KISS1R: Hallmarks of an Effective Regulator of the Neuroendocrine Axis

Running Head: KISS1R: Neuroendocrine Regulator

Robert P. Millar[1] and Andy V. Babwah[2]

[1]Mammal Research Institute, University of Pretoria, Pretoria 0018, South Africa; and Medical Research Council Receptor Biology Unit, University of Cape Town, Cape Town 7539, South Africa;
[2]The Children's Health Research Institute, London, Ontario, Canada; Lawson Health Research Institute, London, Ontario, Canada; Departments of Obstetrics and Gynaecology and Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada N6C 2V5.

Corresponding author and person to whom reprint requests should be addressed:

Dr. Andy V. Babwah

Children's Health Research Institute

Victoria Research Laboratories

800 Commissioners Road East

London, ON,

Canada N6C 2V5

Office: 519-685-8500 ext 55485, Fax: 519-685-8646, e-mail: ababwah@uwo.ca

Abstract

Kisspeptin (KP) is now well recognized as a potent stimulator of gonadotropin-releasing hormone (GnRH) secretion and thereby a major regulator of the neuroendocrine-reproductive axis. KP signals via KISS1R, a G-protein-coupled receptor (GPCR) that activates the G-proteins $G\alpha_{q/11}$. Modulation of the interaction of KP with KISS1R is therefore a potential new therapeutic target for stimulating (in infertility) or inhibiting (in hormone-dependent diseases) the reproductive hormone cascade. Major efforts are underway to target KISS1R in the treatment of sex steroid hormone-dependent disorders and to stimulate endogenous hormonal responses along the neuroendocrine axis as part of in vitro fertilization protocols. The development of analogs modulating KISS1R signaling will be aided by an understanding of the intracellular pathways and dynamics of KISS1R signaling under normal and pathological conditions. This review focuses on KISS1R recruitment of intracellular signaling ($G\alpha_{q/11}$- and β-arrestin-dependent) pathways that mediate GnRH secretion and the respective roles of rapid desensitization, internalization, and recycling of resensitized receptors in maintaining an active population of KISS1R at the cell surface to facilitate prolonged KP signaling. Additionally, this review summarizes and discusses the major findings of an array of studies examining the desensitization of KP signaling in man, domestic and laboratory animals. This discussion highlights the major effects of ligand efficacy and concentration and the physiological, developmental, and metabolic status of the organism on KP signaling. Finally, the potential for the utilization of KP and analogs in stimulating and inhibiting the reproductive hormone cascade as an alternative to targeting the downstream GnRH receptor is discussed.

Introduction

Hypothalamic gonadotropin-releasing hormone (GnRH) has long been recognized as the master regulator of the neuroendocrine-reproductive axis. Diminished GnRH secretion or actions can result in congenital hypogonadotropic hypogonadism (HH), a disorder that is characterized by the absence of spontaneous sexual maturation in the presence of low gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] and sex steroids (estradiol and testosterone) in the absence of anatomical or functional abnormalities of the hypothalamic-pituitary-gonadal axis. HH results from disruption of the migration of GnRH neurons from the olfactory placode to the hypothalamus during embryonic development, or from diminished pulsatile GnRH secretion from the established population of hypothalamic GnRH neurons, presumably due to altered neuronal inputs, or from inactivating mutations in GnRH or its cognate receptor [1--4].

Our understanding of the molecular regulation of GnRH secretion in animal models has grown tremendously in the last decade. At the forefront of this advance was the discovery of three neuropeptides: the opioid peptide dynorphin (DYN), the tachykinin peptide neurokinin B (NKB), and in particular the RF-amide peptide kisspeptin (KP). In goats, sheep, and laboratory rodents, all three peptides are highly coexpressed in a population of neurons in the arcuate nucleus called the KNDy neurons, and it is now well accepted that through their interactions these peptides play a pivotal role in regulating pulsatile GnRH secretion [5--9]. However, in human males, KP-expressing neurons in the infundibular nucleus, a region analogous to the arcuate nucleus of other species, coexpress NKB but surprisingly very little DYN [9, 10]. In human females, however, there is evidence that the same neurons in the infundibular nucleus express KISS1 [11], DYN [12], and NKB [13]. Thus, while there appear to be species and gender differences in the presence and roles of these peptides, there is a consensus that KP, signaling through its

receptor (KISS1R) located on the surface of GnRH neurons, is a direct trigger of GnRH secretion in mammals [14--22]. GnRH then regulates the synthesis and secretion patterns of gonadotropins from pituitary gonadotropes through changes in pulse frequency and amplitude [23--26].

While numerous studies have focused on the role of KP as a potent regulator of GnRH secretion, few studies have explored the mechanisms by which KISS1R mediates KP signals intracellularly in the GnRH neuron as well as in other cell types. Therefore, this review will focus on KISS1R recruitment of intracellular signaling pathways that mediate GnRH secretion and the dynamics of ligand-induced internalization of KISS1R, degradation, and recycling, as well as desensitization.

KP Signals via KISS1R and Gαq/11

KISS1R, which was first designated as the orphan receptor GPR54, was cloned using a degenerate PCR strategy to identify cDNAs encoding G-protein-coupled receptor (GPCRs) [27]. While the novel receptor shared high sequence identities in the transmembrane regions of galanin receptors, KISS1R failed to bind 125I-galanin [27]. In 2001, four independent studies identified KISS1R agonists [28--31]. A study by Clements et al. [28] suggested that the KISS1R cognate ligand is an RF-amide, while the other three studies specifically identified KP as the RF-amide [29--31]. In those studies, the authors demonstrated that KP triggered a robust mobilization of intracellular Ca2+ in KISS1R-expressing cells, suggesting that coupling occurred via Gαq/11 with the resulting activation of phospholipase C, hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), and generation of inositol phosphates (IP). Muir et al. [30] and Kotani et al. [29] also demonstrated that KP does not couple KISS1R to Gαs and that pertussis toxin, an inhibitor of Gαi/o, fails to block the mobilization of Ca2+ [29, 30].

Gαq family members include Gαq, Gα11, Gα14, and Gα15/16 [32]. Gαq and Gα11 are functionally redundant and widely expressed in the body, including the brain. However, the expression of Gα14, and Gα15/16 is restricted to tissues like the kidney and hematopoietic organs, respectively, with almost no detectable expression in the brain [32--34]. Kotani et al. [29] and Wacker et al. [35] demonstrated that KISS1R can also couple to Gα15/16 and thereby generate inositol trisphosphate (IP3) which mobilizes intracellular Ca2+. This finding was not surprising given that Gα15/16 promiscuously couples a large number of GPCRs to phospholipase Cβ (PLCβ) activation and PIP2 hydrolysis [36, 37]. Gα14 has also been reported to couple some Gαi- and Gαs-coupled receptors to PLCβ activation [38]; however, promiscuous coupling by Gα14 does not appear to be as widespread as that displayed by Gα15/16. It is currently unknown whether KISS1R can signal via Gα14. However, from the perspective of this review on KISS1R signaling, coupling to Gα15/16 and Gα14 is not relevant as these are not expressed in the hypothalamus [32--34]. Thus, in hypothalamic GnRH neurons, KP-bound KISS1R signals via Gαq/11 (2, B, and 3 in fig. 1), thereby triggering the activation of PLCβ, hydrolysis of PIP2 to diacylglycerol and IP3, and the mobilization of intracellular Ca2+, events implicated in GnRH neuronal membrane depolarization and GnRH secretion [39--44].

KISS1R Displays Basal Constitutive Signaling and KP-Independent Internalization

In addition to KISS1R displaying KP-triggered signaling and internalization [45], in HEK 293 cells transiently expressing KISS1R, KP-independent Gαq/11 signaling, and internalization are apparent (1 in fig. 1) [45]. In the absence of ligand, signaling is approximately 5% of the maximum KP-induced IP formation, an indirect measure of Gαq/11 activation (A and 1 in fig. 1). Moreover, internalization in the absence of ligand was very high, with about 70% of the labeled cell surface KISS1R internalizing after 5 min at 37°C. This high

basal internalization rate was even greater than that reported for the mammalian type I GnRH receptor (GnRH-RI), where after 90 min at 37°C about 25% of the receptor had internalized [46]. It was also higher than the metabotropic glutamate receptor 1a, a receptor well known for its high constitutive internalization in heterologous cell cultures and primary neurons, where after 5 min at 37°C approximately 50% of the receptor had internalized [47--50]. While KISS1 expression was not detected in HEK 293 cells [45], it appears that basal signaling and internalization occurred constitutively; however, the availability of an inverse KISS1R agonist is required to confirm this.

In an independent study, Min et al. [51] also observed that KISS1R displays a high rate of basal trafficking. Their study was conducted in CHO cells stably expressing KISS1R; thus, the high rate of basal trafficking appears to be cell type-independent. Importantly, there is also in vivo evidence of constitutive KISS1R activity and this comes from studies performed on Kiss1--/-- and Kiss1r--/-- mice [17]. In that study, Lapatto et al. [17] reported that, compared to Kiss1r--/-- female mice, Kiss1--/-- female mice displayed a more variable phenotype and they suggested that it could be the result of modest constitutive Kiss1r activity in the complete absence of endogenous KP. It is unlikely that the variable reproductive phenotype is the result of other RF-amide peptides activating Kiss1r since binding experiments, performed on membranes from CHO cells expressing KISS1R, revealed a high affinity and remarkable selectivity for KP; this is in contrast to the NPFF1/GPR147 and NPFF2/GPR74 receptors which display a high affinity for all RF-amides, including KP [52].

The question arises of whether constitutive KISS1R signaling is physiologically relevant. A putative role could be in maintaining the neuroendocrine axis in a primed state during development. The neuroendocrine axis is highly active during fetal life and in the neonatal period up to approximately 6 months in boys and 1--2 years in girls [53]. It has been suggested that the transient LH surge observed in the period after birth ('mini' puberty) might play a role in regulating the differentiation of spermatogonia and adult germ line stem cells [54, 55]. This is followed by a period characterized by very low-amplitude LH secretion. With the onset of puberty in girls between the ages of 8 and 14 years and in boys between the ages of 9 and 14 years, there is a reactivation of the axis and a concomitant and robust increase in the amplitude of GnRH-induced LH pulses with more modest changes in frequency [56--61]. Thus, keeping the axis in a primed or basal state of activity during many of the childhood years may facilitate its activation when KP-dependent signaling is fully established.

This idea that basal GnRH secretion keeps the pituitary-gonadal axis primed is supported by several studies conducted on laboratory animals and human patients. For example, in the hypogonadal (hpg) mouse it was observed that administration of 40 ng of a GnRH analog could immediately trigger LH secretion from the pituitary, albeit significantly lower than that in normal mice [62]. In the rhesus monkey (Macaca mulatta), the responsiveness of pituitary gonadotropes to GnRH stimulation is clearly enhanced by a preceding chronic pulsatile infusion of GnRH [63]. This important observation led Plant and colleagues [64--68] to employ a standard GnRH priming protocol in their studies where gonadotropin secretion is measured in response to various factors. Studies have also been conducted in men exhibiting gonadotropin deficiency, and in the absence of any pituitary defects it has been reported that GnRH priming improves gonadotropin secretion [69, 70]. More detailed studies in human male patients are generally lacking because males who are diagnosed with GnRH and gonadotropin deficiency often exhibit pituitary and testicular defects [71]. GnRH priming is not only believed to be important for the normal onset of puberty. In reproductively mature female animals, including human females, it has also been proposed that GnRH secreted during progression of the follicular phase increases the number of GnRH

receptors on gonadotropes and this serves to potentiate the LH preovulatory surge [72--74]. Thus, the constitutive activity of KISS1R may act to prime the gonadotrope.

β-Arrestin Coupling in KISS1R Signaling

Traditionally, β-arrestins are recognized as molecules that mediate the homologous desensitization and clathrin-dependent endocytosis of GPCRs [75]; these are discussed further below. Importantly, it is now well established that β-arrestins play much wider roles in regulating GPCR signaling than previously thought. Specifically, they serve as molecular scaffolds that couple GPCRs to a variety of signaling pathways, thus acting as signal transducers in their own right [76--78]. Since the first report that β-arrestins can couple the tyrosine kinase c-Src to the agonist-activated β2-adrenergic receptor (β2AR), eventually leading to G protein-independent activation of ERK1/2, numerous studies have demonstrated that β-arrestins can couple GPCRs to a large array of signaling pathways including p38, c-Jun N terminal kinase-3, AKT, PI3 kinase, and phosphodiesterase 4 [76, 79].

The first evidence that KISSR could signal via β-arrestins (8 in fig. 1) came from the observations that KISS1R physically interacts strongly with both β-arrestin-1 and β-arrestin-2 and that β-arrestin and KISS1R colocalize to clathrin-coated pits [45], structures that have been reported to facilitate the β-arrestin-dependent coupling of GPCRs to G protein-independent pathways (8 in fig. 1) [75]. However, in the case of KISS1R, it remains to be determined whether localization to clathrin-coated pits and even internalization via clathrin-coated vesicles are required for β-arrestin-dependent signaling. It has also been demonstrated that KP-triggered ERK1/2 phosphorylation is significantly diminished in MDA-MB-231 breast cancer cells in which β-arrestin-2 is depleted and in mouse embryonic fibroblasts lacking β-arrestin-1 and β-arrestin-2 [45, 80]. These findings led to the hypothesis that in the GnRH neuron, in addition to coupling to Gαq/11, KISS1R couples to β-arrestin to regulate GnRH secretion. To test this hypothesis, KP-dependent LH secretion, a surrogate marker of GnRH secretion, was measured in mice lacking either β-arrestin-1 or β-arrestin-2 and the results revealed that LH secretion was significantly diminished relative to WT littermates, thus supporting that β-arrestin mediates KP-induced GnRH secretion [81]. This conclusion was only possible because it is well-established that GnRH receptor activity on the anterior pituitary is not regulated by β-arrestin [46, 82--85], and thus the observed reduction in GnRH secretion must have occurred as the result of an absence of β-arrestin-regulated events in the GnRH neuron following KP treatment. It is suggested that KP-triggered β-arrestin signaling is of physiological importance and such signaling might explain why patients bearing some types of Gαq/11-uncoupled KISS1R mutants display a partial gonadotropic deficiency.

KISS1R Gαq/11-Coupled Signaling Undergoes Homologous Desensitization

Homologous desensitization refers to the uncoupling of the GPCR from its cognate G protein in the continuous presence of its agonist. This singular event, which is characteristically rapid, occurs within seconds to minutes, terminates G protein-dependent signaling which is crucial to neuronal signaling, and protects the cell from being overstimulated by an agonist. Homologous desensitization is well understood for β2AR, and based on this a general model of the phenomenon for this GPCR has been proposed. In this model, the ligand (L) binds its GPCR (R) and stabilizes an active conformation of the receptor (R*) that allows recruitment of the signaling G protein (Gαs in this case). The activated receptor then binds one or more of the GPCR serine/threonine kinases (GRKs; of which there are 7), which phosphorylate the receptor at cognate residues on its intracellular loops and carboxyl terminal tail. Phosphorylation promotes high-affinity binding of the arrestin family of proteins, the visual arrestins and the nonvisual

arrestins (β-arrestin-1 and β-arrestin-2) to the receptor and this sterically hinders the interaction between the GPCR and the G protein [75].

In addition to uncoupling the GPCR from its cognate G protein, β-arrestin simultaneously couples and sequesters the desensitized GPCR to clathrin-coated pits via direct interaction. Sequestration into clathrin-coated pits serves multiple purposes. The first is that it prevents recoupling of the desensitized GPCR to its G protein; the second is that it rapidly targets the uncoupled receptor for internalization where it can undergo degradation or resensitization, and the third is that for many GPCRs it provides an environment that allows β-arrestin to couple the sequestered receptor to new signaling pathways, thereby permitting β-arrestin-dependent signaling [45, 75--78, 80].

As stated earlier, assaying the formation of IP by PIP2 hydrolysis is an indirect measure of Gαq/11 activation (A, 1, B, and 3 in fig. 1). In this way it was demonstrated that in HEK 293 cells transiently expressing KISS1R, coexpression of GRK2 [45] or β-arrestin-1 or β-arrestin-2 [unpubl. data] significantly diminished the IP-forming capacity of these cells in response to increasing concentrations of KP following a 4-hour incubation period at 37°C. These results suggest that following KP treatment (2 in fig. 1), GRK2 and β-arrestin uncouple KISS1R from-Gαq/11 and IP generation (4, 5, C, and 6 in fig. 1). This conclusion is reinforced by the observations that KISS1R physically interacts with GRK2 and β-arrestin-1 and β-arrestin-2 and that within 3 min of KP stimulation β-arrestin molecules are recruited to the plasma membrane (5 in fig. 1) where they colocalize and rapidly cointernalize with KISS1R [45]. While it appears that β-arrestin is recruited to KISS1R in response to KP-triggered G protein signaling (5 in fig. 1), it is not known whether the association of GRK2 with KISS1R is also G protein dependent or whether GRK2 is constitutively associated with KISS1R. Nevertheless, it is clear that, in response to the KP-dependent activation of KISS1R, GRK2 acts to rapidly uncouple KISS1R from Gαq/11 [45]. At first consideration, the desensitization of KISS1R appears to be mechanistically identical to that of β2AR. However, unlike β2AR, but similar to the metabotropic glutamate receptor 1a [86, 87] and the calcium-sensing receptor [88], it was observed that following KP treatment GRK2 diminished IP formation in a phosphorylation-independent manner [45].

Since KISS1R colocalized with β-arrestin or clathrin at the cell surface and with β-arrestin or transferrin (a clathrin-coated vesicular cargo) in endosomes in a temporally overlapping manner, it was concluded that, like β2AR, β-arrestin sequestered the G protein-uncoupled KISS1R to clathrin-coated pits (7 and 8 in fig. 1) and thus facilitated KISS1R internalization via clathrin-coated vesicles (9 in fig. 1). However, unlike β2AR but similar to the vasopressin V2 receptor [89], β-arrestin cointernalized with KISS1R in clathrin-coated vesicles (9 in fig. 1) [45] where intracellularly it is believed they eventually dissociate from each other (10 and 11 in fig. 1). Consistent with GPCRs that desensitize and internalize through a β-arrestin- and a β-arrestin/clathrin-dependent mechanism, respectively, KISS1R desensitization and internalization were rapid and after only 5 min at 37°C approximately 80% of the receptor had undergone KP-dependent internalization as assessed by flow cytometry [45]. Notably, KISS1R internalization was rapid as it was about 2.5 times more rapid than β2AR under saturating concentrations of their respective agonists [45].

In a recent study, using CHO cells stably expressing KISS1R, Bianco et al. [90] also demonstrated that KISS1R signaling undergoes desensitization (as assessed by IP formation) and internalization (by measuring radiolabeled KP-10 internalization). Specifically they showed that, following KP treatment, IP formation peaked after 2 h and returned to baseline by 12 h despite the continuous presence of KP (table 1). It must be noted that since this standard IP assay, like that conducted by Pampillo et al. [45], was

conducted in the presence of LiCl, IPs were not rapidly degraded [91] and therefore the results of this study only confirm that KISS1R undergoes KP-dependent desensitization but they do not inform us on the rate of desensitization, which is likely to be more rapid than when using LiCl as an inhibitor of IP degradation. As for internalization, the authors showed that at 37°C after 15 min 50% of the KISS1R was internalized and after 60 min approximately 80% of the KISS1R was internalized as assessed by a loss of cell surface binding of the radioligand [90]. Therefore, consistent with the findings of Pampillo et al. [45], KISS1R internalization was rapid. Min et al. [51] further demonstrated that the endocytic inhibitor phenylarsine oxide significantly inhibited KISS1R internalization and this was consistent with the idea that ligand-dependent KISS1R internalization follows classic clathrin- and dynamin-mediated endocytosis pathways. Taken together, these studies [45, 51, 90] indicate that GRK2 and β-arrestin mediate the rapid and homologous desensitization and internalization of KISS1R via clathrin-coated vesicles.

KISS1R Undergoes Degradation and Recycling

Both in the presence and in the absence of KP, the Kaiser laboratory [51, 90] demonstrated that KISS1R undergoes rapid dynamic internalization and recycling. These authors reported that with each wave of internalization the majority of KISS1R molecules recycled rapidly back to the cell surface (11 in fig. 1) while a small quantity of internalized receptor was degraded via proteasomes (12 in fig. 1). The interpretation of these observations is that if this is characteristic of KISS1R in GnRH neurons, neurons would maintain a pool of KISS1R at their surface and if the recycled receptors were functionally competent they could support sustained or prolonged KP-dependent signaling. This is discussed further in the subsequent section. Importantly, while only a small fraction of the internalized KISS1R had undergone degradation, within an hour of internalization most of the ligand was rapidly processed and degraded [51], thus representing a potential alternative mechanism for the termination of neuropeptide action. It was reported that receptors like the angiotensin II type 1A receptor, neurotensin receptor 1, the vasopressin V2 receptor, the thyrotropin-releasing hormone receptor, and the substance P receptor, which bind β-arrestin-1 and β-arrestin-2 with similar high affinities and cointernalize with β-arrestin in endosomes, are retained intracellularly and recycle very slowly if at all [89, 92]. However, while KISS1R also binds β-arrestin-1 and β-arrestin-2 with similar high affinities and cointernalizes with β-arrestin in endosomes [45], most KISS1R molecules recycle back to the cell surface [51].

While the Kaiser laboratory [51, 90] clearly demonstrated that recycling KISS1R contributed significantly to the dynamic population of cell surface receptors, they considered the possibility that an intracellular pool of nonrecycling receptors also contributed to this receptor population at the plasma membrane. The existence of such an intracellular pool is highly probable given that it was previously observed that KISS1R was present at very high levels intracellularly in at least two unrelated cell-types [45]. Definitive proof of the existence of a nonrecycling intracellular pool of KISS1R eventually came from Min et al. [51], and in that study the authors demonstrated that this intracellular population of receptors did in fact traffic to the plasma membrane independently of KP (13 in fig. 1). This is a major finding since it reveals an important mechanism by which the cell maintains a large population of presumably signaling-competent KISS1R at the cell surface despite receptor internalization and degradation and thereby contributes to the dynamic pool of ligand-responsive KISS1R at the cell surface [51]. The high intracellular content of KISS1R is similar to that described for the GnRH receptor and the relaxin receptor RXFP1 [93--95]. Potentially, intracellular retention of receptors creates and provides a source of receptors needed for rapid availability without transcription or translation.

KP-Dependent Signaling following Chronic KP Exposure Is Resistant to Rapid Desensitization in Cultured Cell Lines

Once it was established that the cell maintains a dynamic population of cell surface KISS1R, it was determined whether this resulted in continuous or prolonged KP signaling. If it did then chronic KP administration should result in the sustained activation of the Gαq/11-coupled signaling pathway [96]. To test this, HEK 293 and the GnRH-secreting GT1--7 cells [97] transiently coexpressing KISS1R and molecular reporters of Gαq/11-coupled signaling were treated chronically with KP-10 and assessed for Gαq/11-dependent signaling. Based on the spatiotemporal properties of PLCβ and PKC activation and intracellular Ca2+ mobilization, it was concluded that chronic KP-10 administration triggers prolonged signaling [96]. For example, in HEK 293 and GT1--7 cells, the initial desensitization of PLCβ activation was observed after about 90 and 45 min, respectively, while for PKC activation it was detected after about 45 min in HEK 293 cells (table 1). To put this into striking perspective, in HEK 293 cells transiently expressing the Gαq/11-coupled endothelin or angiotensin receptors, chronic stimulation by their respective ligands triggered a rapid activation of PKC that was either fully desensitized or nearly so after just 80 s [98].

Thus, the study described above [90] led to the conclusion that the dynamic pool of plasma membrane-bound KISS1R identified by Bianco et al. [90] is a signaling-competent pool capable of responding to chronically administered KP and thereby resulting in sustained signaling via Gαq/11. This conclusion, however, was not consistent with the observations that in HEK 293 cells, just after 5 min at 37°C, approximately 80% of transiently expressed KISS1R had undergone agonist-dependent internalization [45] [51, 90] and presumably desensitization of Gαq/11-coupled signaling [45]. However, when considered together, these studies [45, 51, 90, 96] reveal that it is 'KISS1R-expressing cells', and not the 'receptors', which exhibit prolonged signaling following chronic KP administration. Furthermore, this prolonged signaling is dependent on the rapid desensitization, internalization, and recycling of resensitized KISS1R molecules superimposed on a background of diminished receptor degradation (fig. 1). Because of this dynamic trafficking and signaling, at any point in time, KISS1R is found to be associated with Gαq/11, GRK2, and β-arrestin [45]. Chronic KP signaling is also absolutely dependent on the sustained influx of extracellular Ca2+, which in turn is dependent on the continued presence of exogenous KP and dynamic trafficking of KISS1R (14 in fig. 1) [51, 96]. The net result of this interesting phenomenon is that the cell maintains a dynamic and active pool of receptors at its surface (D in fig. 1), allowing it to display prolonged KP-dependent signaling (2, B, and 3 in fig. 1). Conceivably, this would protect against downregulation of the neuroendocrine axis.

KP-Dependent Signaling in vivo Displays a Wide Range of Temporal Responses following Chronic Exposure to Exogenous KP

Over the last decade, studies conducted on mice, rats, sheep, monkeys, and humans have examined the desensitization of KP signaling following chronic exposure to KP (summarized in table 1) [68, 99--113] and, while most studies detected desensitization during the course of investigation, the temporal aspect of desensitization was surprisingly widely variable, ranging from 1--2 h to greater than 24 h. Based on several studies investigating the desensitization of GPCRs, including KISS1R, there is evidence strongly suggesting that the range of temporal responses following chronic exposure of a receptor-expressing system to its agonist is in part due to differences in ligand efficacy and concentration [108--114] as well as differences in developmental (which would reflect differences in circulating levels of gonadal steroids) and metabolic status [103].

An example of one study reporting that KP signaling is desensitized relatively quickly comes from Plant and colleagues [68] who in 2006 reported that in agonadal juvenile male monkeys continuous intravenous infusion of human KP-10 (100 μg/h for 98 h) elicited a robust LH response that peaked after 1--2 h. However, despite the continuous infusion of KP-10, this was followed by a rapid drop in LH where, 12 h after the infusion, levels were similar to those in vehicle-infused animals (table 1). Thus, the authors concluded that the rapidly diminished response to chronic KP exposure was due to desensitization of KISS1R and was not the result of compromised GnRH secretion or reduced gonadotrope function. In a second study [102], the Plant laboratory extended their investigation to the gonad-intact adult male monkey, a preclinically relevant model that exhibits a highly active HPG axis. Here they found that continuous intravenous infusion of KP-10 (200 or 400 μg/h) triggered a significant rise in circulating LH that peaked 2--3 h after the initiation of treatment and then rapidly declined by 24 h to levels similar to those in control animals (table 1). Those results led the authors to conclude that chronic KP exposure triggers the rapid desensitization of KISS1R signaling in adult male monkeys as in agonadal juvenile male monkeys. They also found that at 400 μg/h there was greater suppression of LH pulses but, since this was observed in the presence of impaired pituitary function, the greater suppressive effect was likely the result of pituitary desensitization at the higher KP-10 concentration rather than greater KISS1R desensitization.

At the other extreme of the temporal spectrum, clinical studies by Millar and colleagues [105, 106] (table 1) showed that chronically administered KP-10 does not result in rapid desensitization of KP signaling but instead elicits prolonged and pulsatile secretion of LH. Specifically, George et al. [105] reported that in healthy men, chronic infusion of KP-10 at the rate of 4 μg/kg/h triggered multiple large LH pulses without evidence of desensitization over a 22-hour period (table 1) (indeed, they have demonstrated in unpublished studies that there is no evidence of desensitization for up to 36 h). The observation that chronic KP-10 administration triggers pulsatile LH release is not confined to healthy individuals. In a subsequent study, the group [106] reported that 12 h of chronic KP-10 infusion administered at a rate of 1.5 μg/kg/h restored pulsatile gonadotropin secretion in male patients with loss-of-function mutations in NKB (TAC3) or its receptor (TAC3R) (table 1). These interesting findings reveal that chronically administered KP-10 can support prolonged gonadotropin secretion and that chronically administered KP-10 is sufficient to elicit a pulsatile release of GnRH, as determined by the observed pulsatile release of LH. While pulsatile LH secretion was not desensitized during the period of investigation, it is predicted that such secretion would eventually desensitize. This is supported by the observation that within 1 day chronic exposure to KP-54 did not result in desensitization of LH, FSH, or estradiol secretion in women exhibiting hypothalamic amenorrhea but sometime after the first day and possibly as late as the 14th day of chronic exposure full desensitization occurred (table 1) [104].

The above studies from the Plant and Millar laboratories clearly illustrate the wide range of temporal responses in gonadotropin secretion in response to chronic KP exposure [68, 102, 105, 106]. While the dose-response study by Plant and colleagues [102] could not establish a relationship between KP concentration and the rate of KISS1R desensitization, several studies using synthetic KP analogs would subsequently do so (table 1) [108, 110--113]. For example, in a recent study from the Okamura laboratory [113], the authors examined the effects of chronically administered KP agonist TAK-683 (an investigational protease-resistant agonist which exhibits improved pharmacokinetics and higher affinities than natural ligands) on LH secretion in goats. An osmotic pump containing TAK-683 (0.03, 0.3, or 3 nmol/h/kg) was subcutaneously implanted (day 0) and TAK-683 was observed to suppress LH secretion as early as on day 1 and with marked suppression on day 5. On day 3, at the lowest concentration suppression was marginal

and LH pulses were still apparent; however, as the dose increased the suppressive effect on LH secretion became strikingly apparent. By day 3, at all concentrations, LH secretion was almost completely suppressed and the potent suppressive effect of continuous TAK-683 treatment was maintained until day 6 (table 1). The results indicated that chronic administration of the KP agonist, in a dose-dependent manner, profoundly suppresses pulsatile LH secretion while additional controls showed it did so without affecting the GnRH content, pituitary function, or GnRH pulse generator activity.

In addition to agonist concentration, the following study showed that agonist efficacy also regulates desensitization responses. When adult male rats were continuously infused over a 4-week period with 0.2--4.0 nmol/h KISS1--305 (a protease-resistant agonist with suboptimal KISS1R agonistic activity in vitro) and 0.1 nmol/h TAK-448 (a derivative of KISS1--305; a protease-resistant agonist with potent agonist activity comparable to KP-10), treatment with 1--4 nmol/h KISS1--305 resulted in castrate levels of testosterone (<0.5 ng/ml) by day 3 but its efficacy over the 4-week treatment period was only sustained at 2 nmol/h and greater. Thus, 2 nmol/h was considered the minimum effective dose of KISS1--305. In contrast, TAK-448, which exhibits greater efficacy, induced testosterone depletion as rapidly as KISS1--305 but at a lower dose (0.1 nmol/h) (table 1). Compared with previous studies using KP-54 in rats [99] or KP-10 in monkeys [102] (table 1), these studies with KISS1--305 and TAK-448 showed a greater reduction in plasma testosterone levels, leading the authors to suggest that the difference in efficacy is probably due to the higher stability of the KP analogs [115--118].

In the rat, desensitization of gonadotropin responses to KP is also influenced by developmental and metabolic states. This has been seen in studies using adult and peripubertal female rats which are fed or underfed and subjected to continuous i.c.v. administration of KP-10 (1 µl/h, 7.5 nmol/day) [103]. In adult fed rats, LH secretion declined on day 3, while FSH secretion remained elevated as long as day 7. In adult underfed rats, the results were reversed and a decline in LH secretion was not detected until day 7 while desensitization in FSH secretion was detected on day 3. In peripubertal fed and underfed rats, LH and FSH secretion remained elevated on day 7, with gonadotropin responses being greater in underfed females (table 1). In man, however, an altered metabolic state does not alter the responsiveness to KP. As discussed earlier, Millar and colleagues [105] reported that chronically administered KP-10 (4 µg/kg/h) to healthy men triggered an increase in LH secretion (5.4 ± 0.7 to 20.8 ± 4.9 IU/l) without evidence of desensitization over a 22-hour period. When the same amount of KP-10 was infused intravenously for 11 hours into obese hypotestosteronaemic men with type 2 diabetes, an altered metabolic state, LH secretion also increased (3.9 ± 0.1 to 20.7 ± 1.1 IU/l) to an almost identical level [119]. Furthermore, like that seen in healthy men, there was no evidence of desensitization in LH secretion over the 11-hour period. Thus, in men displaying a perturbed afferent metabolic and endocrine input, KP responsiveness is preserved.

In conclusion, while a growing number of studies using naturally occurring and synthetic KP analogs show that chronic KP administration results in the desensitization of KP signaling, the temporal responses are dependent on a number of factors including ligand efficacy and concentration and the physiological, developmental, and metabolic status of the organism.

In the Reproductively Mature Female, KISS1R Signaling Is Modulated by Circulating Sex Steroids

In a study from the Terasawa laboratory [120], the authors showed that direct infusion of KP-10 (10 or 100 nM infused over 10 min) into the medial basal hypothalamus and stalk-median eminence region stimulated GnRH release in a dose-responsive manner in both prepubertal and pubertal ovary-intact

female rhesus monkeys. However, when the study was conducted in ovariectomized monkeys, the KP-10-induced stimulation of GnRH release was eliminated in pubertal but not prepubertal monkeys. Furthermore, estradiol add-back to ovariectomized pubertal monkeys resulted in partial recovery of the KP-10-induced GnRH release. Collectively, these results suggest that in the pubertal female monkey, likely at the level of the GnRH neuron, KISS1R responsiveness to KP is estradiol dependent. The idea that ovarian sex steroids modulate KISS1R responsiveness to KP is further seen in the following study from the Seminara laboratory [121]. There the authors characterized the effects of KP on GnRH secretion in healthy women in different phases of the menstrual cycle by intravenously administering KP-10 (0.24 nmol/kg) to women in the early follicular phase, the late follicular (preovulatory) phase, and the mid-luteal phase. The results revealed that the response to KP was dependent on the phase of the menstrual cycle where LH pulses were observed immediately after KP administration in all luteal and preovulatory women, but only half of the early follicular phase women exhibited clear KP responses. Like the study conducted in prepubertal and pubertal monkeys [120], in women, KISS1R responsiveness to KP also appears to be estradiol dependent since the early follicular phase is characterized as a period corresponding to low estradiol levels relative to the late follicular (preovulatory) and mid-luteal phases.

While the above studies suggest that estradiol positively regulates KISS1R signaling in the reproductively mature female, in a study by George et al. [122] the authors found that, compared with the early follicular phase, the gonadotropin response to intravenous KP-10 (0.3 μg/kg) is enhanced in sex steroid-deficient postmenopausal women and suppressed in women taking pharmacological doses of exogenous estrogen and progestogen in the form of sex steroid contraceptives. The authors stated that the diminished response to KP-10 in women taking a combined estrogen/progestogen contraceptive is consistent with an inhibitory negative feedback effect of estrogen at the pituitary level [123]. Clearly, further studies are required to better understand how KISS1R signaling is modulated by circulating sex steroid levels, whether such modulation occurs directly or indirectly, and how such modulation might be achieved mechanistically.

An Effective Neuroendocrine System Requires Protection from Rapid Desensitization

Disruption of the normal amplitude and/or frequency of gonadotropin secretion impacts negatively on fertility as reflected in the high LH pulse frequency in polycystic ovary syndrome and the low LH pulse frequency in hypothalamic amenorrhea. Thus, the neuroendocrine axis has evolved mechanisms that strongly protect against such disruptions. Such mechanisms are evident in the cell biology of the GnRH receptor, which is expressed on anterior pituitary gonadotropes. Gonadotropes maintain an active population of GnRH receptors at their surface so that the pituitary remains responsive to GnRH signals at all times. This is proposed to facilitate a protracted LH surge over several hours, an event required for ovulation in mammals [46]. The mammalian type I GnRH receptor mediates this by having an evolved resistance to homologous desensitization and internalization through the unique absence of a carboxyl terminal tail among GPCRs, which is required to mediate such events [83, 124--126]. Using radiolabeled GnRH, studies have determined that only about 25% of the ligand internalizes after 30 min at 37°C [127, 128], and while direct measurements of receptor internalization confirmed that GnRH-RI internalized very slowly the constitutive internalization rate was unchanged by the presence of GnRH where after 90 min at 37°C only about 25% of the receptor had internalized [46].

In the adult human male, GnRH undergoes pulsatile secretion every 2 h, as interpreted from LH pulsatility [129--139], while in the adult human female, depending on the stage of the menstrual cycle [140--146],

GnRH pulsatile release occurs every hour to as long as every 4--6 h [147]. Pulsatile GnRH secretion therefore seems to have evolved not just to encode specific patterns of gonadotropin secretion but also to safeguard against the desensitization and downregulation of a receptor that once activated remains signaling responsive in a healthy individual for a protracted period. Since the KP/KISS1R signaling system is upstream of GnRH secretion, it is reasonable to conclude that KISS1R must undergo prolonged signaling to support sustained pulsatile GnRH secretion, a requirement for prolonged GnRH signaling. As discussed previously, through the rapid desensitization, internalization, and recycling of resensitized receptors to the cell surface the cell maintains an active and dynamic population of KISS1R at the cell surface that facilitates prolonged KP signaling. Additionally, it is suggested that in vivo, at the GnRH neuron, by regulating the KP concentration and the duration of exposure of KISS1R to KP, signaling can be further sustained to protect against rapid desensitization of the neuroendocrine axis.

KISS1R: An Effective Clinical Target

Moving forward, in developing KP-based clinical therapies [148, 149] it is crucial to fully understand how to effectively administer native KP and analogs (pulsatile administration vs. chronic administration) to achieve a desired outcome (e.g. prolonged KISS1R signaling and elevated gonadotropins for infertility in contrast to termination of KISS1R signaling and diminished gonadotropin and steroid hormones for the treatment of hormone-dependent diseases). Delineating and understanding the dynamics of KISS1R signaling will assist in designing protocols to achieve these stimulatory and inhibitory regimens. This knowledge can then be applied in KP administration under physiological and pathological conditions.

With respect to initiating and prolonging KP signaling, KP must be administered in a manner that ensures pulsatile GnRH secretion that can in turn support the pulsatile gonadotropin secretion needed to maintain fertility. A study conducted on female rhesus monkeys revealed that KP and GnRH release in the stalk-median eminence, as assessed by the a microdialysis method, were both pulsatile and had a tendency to occur with similar timing, leading the authors to hypothesize that a pulsatile GnRH release is associated with pulsatile KP secretion [150]. As every GnRH pulse was not accompanied by a KP pulse (in contrast to GnRH pulses which exhibit very close coincidence with LH pulses), more comprehensive studies are required. Nevertheless, there is support for the conclusion that each GnRH pulse is a result of a KP pulse from studies in the goat [5]. These findings suggest that KP may have to be administered in a pulsatile fashion to trigger physiologically appropriate pulsatile gonadotropin secretion. However, as discussed earlier, Millar and colleagues [105, 106] found that nonpulsatile continuous administration of KP was sufficient to induce pulsatile release of LH. This raises the extraordinary and unanticipated possibility that the GnRH neuron can respond to continuous KP by pulsatile release of GnRH. However, as stated earlier, it is expected that KP signaling would eventually desensitize following chronic KP treatment; thus, therapeutic administration of KP aimed at stimulating reproduction, at least over short periods of 1 to a few days, may not require pulsatile administration unlike the therapeutic use of GnRH which has an absolute requirement for pulsatile delivery.

Based on observations that KP signaling desensitizes following continuous exposure to naturally occurring KPs (table 1), it was immediately apparent that this could be the basis for treating some diseases that require full suppression (e.g. in breast and prostatic cancer therapy) or partial suppression (e.g. in treating endometriosis, polycystic ovarian syndrome, and benign prostatic hyperplasia) of sex steroid levels. However, because the high susceptibility of KP-10 to enzymatic cleavage in vivo and the resulting short half-life [151] limit its potential use as a therapeutic agent, protease-resistant KP agonists that exhibit

improved pharmacokinetics and higher affinities than natural ligands have been developed (table 1) [108, 115--118].

Based on their analysis of two of the first KP-10 analogs developed, i.e. KISS1-305 and TAK-448, Matsui et al. [108] reported that continuous infusion of the analogs reduced testosterone levels to castrate levels in intact adult male rats within 3 days compared to chronic GnRH agonist (leuprolide) which required 14 days to achieve the same result. In healthy human males, continuous s.c. infusion of another KP analog, i.e. TAK-683 (which has properties similar to those of TAK-448), triggered subcastrate levels of testosterone by day 7, while in goats s.c. continuous infusion of TAK-683 suppressed LH secretion after as little as 1 day, with marked suppression by day 3 (table 1) [110, 113]. Because of the remarkable stability of the synthetic agonists in serum, just a single daily s.c. injection of TAK-448 and TAK-683 was sufficient to achieve desensitization of LH and testosterone secretion in intact adult male rats by day 4 (table 1) [111]. When TAK-448 was given s.c. to healthy male subjects aged 50 years or older, castrate levels of testosterone were achieved following 8 days of treatment (table 1), and when given as a 1-month depot formulation to prostate cancer patients near-maximum suppression in testosterone levels was achieved by day 10 (table 1) [112].

It is important to note that while the above studies clearly demonstrate the ability to downregulate the neuroendocrine-reproductive axis through chronic exposure to high-affinity and more stable KP agonists, either as continuous infusions or as single daily injections, these studies also clearly reveal that the kinetics of KISS1R desensitization can be modulated by ligand affinity, efficacy, stability, and concentration [108, 110--114]. Therefore, as we continue to develop KP-based therapies, attention must be paid to ligand delivery (continuous vs. pulsatile), efficacy, and concentration.

Conclusion

This review discussed KISS1R recruitment of intracellular signaling pathways that mediate GnRH secretion as well as the dynamics of ligand-induced internalization of KISS1R, degradation and recycling, and desensitization/uncoupling. These deliberations have illustrated that mechanisms have evolved for the cell to potentially prolong KP signaling as has been found for GnRH signaling. Specifically, we described how at the receptor level, by undergoing rapid desensitization, internalization, and continuous recycling of resensitized receptors, coupled to the trafficking of nonrecycling KISS1R to the cell surface, the cell maintains an active and dynamic pool of activatable KISS1R at its surface, resulting in prolonged KP signaling. We also suggest that the KP concentration and the duration of exposure of KISS1R to KP at the GnRH neuron might be regulated to further sustain KP signaling and protect against rapid desensitization of the neuroendocrine axis. As for the type I GnRH receptor, sustained signaling appears necessary to facilitate a protracted LH surge over several hours, an event required for ovulation in mammals. Studies with KP antagonists [152, 153] have indicated that both pulsatile LH and the LH surge of ovulation are dependent on KP but the mechanism underlying the shift from pulsatile LH to the surge is not known [152]. We have also discussed how KISS1R couples to β-arrestin in addition to coupling to and signaling by Gαq/11, and that both pathways mediate KP-dependent GnRH secretion. However, the specific roles of these two signaling pathways have not been resolved and this may be a valuable undertaking for future research. It is plausible that regulation of GnRH secretion by dual KISS1R-mediated signaling provides a means to maintaining some degree of GnRH secretion and fertility should one signaling pathway become inactivated in physiological and pathological scenarios. Finally, we considered how different therapeutic modalities of administration of KP and super active analogs may be used to stimulate or inhibit the

reproductive hormone cascade, and how the outcomes may differ from targeting the type I GnRH receptor.

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Appendix after References (Editorial Comments)

Legend(s)

Fig. 1. Model of KISS1R signaling and trafficking in the cell. Numbers in parentheses refer the reader to specific illustrated events. At the cell surface, in the absence of KP, KISS1R couples to Gαq/11 (A) and exhibits KP-independent (basal) Gαq/11 signaling (as determined by IP formation) (1). In the presence of KP (2), KISS1R (B) exhibits increased Gαq/11 signaling (as determined by IP formation) (3) but through the rapid actions of GRK2 (4) and β-arrestin, which is recruited from the cytosol to the plasma membrane following KP treatment (5), KISS1R becomes uncoupled from Gαq/11 (desensitization) (C) and IP formation is terminated (6). In addition to uncoupling KISS1R from Gαq/11, β-arrestin simultaneously traffics the desensitized KISS1R to the clathrin-coated pit, resulting in sequestration (7). The sequestered receptor, which remains associated with β-arrestin, undergoes β-arrestin-dependent signaling (8) in addition to internalization via clathrin-coated vesicles (9). Though not depicted here, KISS1R also undergoes KP-independent internalization [45, 51]. β-Arrestin eventually dissociates from internalized KISS1R (10) and the resensitized KISS1R traffics back to the cell surface (11), while a lesser population of KISS1R and KP are targeted for degradation (12). The population of cell surface signaling-competent KISS1R (D) is derived from recycling (11) receptors as well as nonrecycling receptors (13). The net result of KISS1R undergoing rapid uncoupling from Gαq/11, internalization, and continuous recycling to the cell surface, coupled to the trafficking of nonrecycling KISS1R to the cell surface, is that the cell maintains a signaling-competent and dynamic pool of KISS1R at its surface (A and B), resulting in prolonged KP-dependent signaling. Chronic KP signaling is also absolutely dependent on the sustained influx of extracellular Ca2+, which in turn is dependent on the continued presence of exogenous KP and dynamic trafficking of KISS1R (14). CCP = Clathrin-coated pit; CCV = clathrin-coated vesicle.

Fig. 1, Millar and Babwah

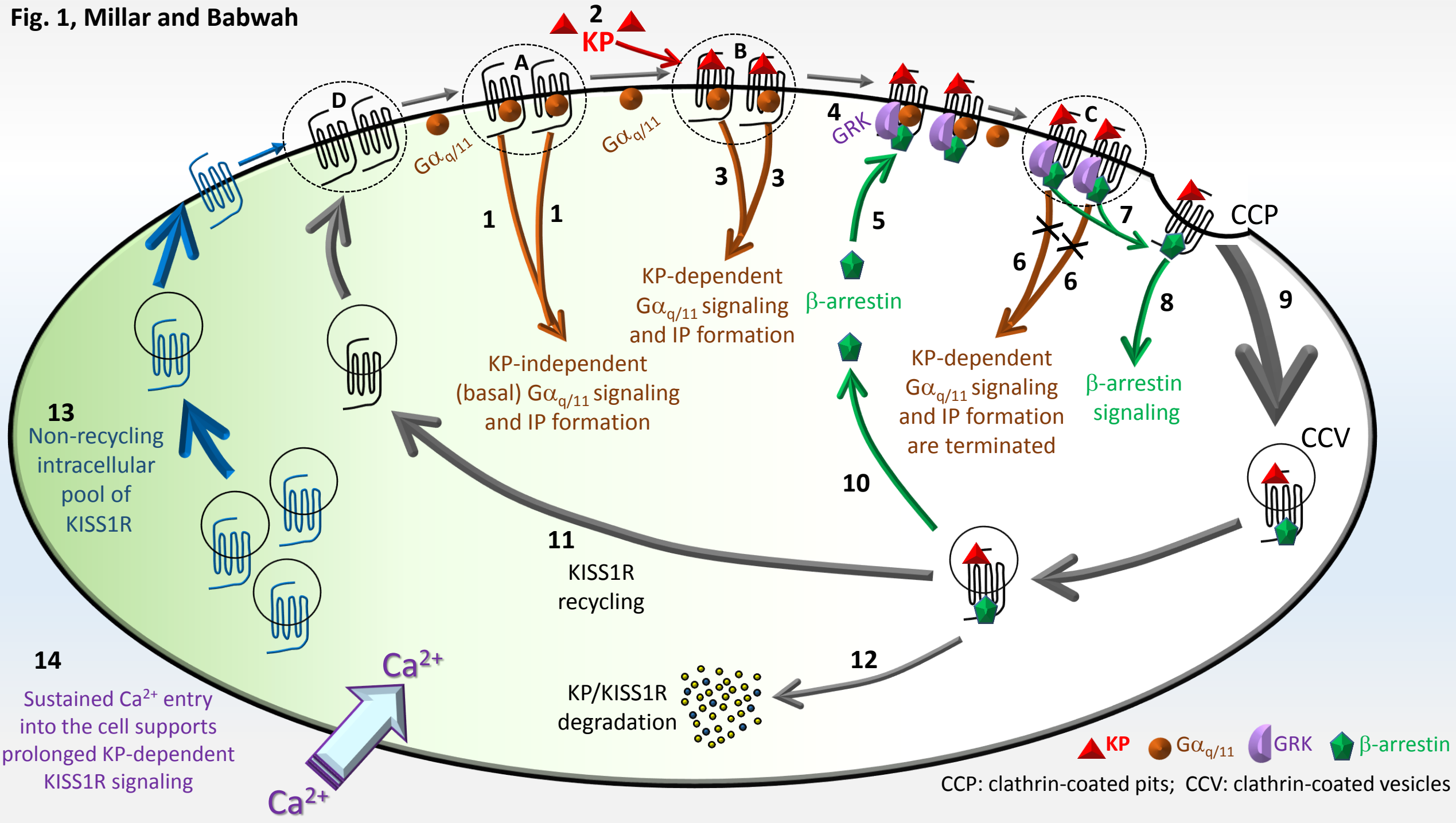

Table 1. Effect of chronic and repeated KP [naturally occurring and stable synthetic agonists] exposure on KP-dependent signaling events

Animal species/cell line	KP administration	KP type and dose	KP-dependent signaling event used for assessing desensitization	Time-point at which desensitization was detected following start of treatment	Reference
Naturally-occurring KPs- *in vivo* studies					
Ovariectomized ewes treated with estradiol implants	Continuous, icv with portable syringe pump	KP-10; flow rate 3 μl/min; concentration of 0.2 μg/min (~9.2 nmol/hr); ~ 37 nmol administered over 4 h infusion period	GnRH and LH secretion; every 10 min	LH: detected within 1-2 h; GnRH: not detected during 4 h infusion period	[100]
Adult male rats	Continuous, sc infusion; Osmotic mini-pumps	KP-54; flow rate 1 μl/h; total of 50 nmol/d (~2 nmol/h)	LH and testosterone secretion measured on d2	Detected after 2 d, but not after 1 d	[99]
Agonadal juvenile male monkeys	Continuous, iv infusion;	KP-10; 100 μg/h (~ 77 nmol/h) for 98 h (~7.5 mmol/98 h)	LH secretion; during first 12 h of continuous infusion at 10, 20, 30, 50, 70, 90, 110, 130, 150, 170, 360, and 720 min into the infusion	Detected after 1-2 h	[68]
Male rats	Repeated administration (four boluses 75 min apart)	KP-10; 30 nmol/kg body wt	LH secretion, every 15 min	Not detected over 5 h assay period	[101]
Gonad-intact adult male monkey	Continuous, iv infusion	KP-10; 200 μg/h (~154 nmol/h) or 400 μg/h (~ 308 nmol/h) for 98 h	LH secretion; during the first 12 h of continuous infusion at 10, 20, 30, 50, 70, 90, 110, 130, 150, 170, 360, and 720 min into the infusion	Detected after 2-3 h	[102]

Female rats (a) adult fed and underfed; (b) peripubertal (PP) fed and underfed	Continuous, icv with osmotic mini-pump pump	KP-10; flow rate: 1 μl/h; 7.5 nmol/d (~0.3 nmol/h)	Start of treatment: D0. Adults: LH and FSH; one measurement/day. PP: single LH/FSH measurements on D7	Adult fed/LH: detected on D3; Adult fed/FSH: not detected by D7. Adult underfed/LH: detected on D7; Adult underfed/FSH: detected on D3. PP fed/LH and FSH: not detected by D7. PP underfed/LH and FSH: not detected by D7 and responses were greater than PP fed on D7.	[103]
Women with hypothalamic amenorrhea	Intermittent administration; data suggest that high KP levels are maintained between injections	KP-54; twice daily iv injections; 6.4 nmol/kg/injection	LH, FSH and estradiol secretion; 11 readings taken over a 240-min period after first and last kp injection on d1 and d14	Detected on d14, but not before first 6 h after first injection on d1	[104]
Healthy men	Continuous iv infusion	KP-10; 4 μg (~3 nmol)/kg/hr	LH secretion; every 10 min during wake period and every h during sleep period	Not detected after 22.5 h (and up to 36 h, unpublished data)	[105]
Male patients with loss-of-function mutations in NKB (TAC3) or its receptor (TAC3R)	Continuous iv infusion	KP-10 1.5 μg (~1.2 nmol)/kg/h	LH and FSH secretion; every 10 min for 12 h over 2 consecutive d	Not detected after 12 h	[106]

Naturally-occurring KPs- *in vitro* studies

Mouse mediobasal hypothalamic explants	Continuous incubation	KP-10; 50 nM	GnRH secretion; measured every h over 6 h assay period	Detected after 5-6 h	[107]
CHO cells stably expressing KISS1R	Continuous incubation	KP-10; 100 nM	Total IP formation following 1,2 4, 12 and 18 h treatment of cells	Detected after 2 h; presumably occurred earlier	[90]
GT1-7 and HEK 293 cells transiently expressing KISS1R	Continuous incubation	KP-10; 100 nM	Spatiotemporal properties of PLCβ and PKC activation and intracellular Ca^{2+} mobilization	Detected between 45-90 min	[96]

Synthetic KP agonists- *in vivo* studies

Adult male rats	Continuous sc infusion with osmotic mini-pump	KISS1-305: 0.2-4.0 nmol/h; TAK-448: 0.1 nmol/h	Testosterone secretion	KISS1-305: Castrate levels achieved by d3 at 1-4 nmol/h; TAK-448: detected by d3 and depleted testosterone content by wk 4	[108]
Healthy human males	Administered as a single sc dose on D1 followed by a 13 d continuous infusion via an ambulatory pump from D2 to D14.	TAK-683: single sc dose on D1 (0.03-1.0 mg), followed by continuous infusion from D2-14 (0.01-2.0 mg/d) to simulate a depot formulation	Testosterone secretion	Sub-castrate levels achieved by D7 with D2-14 concentrations of 2.0 mg/d	[110]

Adult male rats	single daily sc injection; and continuous sc infusion with osmotic mini-pump	TAK-448 and TAK-683 (0.008-8μmol/kg) administered for 7 d. Continuous sc infusion of TAK-448 (about 0.7 nmol/kg/day) and TAK-683 (about 2.1 nmol/kg/day)	LH and testosterone secretion	Detected by D4 with repetitive single sc injections. With continuous infusion, castrate levels of testosterone detected within 3-7 d.	[111]
Healthy human male subjects aged 50 years or older and prostate cancer patients aged 40-78 years,	Healthy subjects: 14 d sc administration; D1: bolus injection; D2-14: continuous infusion using a standard insulin infusion pump. Cancer patients: 1 mth depot formulation.	TAK-448; healthy subjects: on D1 given 0.1 mg sc bolus; on D2-14-day sc infuion (total daily doses of 0.01, 0.1, 0.3, or 1 mg/d); prostate cancer patients: 1-mth depot formulation (6, 12, or 24 mg)	Testosterone secretion	Healthy subjects: at doses above 0.1 mg/d, sub-baseline testosterone levels achieved by 60 h and sub-castration level by d8. Cancer patients: significant decline in testosterone levels detected by D10 in all patients dosed with 12 or 24 mg TAK-448 sc-depot injections. Suppression was maintained in 4/5 patients during the treatment period.	[112]
Goats	Continuous sc infusion; osmotic mini-pumps	TAK-683; 0.03, 0.3 or 3 nmol/h/kg; flow rate 10 μl/h	LH secretion; every 3 h	Detected in as little as 1 d and with marked suppression by d3	[109]