neurons. To generate GnRH pulses, KNDy neurons presumably project to GnRH neural terminals and produce Kp pulses, which in turn lead to pulsatile GnRH release and eventually stimulate the pituitary. Although unknown, Kp-independent other neurons may support GnRH neural activity. **b** KISS1R antagonists (Kp-ant) block KISS1R on GnRH neural terminals, which in turn suppress GnRH release. KISS1R antagonists may not be able to suppress Kp-independent neural inputs; however, further studies are required for clarification. **c** KISS1R agonists (Kp-a) acutely stimulate GnRH release. Abrupt elevations in plasma LH levels were generally observed, whereas infusions of small quantities of Kp-10 may increase GnRH/LH pulse frequencies without resulting in desensitization. **d** Continuous administration of KISS1R agonists paradoxically suppresses GnRH release, potentially because of desensitization of KISS1R on GnRH neural terminals. Studies of Kp agonist analogs in male rats also suggest small and nonpulsatile leakage of GnRH.

Unrecovered Hypothalamic GnRH Contents

Desensitization of GnRH neurons could precede complete loss of responsiveness to further Kp stimuli, but our study in male rats suggested that this is not the case. As mentioned above, continuous administration of Kp agonist analogs leads to rapid declines in c-Fos immunoreactivity in GnRH neurons. However, c-Fos immunoreactivity was again induced in the majority of GnRH neurons in response to a single high dose of Kp agonist analogs [63] (fig. 2). This observation suggests that GnRH neurons maintain responsiveness to high doses of Kp agonist analogs, at least in terms of c-Fos immunoreactivity induction; therefore, desensitization of GnRH neurons does not indicate ‘complete abolishment of the responsiveness to Kp’ but actually indicates ‘significant attenuation of the responsiveness to Kp’. Furthermore, hypothalamic GnRH peptide levels were rapidly and dramatically reduced. The GnRH content did not recover during the 4-week dosing period, although *Gnrh* mRNA expression remained unchanged (fig. 2), suggesting the involvement of a post-translational mechanism. Bilateral orchectomy (ORX)

also dramatically reduced hypothalamic GnRH content without changing *Gnrh* mRNA expression, whereas pituitary gonadotropin expression and plasma gonadotropin levels were significantly increased [63, 71], presumably due to increased pulsatile GnRH release [72–74] (fig. 2). This contrasts with the Kp effects which differed in the reduction of gonadotropins. Decreased and unrecovered GnRH contents after continuous administration of Kp agonist analogs may reflect continuous release of GnRH, but in this case we suggest low-level and nonpulsatile leakage, which is biologically insignificant for gonadotropin secretion but could prevent recovery of hypothalamic GnRH content (fig. 3) [63]. This hypothesis requires remaining KISS1R on the cell surface after chronic exposure to Kp agonist analogs, and it may be supported by previous findings suggesting intracellular KISS1R pools and dynamic receptor recycling even after chronic exposure to the ligand [75, 76]. In addition, this continuous stimulation of GnRH neurons and subsequent nonpulsatile low-level leakage may disrupt Kp-independent GnRH release as suggested by Chan et al. [77].

KISS1R Antagonists

Both peptide and small-molecule KISS1R antagonists have been reported, and their in vivo efficacy has been investigated. In 2009, Roseweir et al. [78] reported Kp antagonist analogs, and their work has been described in detail elsewhere [79]. In brief, Roseweir et al. [78] tried to identify key amino acid residues that were critical for in vitro receptor binding and antagonistic activity, while avoiding residual agonistic effects. Initially, they identified critical amino acid residues within the five C-terminal amino acids using N-terminally truncated peptides and revealed that a length of 10 amino acids is important for efficient receptor binding. They also found that the C-terminal RF-amide structure as well as Asn⁴⁶, Trp⁴⁷ and Phe⁵⁰ are critical for receptor binding. In contrast to Kp agonist analog studies, the substitution of Phe⁵⁴ with Trp⁵⁴ was not efficacious in their antagonist screening, whereas our Kp agonist analogs as well as those reported by Niida et al. [80] demonstrated that RW-amide structures result in good Kp agonist analogs [35]. In subsequent screening based on in vitro antagonist assays, Roseweir et al. [78] identified that replacements of Ser⁴⁹ and Leu⁵² with D-amino acids or Gly⁴⁹ and D-Trp⁵² were the key for antagonism. They also demonstrated that Asn⁴⁶-Trp⁴⁷ residues were important because deletions in these positions resulted in attenuated antagonistic activity. Thus, they identified 4 potential Kp antagonist analogs, and the peptide 234 with ac-[D-Ala⁴⁵, Gly⁴⁹, D-Trp⁵²] Kp-10 has been most extensively tested in several animals, showing the suppression of GnRH/LH pulses in intact or gonadectomized animals, delay in vaginal opening or inhibition of preovulatory LH surges [25, 78, 80, 81]. These findings are in accordance with previous data demonstrating that Kp regulates both steroid hormone negative (i.e. GnRH pulses) and positive (i.e. preovulatory surge) feedback effects as well as the onset of puberty [27].

Kobayashi et al. [82, 83] reported small-molecule antagonists of the KISS1R receptor, including 2-acylamino-4,6-diphenylpyridine derivatives. Of several derivatives, they evaluated the in vivo efficacy of compound 15a in castrated male rats and showed reduced plasma LH levels [83]. Although these KISS1R antagonists reduce gonadotropin levels, it appears that the suppression is marginal compared to more profound suppressions by continuous administration of Kp agonist analogs. One possible explanation may be insufficient drug exposure of Kp antagonists at the target site. Another possibility is that a Kp/KISS1R-independent mechanism may be involved in GnRH/gonadotropin release, which cannot be suppressed by Kp antagonists [77] (fig. 3).

Future Perspectives and Potential Therapeutic Utility

Following the discovery of the role of Kp in reproductive functions, numerous physiological and pharmacological effects of Kp have been delineated during the past decade, suggesting that KISS1R agonists and antagonists may provide novel treatment approaches to control HPG functions via stimulatory or inhibitory mechanisms of action. Notably, clinical studies with Kp or Kp agonist analogs have reported excellent safety [10–12, 42, 45, 49, 50, 65]. However, several effects remain uncharacterized to date, as mentioned above, such as the sexual dimorphic effects of Kp or the complex Kp responses in women. In addition, Tanaka et al. [84] recently demonstrated that the continuous administration of TAK-683 in ovariectomized goats suppressed LH pulses but induced surge-like LH release following estradiol infusion, as shown in control animals, suggesting that continuous exposure to Kp agonist analogs differentially affected pulsatile and surge mode secretions of GnRH and LH in female goats. Moreover, recent data strongly suggest that Kp neurons in ARC, which are responsible for receiving steroid hormone negative feedback, also express other neurotransmitters such as neurokinin B (NKB) and dynorphin, and these findings led us to describe ARC Kp neurons as KNDy neurons (Kp, NKB and dynorphin) [85–90]. We will not describe KNDy neurons in detail here; however, emerging data on KNDy neurons have provided important clues to the neural mechanisms of GnRH pulse generation systems (detailed review articles of the role of KNDy neurons are present in this special issue by Grachev et al. [91] and Goodman et al. [92]). In brief, KNDy neurons apparently express the NKB receptor (NK3R) [89, 93] and lack KISS1R [21, 94, 95]. The expression of the κ -opioid receptor (KOR) remains controversial [96]. On the other hand, GnRH neurons express KISS1R [13, 14], with little evidence regarding the expression of NK3R [93, 96] or KOR [97, 98]. Human inactivating mutations in *KISS1*, *KISS1R*, *NKB*, or *NK3R* lead to severe hypogonadism and pubertal disorders [4, 5, 99, 100]. In contrast, presumable gain-of-function mutations in *KISS1* and *KISS1R* have been identified in patients with central precocious puberty [101, 102]. In animals, NKB and senktide (an NKB agonist), stimulate GnRH/LH secretion and activate KNDy neurons [96, 103–105], presumably leading to increased pulse frequency, as shown in MUA volley [103]. In contrast, endogenous opioids have been known to suppress GnRH secretion and recent evidence (e.g. in goats) suggests that dynorphin suppresses MUA volley, whereas the KOR an-

tagonist norbinaltorphimine increases it [103]. Thus, a KNDy neuron model has been established; Kp serves as the stimulatory output from KNDy neurons to GnRH neurons, whereas KNDy neurons form KNDy-KNDy neural circuits to control their pulse-generating activity via tonic NKB and inhibitory dynorphin either directly or indirectly [85–90]. Notably, the effects of NKB or its agonists on LH secretion remain controversial, particularly in rodents, because some groups have demonstrated that NKB agonists suppress LH secretion [106, 107]. Interestingly, this effect also appears to be dynorphin-KOR dependent [104]. Therefore, agonists/antagonists of NK3R or KOR may also provide novel therapeutic approaches to precisely regulate the HPG axis in addition to the Kp/

KISS1R system. Moreover, KNDy neurons may have other physiological roles such as thermoregulation [108]. Additional physiological and pharmacological studies will provide deeper knowledge of these neural systems and may eventually provide novel therapeutic approaches focused on the Kp/KISS1R system.

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