THE MULTIPLE FACETS OF KISSPEPTIN ACTIVITY IN BIOLOGICAL SYSTEMS

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THE MULTIPLE FACETS OF KISSPEPTIN ACTIVITY IN BIOLOGICAL SYSTEMS

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In 1996 a cDNA called *KISS1* (KI in reference to the place of discovery-Hershey Pennsylvania, home of the famous Hershey Chocolate Kisses- and SS as suppressor sequence) was identified in non-metastatic melanoma cell lines. Its 54 amino-acid product, Kisspeptin-54 (Kp-54), was originally called metastin for its ability to inhibit cancer metastasis through the activation of a G coupled receptor, previously known as GPR54 and currently renamed the Kisspeptin receptor (KISS1R). Shorter active peptides all capable of binding to KISS1R have been identified and the physiological activities of these Kisspeptins are now known to extend not only to the suppression of metastasis.

Kisspeptins currently represent evolutionarily conserved biological modulators, with a recognized role in the central control of sex maturation, reproduction and fertility. Consequently, the focus on the central role of the Kisspeptins has led to neglecting their possible activities in peripheral tissues. Increasing data reveals that Kisspeptins and KISS1R have a wider expression and possibly a broader spectrum of action in several peripheral tissues such as the gonads, adipose tissue, and liver with direct consequences on gamete quality and fertility rate, pregnancy, energy homeostasis and body weight control.

In this respect, the Kisspeptin system may represent a promising prognostic/diagnostic biomarker and therapeutic target for cancer and other human diseases such as infertility and metabolic disorders.

This Research Topic provides a comprehensive picture of the recognized and the emerging role of the Kisspeptin system. Such a volume is very timely and useful to the wide community of researchers in the field and for the scientific community at large.

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SECTION IV

EXTRA-HYPOTHALAMIC ACTIVITY OF KISSPEPTIN IN MAMMALIAN AND NON MAMMALIAN VERTEBRATES

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EDITORIAL
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Editorial: The Multiple Facets of Kisspeptin Activity in Biological Systems

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Editorial on the Research Topic

The Multiple Facets of Kisspeptin Activity in Biological Systems

In 1996, as part of a screen for anti-metastatic genes, a novel gene termed *Kiss1* was found to be expressed in non-metastatic melanoma cell lines (1). Its 54 amino-acid product, Kisspeptin-54 (Kp-54), was originally called metastin for its ability to inhibit cancer metastasis through the activation of a G protein coupled receptor, previously known as GPR54, and currently renamed the Kisspeptin receptor (KISS1R) (2, 3).

Kp-54 is the longest cleavage product of the Kisspeptin precursor protein, but there are shorter active peptides [i.e., Kp-14, Kp-13, and Kp-10], all capable of binding to KISS1R (3). Since the tissue distribution of KISS1R was very similar in mammalian and non-mammalian vertebrates, especially in the brain, it was suggested that kisspeptins not only acted as metastasis suppressors, but also were a new family of evolutionarily conserved biological modulators (4).

In both rodents and humans, genetic ablation or inactivating mutations of the *Kiss1*/*Kiss1R* genes cause lack of sexual maturation and hypogonadotropic hypogonadism. Conversely, functionally activating mutations of *Kiss1*/*Kiss1R* genes cause precocious puberty (5, 6). Thus, most studies have focused on the involvement of Kisspeptin activity in the central control of reproduction, through the regulation of hypothalamic Gonadotropin Releasing Hormone (GnRH) neurons, which depends on the hormonal milieu, energy homeostasis, and environmental factors (7).

The twelve articles in this Research Topic provide a comprehensive insight into the wider physiological actions of Kisspeptin beyond the reproductive system including cancer, metabolism and neuroscience and also highlight the role of Kisspeptins in non-mammalian species.

Two mini reviews and a review article focus on Kisspeptin and cancer suggesting that the Kisspeptin system is a potential therapeutic agent and/or a prognostic marker for some types of cancer. Ciaramella et al. summarize the latest data concerning the role of Kisspeptin signaling in the suppression of metastasis in cancer, whereas Fratangelo et al. point out additional Kisspeptin activity in tumors especially those with drug resistance. Indeed, new evidence reveals that Kisspeptins can exhibit dual roles in cancer either acting as suppressors of tumorigenesis and metastasis in many cancers or as enhancers in others. In this respect, Guzman et al. highlight the importance of studying cancer in context with attention toward the micro-environment and the steroid receptor status of the cancer cell.

Moving toward the hypothalamic activity of Kisspeptin, the review submitted by Terasawa et al. concerns the contribution of Kisspeptin and Neurokinin B (NKB) signaling in the pubertal increase in GnRH release in female non-human primates. Reciprocal signaling pathways between Kisspeptin and NKB neurons are indispensable to facilitate the pubertal increase in

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GnRH necessary for the reproductive function in females. In contrast, Kisspeptin as the gatekeeper of reproduction and sex maturation in non-mammalian vertebrates which exhibit more complex GnRH and Kisspeptin systems with multiple forms of ligands and receptors (4, 8), is not as clear as in mammals. In this respect, Ohga et al. summarize Kisspeptin studies in the teleost chub mackerel and elucidate the possible role in fish reproduction and gonadal development. Furthermore, the research article by Pasquier et al. concerns the cloning, sequencing, and differential expression of *Kiss1* and *Kiss2* in both the brain and peripheral tissues from the European eel. A dual inhibitory effect of homologous Kisspeptins on the expression rate of both pituitary LHβ and GnRH receptor2 has been also reported.

A large body of data has established that KISS1 neurons are capable of integrating information about the hormonal milieu, environmental factors, stress signals, metabolism and energy balance, and conveying this information to GnRH secreting neurons. Consequently, the focus on the central role of the Kisspeptins has led to neglecting their possible activities in peripheral tissues. Increasing data reveal that Kisspeptins and KISS1R have a wider expression and possibly a broader spectrum of action in several peripheral tissues such as the gonads, adipose tissue, and liver with direct consequences on gamete quality and fertility rate, pregnancy, energy homeostasis, and body weight control (9).

These points have been fully assessed in this Research Topic. The intricate neuronal networks and the related environmental factors capable to modulate the reproductive axis via KISS1 neurons have been summarized by Yeo and Colledge. These authors also describe the main experimental approaches for investigating functional inputs to KISS1 neurons in the arcuate nucleus. Furthermore, the regulation of the hypothalamic Kisspeptin-KISS1R signaling by metabolic cues and in other situations of energy imbalance like diabetes and obesity has been extensively analyzed by Wahab et al. The direct impact of Kisspeptin on peripheral metabolic tissues has only recently been recognized and emerging data from animal models and clinical studies have been summarized in two review articles. The first one, by Wolfe and Hussain, focuses on the endocrine role of Kisspeptin in the central and local regulation of metabolic functions; the participation of liver-derived Kisspeptin in islet hormone cross-talk and the peripheral sources of circulating Kisspeptin (i.e., placenta and adipose tissues). The second one by Dudek et al. focuses on the local activity of Kisspeptin in peripheral organs such as the pancreas, liver and adipose tissue and the dysregulation of the Kisspeptin system in metabolic diseases (e.g., obesity and diabetes) in humans, situations that are often linked to reproductive disorders and infertility.

Finally, this Research Topic closes with a focus on extra-hypothalamic activity of Kisspeptin in both mammalian and non-mammalian vertebrates. In particular, the review by Ogawa and Parhar provides a survey of Kisspeptin signaling within the habenula of fish, the brain area involved in the neuromodulatory processes of emotional and goal-directed behaviors. Lastly, Stephens and Kauffman summarize what is currently known about the regulation, development, neural projections, and potential functions of Kisspeptin neurons located within the medial amygdala and discuss both the related signaling and the possible implication in many diverse functions and behavioral processes.

Taken together, this Research Topic fills several gaps in Kisspeptin knowledge and provides exciting tools for future directions devoted to the use of Kisspeptin as prognostic/diagnostic biomarkers and therapeutic target for the treatment of cancer and other human diseases like infertility in both sexes and metabolic disorders.

AUTHOR CONTRIBUTIONS

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

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MINI REVIEW
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Kisspeptin and Cancer: Molecular Interaction, Biological Functions, and Future Perspectives

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Cancer disease is the second leading cause of death in the world and one of the main fields of medical research. Although there is now a greater understanding of biological mechanisms of uncontrolled cell growth, invasiveness and metastasization, the multi-step process of cancer development and evolution is still incompletely understood. The inhibition of molecules activated in cancer metastasization is an hot topic in cancer research. Among the known antimetastatic genes, KiSS-1 is involved in the metastatic cascade by preventing growth of metastasis. Moreover, loss of KiSS-1 protein expression by tumor cells has been associated with a more aggressive phenotype. *KiSS-1* gene encodes a 145-amino acid protein, which following proteolytic cleavage, generates a family of kisspeptins (Kp-10, -13, and -14), that are endogenous agonists for the G-protein-coupled receptor (GPR54). The antitumor effect of KiSS-1 was primarily associated with the inhibition of proliferation, migration and cell invasion and, consequently, the reduced formation of metastasis and intratumoral microvessels. In this review, we highlight the latest data on the role of kisspeptin signaling in the suppression of metastasis in various cancer types and the use modulators of KiSS/GPR54 signaling as potential novel therapeutic agents for the treatment of cancer.

Keywords: kisspeptin, metastasis, cancer, GPR54, prognostic biomarkers

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INTRODUCTION

Cancer is defined as a heterogeneous group of diseases, characterized by uncontrolled cell growth.

Currently, cancer disease is still one of the main causes of death, 7.6 million cancer deaths occurred in 2008 and it is expected that cancer related mortality could reach 13.1 million deaths by 2030 (1). The cancer mortality rate is caused in 90% of cases but the development of metastasis, that are the clinical result of the extremely invasive behavior of cancer.

Metastasization is the process through which primary tumor cells colonize distant second sites: a tumor cell that grows in a microenvironment, in order to become able to move and proliferate in another location, begins a chain of events defined as a "metastatic waterfall." In particular, uncontrolled cell growth and tumor progression are the result of a multi-step complex process including inactivation of tumor suppressor genes. Tumor cells are characterized by epithelial to mesenchymal transition (EMT), a complex biological and morphological change that reduce their dependency from intracellular connection. Thus, mesenchymal tumor cells are able to infiltrate distant organs escaping the recognition from the immune system, until they create a macroscopic mass in second sites (2).

In the recent years, oncology research has focused its interest in identifying the metastases suppressor genes. At present, about 30 metastasis suppression genes have been isolated which provide useful candidates for cancer specific therapeutic strategies involving gene transfer, gene expression induction, exogenous administration of a genetic product, targeting of metastasis suppressors, and signaling (3).

Among the known metastasis suppressor molecules, in this review, we highlight the KiSS-1 data suggests that its unique mechanism of action is capable of delaying the metastatic cascade by preventing growth and colonization of metastatic cells in distant sites. This mechanism is different from other antimetastatic genes, which block cellular detachment and migration from the primary tumor (4). The *KiSS-1* gene is located in the long arm of human chromosome six and encodes a 145-amino acid protein, which is subsequently cut to generate a C-terminal amidated 54-amino acid peptide kisspeptin-54 (Kp-54, called metastin after its capability to blockade metastasis), that is further cleaved into even shorter peptides (Kp-10, -13, and -14 amino acids long), generally defined as kisspeptins (Kps). All C-terminal cleavage fragments Kp-54, -14, -13, and -10 possess biological activity and are the endogenous ligands for the G-protein-coupled receptor (GPR54) (5) (**Figure 1**). Furthermore, it is demonstrated that treatment with KiSS-1 derived polypeptides reduced metastasis in mice engrafted with melanoma cells with GPR54 over-expression (6).

In this review, we present findings from preclinical and clinical settings and confer strategies, whereby KiSS-1 and its receptor GPR54 mediated signaling pathway which may be exploited as anticancer therapy in metastatic cancers.

KISSPEPTIN IN CANCER PROLIFERATION AND METASTASIZATION

The antimetastatic action of the Kp was observed for the first time in human melanoma cell lines. The experiments based on hybridization and differential display showed the presence of an unregulated gene in cells transfected with chromosome six and this gene acted as a suppressor of metastasis; in fact, mapping demonstrated that this gene was not localized on chromosome six but on chromosome 1q32, and it was the *KiSS-1* gene (7, 8). Activation of the Kps/GPR54 system has been demonstrated to have a multiplicity of effects on cancer cell biology, including suppression of motility, culture scratch repair, proliferation, metastasis, and invasion of human cells *in vitro*. Until now, the specific mechanism for the antimetastatic function of Kps is still uncertain but various intracellular signaling pathways triggered by Kp have been identified that might be involved (9).

GPR54 belongs to the group A G-protein-coupled receptors that are associated with Gq/11, determining activation of the phospholipase C (PLC) signaling pathway and subsequently Ca_2 recruitment. Stimulation of GPR54 by Kps also leads to phosphorylation of focal adhesion kinase (FAK) causing the formation of excessive focal adhesions and stress fibers (5), thus, explaining the action of Kps on inhibiting chemotaxis. Moreover, GPR54 activation reduces calcineurin activity (10), which contributes to the metastasis suppression. Until now, the antimetastatic activity of Kps has been demonstrated in several tumors including melanoma (6), thyroid, ovary, bladder, gastric, pancreas, and lung cancers (10–15). For some of these tumors, a unified antimetastatic mechanism regulated by the Kps/GPR54 system is related to the inhibition of the activity of the matrix metallo proteinase 9 (MMP-9) and the consequent blockade of tumor cell migration and invasion (16).

A molecular mechanism of Kp action is an inhibition of cellular proliferation during intracellular Ca^{++} replaces discharge with release and activation of protein kinase C (17). A possible mechanism of Kp action is through an increase in intracellular Ca^{++} which inhibits cell proliferation and increases cell differentiation and apoptosis. Thus, *KiSS-1* gene acts as a cancer suppressor gene

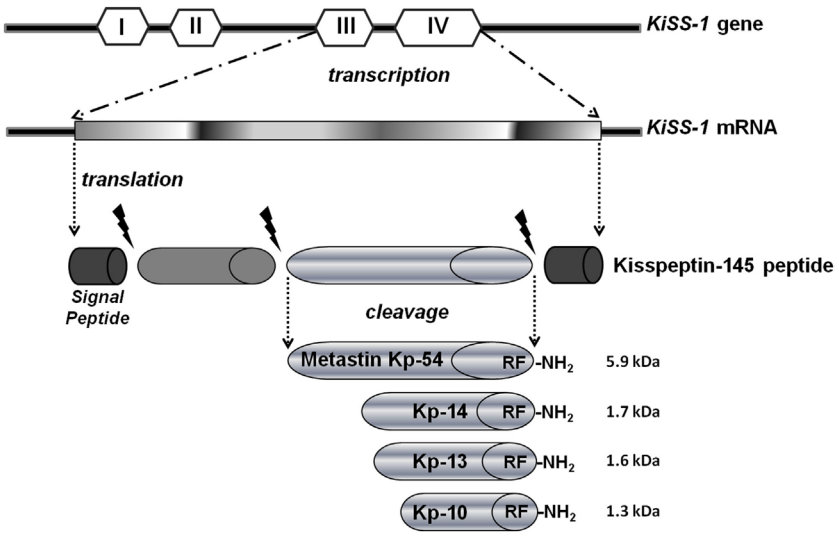

FIGURE 1 | Kisspeptins (Kps) formation.

and activation of Kp signaling cascade inhibits cancer cell invasion, metastasis formation, and tumor recurrence (13).

Contradictory information on the function of KiSS-1 and GPR54 in carcinogenesis may derive from the presence of alternative forms of these genes or their different epigenetic regulation. There is currently no confirmed data on the methylation status of *KiSS-1* and *GPR54* genes in cancer, but the study of this epigenetic modification could potentially elucidate the variations in expression patterns examined in different studies on human cancer tissues, as subsequently described (**Figure 2**).

EXPRESSION OF KiSS-1 AND GPR54 IN HUMAN CANCERS

According to various clinical data, a decrease in KiSS-1 and/or GPR54 expression has been shown to be associated with poorer prognosis in cancer patients and, therefore, the expression of KiSS-1 and/or GPR54 might be useful predictive biomarkers in medical outcomes.

From a case study on gastric patients, classified as low or high KiSS-1 expression levels, a down regulation of KiSS-1 was associated with recurrent cancer invasion and linked to shorter survival in several studies, suggesting that KiSS-1 could develop into a novel negative predictive factor for gastric cancer (13).

Similarly, in ovarian cancer patients, lower *KiSS-1* gene expression was related to more resistant ovarian cancers, cell invasion, the presence of macroscopic residual tumor following surgical resection, and the patient's worse prognosis (11).

Similar data were detected in an analysis of urinary bladder cancer samples: loss of KiSS-1 expression was found in all invasive cancers and the surrounding normal uroepithelium showed higher levels of KiSS-1 expression. Moreover, KiSS-1 expression was also shown to be negatively correlated with the histopathological stage, with lower expression of the *KiSS-1* gene observed in cases with positive vascular invasions (12), confirming that KiSS-1 expression having a predictive value and correlating with negative prognostic features and worse patients' outcome. In esophageal squamous cell carcinoma, lower KiSS-1expression is found in more of 85% of tumors with lymph nodes metastases, without any correlation with local invasion or primary tumor size, reinforcing the idea that Kp signaling plays a role in metastatic diffusion of cancer also in esophageal carcinoma (18).

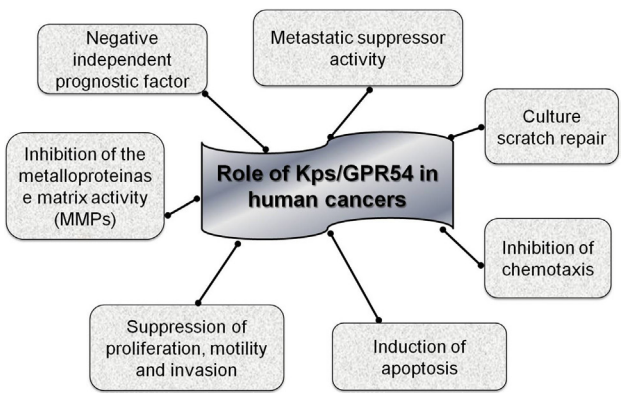

FIGURE 2 | Kps/GPR54 in human cancer.

A role of Kps has also been shown in breast cancer although the results are somewhat contradictory. Some chapters show that KiSS-1 mRNA and protein expression are deficient in node-positive cancers, and establish a considerable negative association with axillary lymph node attachment (19). In contrast, the results obtained by another study of KiSS-1 expression in high-risk breast cancer patients on surgical samples support the hypothesis that the *KiSS-1* gene is a metastasis suppression gene. In fact, it has been observed that the KiSS-1 transcriptional activity is present in node-negative breast cancer patients, primary breast cancer, and metastatic deposits, but higher expression, due to the molecular alteration, occurs essentially in localized tumors with no nodal involvement, thus confirming that restoring the function of the *KiSS-1* gene could be a promising approach to stop micrometastatic growth and prevent metastatic diffusion in distant sites (20). In other reported cases, the level of KiSS-1 mRNA and protein was higher in primary localized breast tumors than in breast cancer that was metastasized to other sites such as in the brain.

In contrast, a reduction of KiSS-1 expression in brain metastases has observed, thus suggesting that a loss of KiSS-1 may influence the formation of distant metastases; we speculate that further investigations on KiSS-1 expression levels in metastastic lesions and primary tumors in breast cancer patients could confirm the antimetastatic role of Kp (21).

No clinical data are available with regard to the correlation between KiSS-1 and GPR54 expression levels and the resistance to treatment. Only in metastatic prostate cancer patients, a preliminary data that increased expression of KiSS-1 is able to sensitize cancer cells to chemotherapies (22, 23); in the same setting of patients, expression of KiSS-1 is inversely correlated with tumor differentiation and clinical staging.

PROMISING ROLE OF KISSPEPTIN IN CANCER THERAPEUTICS

Several reports have attempted to demonstrate whether the secretion of Kps is necessary for metastasis inhibition. It has recently been shown that in cell lines, after transfection and blockade generation of metastases, the expression of the *KiSS-1* gene induces the production of the GPR54 receptor.

This autocrine feedback signal could represent a mechanism of perpetuation of antimetastatic effect of Kp (24).

The primary cancer cell lines derived from skin and lung had different levels of GPR54 expression, particularly, it has been demonstrated that lung cancer cells produce more growth inhibitory signals than skin cell cancer from primary cultures. These results confirm why melanoma cells expressing KiSS-1 are able to grow in the skin, but fail to develop after they have already distributed (25, 26).

Since the active peptides of KiSS-1 are secreted proteins, their effects can be simulated experimentally by delivery to the bloodstream. The idea that these molecules, Kps and synthetic derivatives, can be used in clinical setting as drug is very promising because as they are natural peptides, they should not generate

serious toxic effects in humans (27). For example, their application could be compared to the use of insulin in diabetic patients.

A therapeutic option for use of Kps as treatments in humans could be tested if their injection in the human body could reach a systemic distribution and access tumor cells. The administration of Kps for therapeutic purposes has already verified as safe in humans: in fact, a single dose of Kp-54 administered subcutaneously did not cause any significant adverse effects. However, regular administration of this dose could affect the normal endocrine function and beginning of puberty due to the activation of the release of gonodotropin-releasing hormone (GnRH) (28). KiSS-1-based treatments could be hypothetically simple if metastatic tumor cells express the KiSS-1 receptor; but the limitation is that most tumor cells do not express GPR54, so it could be a challenging aim for future researches to test multiple cancer treatment with *KiSS-1* gene in the absence of its potential biomarker GPR54 (24).

Collectively, available preclinical data suggested that induction of *KiSS-1* expression gene and the therapeutic use of Kps could block metastasization. In particular, the current hypothesis is that KiSS-1 expression alone might have the ability to inhibit metastatic growth in multiple organs, by targeting disseminated cells and their interactions with the microenvironment, thus deserving future studies for Kps as novel potential anticancer molecular agents.

AUTHOR CONTRIBUTIONS

VC and CD wrote the chapter and made bibliography research. FC and FM revised the manuscript.

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Controversial Role of Kisspeptins/KiSS-1R Signaling System in Tumor Development

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KiSS-1 was first described as a metastasis suppressor gene in malignant melanoma. *KiSS-1* encodes a 145 amino-acid residue peptide that is further processed, producing the 54 amino acid metastin and shorter peptides collectively named kisspeptins (KPs). KPs bind and activate KiSS-1R (GPR54). Although the KPs system has been extensively studied for its role in endocrinology of reproductive axis in mammals, its role in cancer is still controversial. Experimental evidences show that KP system exerts an anti-metastatic effect by the regulation of cellular migration and invasion in several cancer types. However, the role of KPs/KiSS-1R is very complex. Genomic studies suggest that KiSS-1/KiSS-1R expression might be different in the various stages of tumor development. Furthermore, overexpression of KiSS-1R has been reported to elicit drug resistance in triple negative breast cancer. In this review, we focused on multiple functions exerted by the KPs/KiSS-1R system in regulating tumor progression.

Keywords: kisspeptin, KiSS-1R, tumor development, tumor invasion, metastasis

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INTRODUCTION

KiSS-1 gene, located on human chromosome 1q32, encodes a precursor peptide of 145 amino acids that subsequently is processed by proteolytic cleavage into shorter peptides collectively defined as kisspeptins (KPs): KP-10, KP-13, KP-14, and KP-54 (metastin). KPs bind to G-protein-coupled receptor 54 (GPR54) also named KiSS-1R that activates the G proteins $G\alpha_{q/11}$ (1, 2).

High levels of KiSS-1 have been found in the placenta and in the brain (3) and high expression levels of KiSS-1R were observed to in the placenta, pituitary, pancreas, and spinal cord (2). Low levels of KiSS-1R are present in lymph nodes, peripheral blood lymphocytes, adipose tissue, and spleen (1, 3).

KiSS-1/KiSS-1R coupled to $G\alpha_{q/11}$ activates phospholipase C (PLC). PLC activation promotes the hydrolysis of phosphatidylinositol-4,5-bisphosphate which, in turn, leads to the production of two potential "second messengers" inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The activation of DAG leads to the activation of protein kinase C, ERK1/2, and p38 phosphorylation, while IP3 induces release of intracellular Ca^{++} (2, 4–6) (**Figure 1**).

G-protein-coupled receptors are regulated by β-arrestins, which not only desensitizes G-protein signaling, but also acts as molecular scaffolds and activates a series of signaling pathways including ERK 1/2, p38, PI3K/Akt, and cJun N-terminal kinase 3 (7, 8).

G-protein-coupled receptor serin/threonine kinase GRK2 and β-arrestins promote KiSS-1R desensitization by internalization *via* clathrin-coated pits (9, 10).

The anti-metastatic role of KP has been identified by early studies performed in melanoma and breast cancer cells before its role in regulating the reproductive functions. Although the

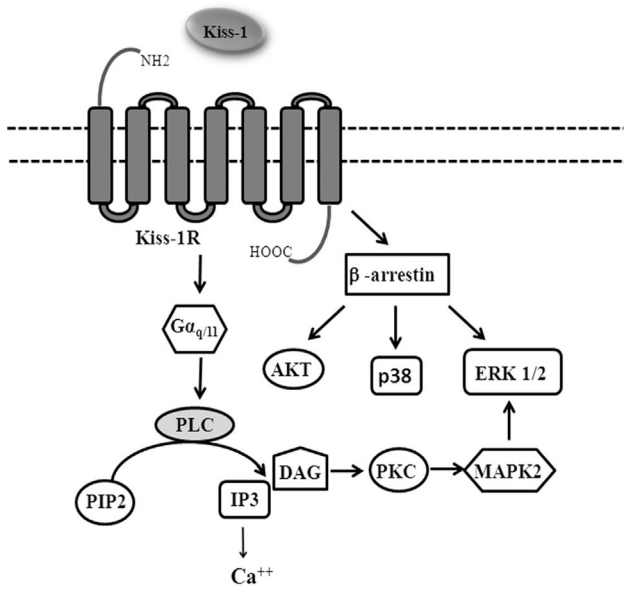

FIGURE 1 | Signaling pathways activated by KiSS-1R.

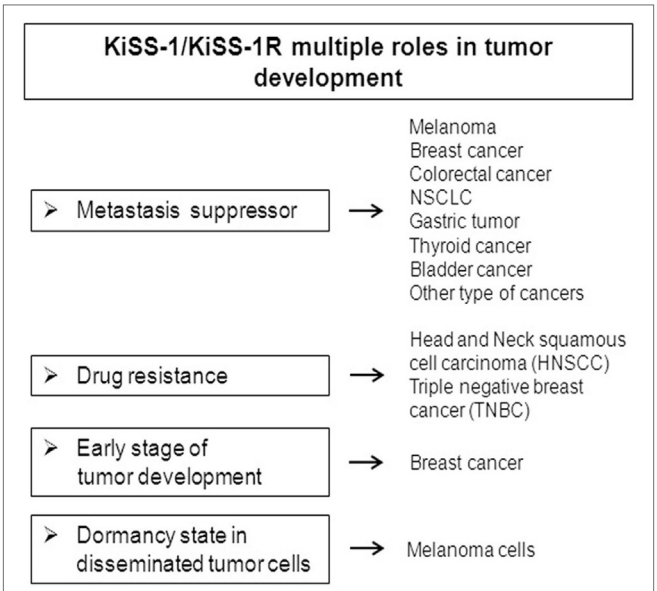

FIGURE 2 | Multiple functions exerted by the KiSS-1/KiSS-1R system in regulating tumor progression.

anti-metastatic role has not been studies extensively as compared to the reproductive function, nevertheless a large body of literature documented the involvement of the KiSS-1/KiSS-1R system in supporting tumor progression. Furthermore, recent studies have demonstrated that plasma levels of KPs are increased in colorectal cancer (CRC) and small renal tumor patients, suggesting that KPs may be considered plasmatic biomarkers in these tumors (11, 12).

In this review, we focused on multiple functions exerted by the KPs/KiSS-1R system in cancer (**Figure 2**).

KiSS-1 AS A METASTASIS SUPPRESSOR GENE

First Evidence in Melanoma and Breast Cancer

KiSS-1 was primarily identified as a human melanoma metastasis suppressor gene using subtractive hybridization between the metastatic human melanoma cell line C8161 and non-metastatic variants generated after microcell-mediated transfer of chromosome 6 into C8161 cells to suppress their ability to metastasize (13, 14).

Transfection of *KiSS-1* into metastatic human melanoma cell lines suppressed metastasis in athymic nude mice by 50–95% (13). Thus, since *KiSS-1* map to the long arm of chromosome 1 and its expression suppresses metastasis of melanoma, it was hypothesized that some regulators of *KiSS-1* are encoded by chromosome 6 (14). Indeed, a region of 40cM between 6q16.3 and q23 was identified as an important regulatory region of *KiSS-1* (15). Moreover, Shirasaki and co-workers documented the loss of 6q16.3-q23 in more than 50% of melanoma metastases and that loss of heterozygosis of this region associates with the loss of *KiSS-1* (16).

Only 1 year after the first paper on melanoma, Lee and colleagues conducted another study on breast cancer demonstrating that human breast carcinoma MDA-MB-435 cells transfected with *KiSS-1* expression vector exhibit a lower metastatic, but not tumorigenic potential in comparison with control cells. This study was based on the observations that 1q chromosome is frequently deleted in late stage of human breast carcinomas and that *KiSS-1* is located on chromosome 1q32-q41 (17).

The studies that followed to better underline the anti-metastatic role of *KiSS-1* in breast cancer, instead, revealed the existence of controversial data for this type of tumors.

In fact a study conducted in 2005 by Martin and colleagues demonstrated that *KiSS-1* may not be functioning as a metastasis suppressor in breast cancer cells (18).

They found that the expression of *KiSS-1* mRNA had significantly increased in primary tumors in comparison with normal mammary tissues; they also found that levels of KiSS-1 expression were higher in metastatic disease patients compared to healthy individuals, and that this was associated with poor patient prognosis.

This study contrasted the reports which supported the anti-metastatic potential of *KiSS-1* in breast cancer. The analysis of *KiSS-1* mRNA levels in fresh frozen tissue samples from ductal invasive breast carcinomas revealed a significant reduction of KiSS-1 expression in brain metastases as compared to the primary tumors (19). These results were confirmed in 2012 by Ulasov and co-workers, who found a statistically significant downregulation of *KiSS-1* mRNA and protein in brain metastasis compared to primary tumors (20).

So, even though several studies have identified the *KiSS-1* gene as a metastasis suppressor in breast cancer, the existence

of contradictory data underlines the need to better elucidate its biological role in this particular cancer.

Breast tumors are divided into estrogen receptor α (ERα)-positive and ERα-negative tumors and the role of KiSS-1 and KiSS-1R in the positive group are conflicting.

Estradiol (E2) through its receptor ERα is involved in mammary ductal normal growth and development. It is well established that estrogen is associated with increased breast cancer risk (21).

It has been demonstrated that E2 negatively regulates KiSS-1 and KiSS-1R expression. They showed that in ERα-positive primary tumors, KiSS-1 levels are lower than ERα-negative tumors (22, 23). In contrast, Jarzabek and colleagues demonstrated that ERα-positive breast tumors express higher levels of both KiSS-1 and KiSS-1R than ERα-negative tumors (24).

Recently, *KiSS-1R* has been documented to induce invasion of triple negative breast cancer (TNBC) cells which lack ERα, progesterone receptor, and human epidermal growth factor receptor 2; also, *KiSS-1* mRNA and *KiSS-1R* mRNA and protein were found to be upregulated in TNBC tissues as compared to normal breast tissue (25).

So far, it is not possible to assign a clear role to KiSS-1/KiSS-1R system in regulating the progression of ERα-positive and ERα-negative breast tumors as the complex cross-talk between KiSS-1 expression and signaling pathways regulated by ERα deserve further investigation.

Studies on Other Tumors

Since the development of metastases is one of the most dangerous complications of solid tumors, many efforts are conducted to discover and characterize new potential anti-metastatic targets.

Colorectal cancer is identified as one of the most frequent and deadly types of cancer; it represents the second most common tumor among women and the third most common among men (26).

A major complication of CRC is disease progression *via* liver metastases.

Metastases from CRC are strictly associated with matrix metalloproteinase (MMP)-9 expressions (27, 28).

The analysis of KiSS-1 and KiSS-1R expression in colorectal liver metastases showed the existence of a correlation with the patients' prognosis. CRC tissues with low levels of KiSS-1 express high levels of MMP-9 and metastasize more frequently to distant sites (29).

Accordingly, Chen et al. showed that *KiSS-1* gene represses the metastatic potential of CRC cells by inhibiting the expression of MMP-9. Overexpression of *KiSS-1* suppressed the proliferation and the invasiveness of HCT-119 CRC cells and enhanced their apoptosis by reducing the expression of MMP-9 through blocking PI3K/Akt/NF-κB pathway (30).

The analysis of KiSS-1 and KiSS-1R expression in normal and malignant tissue samples from 111 patients with colorectal adenocarcinoma showed that KiSS-1 expression levels were much higher in the normal than in the malignant colonic mucosa (31).

Regarding malignant tissues, it has been shown that the expression level of KiSS-1 had a negative correlation with Dukes staging, TNM (tumor, lymph node, and metastasis) staging, tumor size, and lymph node involvement. Reduction of KiSS-1R was also linked to poor prognosis of the patients (32).

Studies of comparison of the miRNA expression profiles in CRC tissues and hepatic metastasis revealed that down-regulation of miR-199b associates with distant metastasis in CRC and a longer median survival. Through human tumor metastasis PCR array, Shen et al. identified *KiSS-1* as one of the downstream targets of SIRT1: silencing of SIRT1 upregulates KiSS-1 expression by enhancing the acetylation of the transcription factor CREB which, in turn, may be activated through its binding to the promoter of *KiSS-1*. Thus, miR-199b regulates SIRT1/CREB/KiSS-1 signaling pathway and might serve as a prognosis marker for patients with CRC (33).

So KiSS-1 and MMP-9 could be considered as prognostic markers in patients with CRC.

An inverse correlation between KiSS-1 and MMP-9 has been demonstrated also in non-small cell lung cancer (NSCLC) patients. It has been reported that *KiSS-1* and MMP-9 mRNA and protein correlate with disease stage, metastasis, and survival of the patients. In particular, KiSS-1 expression was found to be lower in the metastatic tissues as compared to the primary tumors, supporting the notion that *KiSS-1* may be considered as a metastasis suppressor in NSCLC (34).

The potential use of KiSS-1 and KiSS-1R as favorable prognostic markers in NSCLC has been confirmed by Sun et al. in 2013 (35). They analyzed 56 NSCLC specimens divided into low stage (locally advanced) and metastatic (advanced) disease and they found an inverse correlation between KiSS-1 and KiSS-1R expression and NSCLC progression. In particular, they assessed that the expression levels of KiSS-1 and KiSS-1R were lower in cancer tissues compared to normal tissues; moreover, KiSS-1 and KiSS-1R expression was lower in patients with advanced stage compared to patients with low stage of NSCLC. Additionally, they found that cells collected from low stage disease showed high apoptotic ratio and arrest in G1 phase, suggesting a role of the KiSS-1/KiSS-1R system not only in invasion and migration, but also in apoptosis and cell cycle processes.

Furthermore, studies on cisplatin-resistant NSCLC cells demonstrated that overexpression of exogenous *KiSS-1* significantly decreases their invasive capability *in vitro* and *in vivo* (36).

KiSS-1 has been shown to inhibit the proliferation and invasion also of gastric carcinoma cells *in vitro* and *in vivo* through the downregulation of MMP-9 (37).

A study on 40 gastric cancers divided into two groups according to their high or low *KiSS-1* mRNA expression levels and compared with their adjacent normal gastric mucosa, demonstrated that KiSS-1 may represent an independent prognostic factor for gastric cancer patients. Indeed, the low expression of *KiSS-1* in tumor tissues was documented to correlate with the propensity of gastric cancers to invade, metastasize, and relapse as well as to worse overall and disease-free survival (38). Immunohistochemical analysis of tissue microarrays from 71 patients with gastric cancer revealed a statistically significant reduction of KiSS-1 in lymph node and liver metastases compared with primary tumors (39).

A potential role of the *KiSS-1* gene product, metastin, and its receptor in modulating the biological behavior of thyroid carcinomas has been suggested.

Ringel and co-workers demonstrated that metastin is expressed in normal thyroid and in papillary thyroid carcinomas, while KiSS-1R is overexpressed in papillary thyroid cancer but not in normal thyroid and in follicular adenomas. The expression of KP and its receptor are less common in follicular carcinomas. The authors suggest that the high ability of follicular carcinomas to metastasize is due to their low expression of KiSS-1 product and KiSS-1R. They also show that KiSS-1R activates MAPK, but not Akt in thyroid cancer cells (40). Moreover, KiSS-1 expression is significantly higher in advanced tumors with extrathyroidal invasion compared to thyroid tumors in the early stages. Decreased expression of KiSS-1R seems to attenuate signaling of the KiSS-1/KiSS-1R system, possibly leading to tumor growth (41).

Another type of cancer in which the expression of *KiSS-1* is correlated with stage and tumor grade is bladder cancer. Sanchez-Carbayo and co-workers found lower levels of *KiSS-1* in bladder cancer tissues as compared to normal counterpart; also, they documented that the loss of *KiSS-1* is associated with bladder cancer progression and clinical outcome (42).

Successively, Cebrian and co-workers identified an epigenetic silencing of *KiSS-1* due to a CpG island hypermethylation near to its promoter region, which correlates with lower levels of *KiSS-1* transcripts in invasive bladder cancer as compared to superficial tumors. These findings highlight the value of *KiSS-1* as a prognostic biomarker (43).

The occurrence of an epigenetic regulation of *KiSS-1* expression which favors bladder cancer invasion was confirmed by Zhang and co-workers. They found that ubiquitin-like with PHD and RING finger domains 1 increase the methylation of CpG nucleotides of *KiSS-1*, thus reducing its expression (44).

Finally, *KiSS-1* overexpression has been documented to regulate apoptosis by increasing caspase 3 and Bax and decreasing Bcl-2 and Bax mRNA levels in human osteosarcoma MG-62 and U2OS cells. In these cell lines, *KiSS-1* overexpression reduces the extent of both cell proliferation and invasiveness (45).

It has been shown that KiSS-1 exerts an anti-metastatic role in human hepatocellular and renal carcinoma by inhibiting metalloproteinase MMP-9 and MMP-2 activity (46, 47).

In human head and neck squamous cell carcinoma (HNSCC) tumors, loss of *KiSS-1* expression has been associated with high metastatic potential compared with non-metastatic tumors (48).

ROLE OF KiSS-1R IN THE ACQUISITION OF DRUG RESISTANCE

Recent papers attribute to KiSS-1/KiSS-1R complex a diverse function from that observed in other tumor types.

Genetic reconstitution of *KiSS-1* in cisplatin-resistant HNSCC cells has been shown to induce alterations in cisplatin metabolism thus restoring platinum sensitivity (48).

In TNBC, Blake and co-workers documented that KiSS-1R signaling promotes drug resistance in ERα-negative breast cell lines and in TNBC cells by increasing the expression of the efflux drug transporter breast cancer-resistance protein and also by promoting tyrosine kinase (AXL) expression and activity. The authors demonstrated that KiSS-1R activity is necessary to promote drug resistance in ERα-negative breast cell lines and in TNBC cells, since KiSS-1R antagonist restored cell sensitivity to doxorubicin. These findings suggest the possibility to consider KiSS-1R as a possible novel therapeutic target to restore drug sensitivity in patients affected by TNBC (25).

ROLE OF KiSS-1 IN EARLY STAGE OF TUMOR DEVELOPMENT

An additional role of KiSS-1/KiSS-1R was demonstrated by Cho et al. in the early steps of breast cancer development. Using transgenic mice expressing the polyoma middle T antigen under the control of MMTV (mouse mammary tumor virus) long terminal repeat promoter (MMTV-PyMT), the authors found that heterozygous mouse for *KiSS-1 or KiSS-1R* showed delayed hyperplasia, resulting in a late breast cancer initiation, progression, and lung metastasis. Also, they showed that *KiSS-1 and KiSS-1R* silencing in pubertal breast epithelium inhibits mammary gland hyperplasia (49). These findings highlight the role of KiSS-1/KiSS-1R complex in the early phase of breast tumor development.

KiSS-1 INDUCES A DORMANCY STATE IN DISSEMINATED TUMOR CELLS

Nash et al. hypothesized that secreted KiSS-1 was able to keep the disseminated melanoma cells in a state of dormancy inducing a suppression of metastatic colonization to multiple organs. The authors showed that mice injected intravenously with cells expressing KiSS-1 without the secretion sequence developed lung metastasis. Also, the mouse survival fell quickly after the interruption of the dormancy and the reactivation of proliferation. Thus, the possibility to keep tumor cells in a dormant state and obtain high survival through the use of exogenous KiSS-1 therapy has been suggested to represent an important goal for the treatment of tumor patients. In addition, the capability of KiSS-1 to maintain the cells in a dormancy state was documented to occur in the absence of its cognate receptor, raising the possibility that additional KiSS-1 receptors and/or paracrine signals exist with the potential to be selectively targeted (50). Similar hypothesis was formulated by Beck et al., suggesting that many tumor cells do not express KiSS-1 receptor, so a paracrine control may exist between tumor cells expressing KiSS-1 and stromal cells expressing KiSS-1R. KiSS-1 may activate or induce stromal cells to produce secreted factors in the surrounding extracellular milieu that, directly or indirectly, elicit dormancy in metastatic cells (51).

CONCLUSION

Although initially the KiSS-1/KiSS-1R complex was described to be involved in the onset of puberty, sexual maturity, and pregnancy through direct regulation of the hormone releasing the gonadotropin produced by the hypothalamus, other multiple roles have been proposed for this complex in the tumor development process.

Metastasis is a main cause of death in cancer patients and involves a multi-step process encompassing detachment of cancer cells from a primary tumor, invasion of adjacent tissue, transvasation of blood vessels, and spread through circulation, distant organs colonization. *KiSS-1* has been described as a gene suppressor of metastasis in melanoma and more recently in other types of cancer, such as breast cancer, CRC, lung, thyroid, bladder, gastric, and other cancers.

In this mini-review, we highlighted other roles of the KiSS-1/KiSS-1R complex in addition to the role of suppressor gene of metastases. One of the main functions that have been found is the involvement of this complex in drug resistance. The development of drug resistance is still one of the main obstacles in effective cancer treatment. Therefore, there is still an unmet need to identify the molecules that could be targeted in order to overcome resistance in the treatment of tumors and the KiSS-1/KiSS-1R complex may represent a potential target for the treatment of drug-resistant tumors. In fact, it has been described that the KiSS-1/KiSS-1R system is involved in the sensitivity to traditional drugs, since the reconstitution of *KiSS-1* in cisplatin-resistant head and neck cancer cells restores platinum sensitivity; in addition, KiSS-1R has been reported to be involved in the process of drug resistance in TNBC.

Furthermore, a potential role of the KiSS-1/KiSS-1R complex has been proposed in the early stage of breast cancer development so this complex is not only involved in the advanced stages of the tumor, but could also represent a good target to hit tumors at an initial and final stage of development.

Finally, a role played by KiSS-1 in the dormancy of disseminated tumor cells and in the suppression of multiple organ metastases was described, representing the possibility of maintaining the tumor in an asymptomatic state. This could be a valid method to intervene on the block of metastatic development through the treatment with KiSS-1 that inducing tumor cell dormancy could block the metastatic process. The perspective that KiSS-1 can be used in clinical treatment is really favorable because KiSS-1 is a natural product that can be administered at high levels to humans without toxicity.

AUTHOR CONTRIBUTIONS

MM: conception of the work. FF and MM: performed extensive literature search. MM and FF: manuscript drafting. MM and MC: critical revision of the work. MM: final version approval.

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REVIEW
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KISS1/KISS1R in Cancer: Friend or Foe?

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The *KISS1* gene encodes KISS1, a protein that is rapidly processed in serum into smaller but biologically active peptides called kisspeptins (KPs). KISS1 and the KPs signal via the G-protein coupled receptor KISS1R. While KISS1 and KPs are recognized as potent positive regulators of the reproductive neuroendocrine axis in mammals, the first reported role for KISS1 was that of metastasis suppression in melanoma. Since then, it has become apparent that KISS1, KPs, and KISS1R regulate the development and progression of several cancers but interestingly, while these molecules act as suppressors of tumorigenesis and metastasis in many cancers, in breast and liver cancer they function as promoters. Thus, they join a small but growing number of molecules that exhibit dual roles in cancer highlighting the importance of studying cancer in context. Given their roles, KISS1, KPs and KISS1R represent important molecules in the development of novel therapies and/or as prognostic markers in treating cancer. However, getting to that point requires a detailed understanding of the relationship between these molecules and different cancers. The purpose of this review is therefore to highlight and discuss the clinical studies that have begun describing this relationship in varying cancer types including breast, liver, pancreatic, colorectal, bladder, and ovarian. An emerging theme from the reviewed studies is that the relationship between these molecules and a given cancer is complex and affected by many factors such as the micro-environment and steroid receptor status of the cancer cell. Our review and discussion of these important clinical studies should serve as a valuable resource in the successful development of future clinical studies.

Keywords: kisspeptin, KISS1R, cancer, metastasis, prognostic marker, metastasis promoter, metastasis suppressor

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INTRODUCTION

Cancer is the second leading cause of mortality and accounts for about 1 in every 6 deaths globally (http://www.who.int). At any time in development, the body contains abnormal cells with malignant potential or neoplastic characteristics. In the healthy host, these cells are detected, and destroyed by the immune system. However, under various physiological conditions these cells can escape the body's immune surveillance resulting in life-threatening tumorigenesis and deadly metastatic disease. The body produces molecules that can either inhibit

or promote the development of a tumor and its metastatic potential and identifying and studying these factors are essential in the treatment of cancer. In 1996, Welch and colleagues at The Pennsylvania State University College of Medicine, Hershey, Pennsylvania, conducted studies to identify the gene(s) responsible for the suppression of metastasis in chromosome 6/melanoma cell hybrids. Their brilliant work led to the discovery of a novel cDNA-designated KiSS-1 (1). This name was chosen to indicate that the cDNA encoded a metastasis suppressor sequence and also to recognize its discovery in the town of Hershey, Pennsylvania, the home of world-famous Hershey's chocolates known as Kisses. Out of that discovery and clever branding was born the era of kisspeptin biology.

In the original study from the Welch laboratory, Lee et al. (1) reported that among a panel of melanoma cells, KiSS-1 (later renamed *KISS1*) was expressed only in non-metastatic melanoma cells and when exogenous *KISS1* was expressed in C8161 melanoma cells, metastasis was suppressed in an expression-dependent manner. Thus, they concluded that *KISS1* suppresses the metastatic potential of malignant melanoma cell. Since this initial study (1), KISS1 (145 amino acid), the primary and full-length product of the *KISS1* gene and its derivatives (54, 14, 13, and 10 amino acids) called kisspeptins, as well as their cognate receptor KISS1R (previously called GPR54), have been reported to exhibit anti-metastatic and/or anti-tumoral roles in numerous cancers e.g., bladder (2, 3), ovary (4), colorectal (5–7), pancreas (8), pituitary (9), prostate (10), and thyroid (11), as previously reviewed (6, 12–14). Mechanistically, these molecules may exert their anti-metastatic effect on these cancers via a repression of matrix metalloproteinase MMP-9 activity and inhibition of cancer cell migration and invasion (14, 15). Interestingly, based on studies conducted on the human breast carcinoma MDA-MB-435 cell line, *KISS1* was also reported to suppress breast cancer metastasis (16). However, it is now recognized that MDA-MB-435 cells display a gene expression profile more closely resembling melanoma cancer cells, raising the possibility that these are melanoma and not breast cancer cells (17, 18). Based on the use of established cell models of breast cancer and breast cancer patient data, several studies from our laboratories (19–22), and those of others (23–25) would later show that KISS1 and its receptor KISS1R, in striking contrast to most cancers, promote breast cancer invasion, and metastasis.

The finding that the same molecule can act as both a promoter and suppressor of tumorigenesis and metastasis is not unique to KISS1 or KISS1R as other molecules such as NFκB (26), c-Myc (27), AMP-activated protein kinase (AMPK) (28), transforming growth factor β (TGF-β) (29), SKY (30), and hyaluronidase (31) have been reported to play dual roles. Growing evidence suggest that the role any of these molecules assumes is dependent on many factors including the cancer type (e.g., melanoma vs. breast), stage of development (e.g., pre-malignant vs. malignant) and composition of its microenvironment (e.g., steroidal milieu and the absence and presence of other signaling molecules that might facilitate suppressor or promoter pathways). Thus, it is pivotal to study cancer in context (32). To date, in addition to playing a promoter role in breast cancer metastasis, KISS1 and KISS1R also appear to promote hepatic cell carcinoma (33).

Since the seminal discovery that *KISS1* regulates metastasis of melanoma cells (1), a large and growing body of studies has described roles for KISS1 and KISS1R mRNA and protein in regulating tumor growth and metastasis (6, 12–14). These studies are of tremendous importance as we forge forward toward developing these molecules as either prognostic markers and/or therapeutic targets in the treatment of various types of cancers. The goal of this review is therefore to summarize and discuss the key clinical evidence in support of the finding that KISS1 and KISS1R act as both promoter and suppressor of tumorigenesis and metastasis (**Table 1**).

METASTASIS PROMOTER ROLES

Breast Cancer

Breast cancer is the leading cause of cancer related deaths in women world-wide and metastasis is responsible for the majority of cancer deaths (44). Triple negative breast cancer (TNBC) comprises a heterogeneous group of tumors defined as a basal-like subtype lacking estrogen receptor alpha (ESR1), progesterone receptor (PGR), and human epidermal growth factor receptor 2 (ERBB2) (45). TNBC represents 15–20% of all breast cancers and occurs frequently in women under 50 years of age, in women with African ancestry or in Hispanic women. Unfortunately, these patients do not benefit from hormone receptor or HER2-targeted therapies, leaving chemotherapy as the main systemic treatment option (45). The prognosis for TNBC patients remains poor since patients are often high grade and either metastatic at time of diagnosis or succumb to metastasis within 3 years of diagnosis (45).

Spanning a little over one decade, several studies have highlighted a detrimental role for KISS1/KISS1R in breast cancer. Martin et al. (34) first demonstrated that KISS1 mRNA and protein levels were found to be elevated in ERα-negative invasive ductal carcinoma compared to ERα-positive tumors, demonstrating their positive correlation with tumor progression and poor patient prognosis. This study also revealed that breast tumors that were positive for lymph node metastasis showed higher *KISS1* levels compared to the lymph node negative tumors. Using immunohistochemistry, studies have shown that KISS1 and KISS1R is localized within the ductal carcinoma *in situ* and in invasive ductal carcinoma, and that there was higher cytoplasmic staining in tumors as well as surrounding cells, compared to normal tissue (19, 34, 35). Marot et al. (23) demonstrated that patients with high *KISS1* and *KISS1R* expression in breast tumors had the shortest relapse-free survival relative to tumors expressing low levels of these genes. This study showed that treatment of

Abbreviations: AMPK, AMP-activated protein kinase; BCRP, Breast cancer resistance protein; CRC, Colorectal cancer; DAG, Diacylglycerol; EGFR, Epidermal growth factor receptor; EMT, Epithelial-to-mesenchymal transition; ESR1, Estrogen receptor alpha; EOC, Epithelial ovarian cancer; HER2, Human epidermal growth factor receptor 2; HCC, Hepatocellular carcinoma; HPG, Hypothalamus-pituitary-gonadal; KP, Kisspeptin; KISS1R, Kisspeptin receptor; MMP, Matrix metalloproteinases; PGR, Progesterone receptor; RCC, Renal cell carcinoma; TGF-β, transforming growth factor β; TNBC, Triple negative breast cancer.

TABLE 1 | KISS1/KISS1R expression in patient tumors.

Type of cancer	Clinical evidence: KISS1/KISS1R expression and plasma kisspeptin levels	Sample size	References
Breast cancer	Increased KISS1 mRNA and protein in primary breast tumors and lymph node metastasis.	normal: 33 Tumor: 124	(34)
	Increased *KISS1* mRNA with the grade of the breast tumors; ERα-positive breast tumors; expressed sevenfold less *KISS1* than ERα-negative breast tumors.	Tumor: 92	(23)
	Increased KISS1/KISS1R protein vs. Control	Non-tumor: 11 (fibrocystic disease) Tumor: 43 resected breast adenocarcinomas	(35)
	KISS1 protein expression is positively associated with lymph node positive status.	Breast tumor microarray ($n = 48$)	(25)
Triple negative breast cancer (TNBC)	Increased KISS1/KISS1R mRNA and protein in primary TNBC tumors compared to healthy breast.	Healthy: 13 TNBC: 20	(19)
Hepatocellular Carcinoma (HCC)	Increased *KISS1R* mRNA expression in HCC vs. normal; no change in *KISS1* levels.	Normal: 8 HCC: 60	(33)
	KP-54 immunoreactivity associated with tumor grade, stage, poor prognosis	Normal: 10 HCC: 142	(36)
	Lower KISS-1 protein in HCC vs. normal.	Normal: 16 HCC: 150 patients	(37)
Pancreatic Cancer	High level of KP-54/KISS1R immunostaining associated with longer survival.	Tumors: 53 Normal: not reported	(38)
	Lower *KISS1* mRNA in pancreatic cancer tissue vs. control. Higher *KISS1R* expression in pancreatic tumors vs. matched controls.	Adjacent normal: 5 Tumors: 30	(8)
Colorectal Cancer (CRC)	Decreased *KISS1* expression associated with lymph node metastasis and poorer prognosis.	CRC:175	(5)
	Decreased expression of *KISS1/KISS1R* in tumor tissues compared with normal.	Normal: 80 CRC: 94	(6)
	Mean KP-54 (CRC): 86.2 ± 20.5 ng/ml; Mean KP-54 (control) patients: 49 ± 12.7 ng/ml	Controls: 59 CRC: 81	(39)
	Decreased *KISS1* mRNA expression and KP-54 protein expression - increased depth of invasion and lymph node metastasis.	Normal: 142 CRC: 126	(7)
Bladder Cancer	*KISS1* was found to be hypermethylated in ∼83% of tumors, epigenetic-induced loss of *KISS1* expression associated with poor survival	804 primary bladder tumors	(2)
	Decreased or lost *KISS1* expression in invasive bladder tumors ($n = 173$) vs. controls	Normal urothelium: 25 Tumors: 173	(40)
Renal cell Carcinoma (RCC)	Higher *KISS1R* mRNA in RCC compared to non-neoplastic renal cortex	Non-neoplastic: 25 Tumors: 25	(41)
Ovarian Carcinoma	Lower KISS1 immunostaining in primary epithelial ovarian cancer (EOC) biopsies vs. control; patients with KISS1-negative tissues had a lower survival rate vs. to KISS1-positive patients	EOC: 207 Control: 60	(42)
	Favorable prognosis and overall survival associated with KISS1 and KISS1R immunoreactivity	Ovarian carcinomas tissue microarray: 518	(4)
	Median plasma kisspeptin: stage 1 ($n = 9$): 17.4 pmol/L, stages 2-4 ($n = 23$): 7.8 pmol/L, control: 11.4 pmol/L	Cancer patients: 31 Healthy volunteers: 31	(43)

Changes in the expression of KISS1/KISS1R transcripts, protein and circulating kisspeptins in cancer patients compared to healthy subjects.

ERα-positive MCF7 and T47D breast cancer cells with tamoxifen, a selective estrogen receptor modulator (with antagonistic role in breast tissue), stimulated *KISS1/KISS1R* expression, implicating that ERα signaling downregulates KISS/KISS1R levels. This negative regulation of *KISS/KISS1R* expression by ERα is well-documented in the ARC KISS1 neurons in the hypothalamus (46). Along with the results reported by Martin et al. it appears that the increase in KISS1R correlates better with a metastatic capacity rather than with tumor growth. Next, Papaoiconomou et al. (35) found higher expression of KISS1 and KISS1R protein in breast cancer tissues (ductal carcinomas and lobar carcinomas) compared to non-cancerous fibrocystic mammary tissues. However, a significant correlation was not found between KISS1 and KISS1R expression and tumor grade, tumor size,

lymph node positivity, histological type or ER status, results possibly due to the study's small sample size ($n = 43$).

Recently, we demonstrated that KISS1/KISS1R mRNA and protein expression was upregulated in primary TNBC tumor biopsies compared to healthy breast tissue (19) (**Table 1**). We found localization of KISS1 and KISS1R in invasive ductal carcinoma tumors using immunostaining. Furthermore, immunolocalization of endogenous KISS1R was found to be enhanced at the leading edge of migratory tumor cells (21). We also demonstrated that KISS1R signaling induces the drug resistant phenotype in TNBC cells, by inducing the expression of efflux drug transporter, breast cancer resistance protein (ABCG2) and by activating the tyrosine kinase, AXL (19). The P13K/AKT and Ras/ERK pathways are the most dysregulated signaling cascades in human carcinomas that are related to tumor drug resistance, survival, and proliferation (47). We found that KISS1R signaling in the drug-resistant cells led to an increase in the expression of AKT, ERK, and the anti-apoptotic protein, survivin. Specifically we and others found that ERα negatively regulates KISS1 (23), KISS1R expression and KISS1R-induced cell invasion (21). This may, in part, account for switching from the metastasis suppressor to metastasis promoter roles of the KISS1/KISS1R system in breast cancer. Thus, when ERα expression is lost (e.g., in TNBC), this results in increased transcription of KISS1/KISS1R, and increased receptor signaling and the induction of epithelial to mesenchymal transition (EMT), allowing epithelial cells to acquire invasive characteristics (14). KISS1R signaling stimulates TNBC cell invasion by inducing invadopodia formation by activating key invadopodia proteins, cortactin, cofilin, and membrane type I matrix metalloproteases (MT1-MMP), via a β-arrestin2, and ERK1/2-dependent mechanism (20). Additionally, KISS1R signaling can activate the epidermal growth factor receptor (EGFR) to promote TNBC invasion, via a β-arrestin2 and MMP-9 pathway (22). Interestingly, it appears that KISS1/KISS1R also mediate the effects of pro-invasive factors in TNBC, as it was recently reported that TGFβ-induced cancer cell invasion is dependent on KISS1 (25). Downregulation of KISS1 blocked TGFβ-mediated cancer cell invasion as well as MMP-9 expression and activity in TNBC cells, but not ERα-positive breast cancer cells. Through an immunohistochemical analysis of a tumor microarray, this study also showed that lymph node positive status is associated with high KISS1 protein levels.

In additional support for the pro-metastatic roles of KISS1R in breast cancer, a landmark study by Cho et al. (24) provided *in vivo* evidence that relative to wild-type mice, *Kiss1r* heterozygosity triggered a haploinsufficient phenotype where breast tumor initiation, growth and metastasis were delayed. This study also demonstrated that KISS1/KISS1R signaling occurs in an autocrine manner in breast epithelial cells to promote in breast tumor development in an animal model. Consistent with these findings, we observed that kisspeptin treatment of normal human mammary epithelial MCF10A cells induced cell transformation, resulting in a malignant phenotype. Kisspeptin treatment or exogenous expression of KISS1R in MCF10A cells induced EMT and stimulated cell invasiveness by inducing the expression of mesenchymal markers (N-cadherin, Snail/Slug) and loss of E-cadherin from cell-cell junctions (21). Finally, we also reported that in chick embryo assays, ERα-negative SKBR3 breast cancer cells expressing exogenous KISS1R exhibited increased invasion (21). Taken together, these *in vitro*, animal model studies and clinical findings provide substantial support that KISS1 and KISS1R promote metastasis in breast cancer.

Liver Cancer

Primary liver cancer is one of the leading causes of cancer deaths in the world where its incidence has increased by 75% between 1990 and 2015 (48). In the US, liver cancer cases have more than tripled since 1980, and cancer death rates have increased by almost 3% per year since 2000 (www.cancer.org), despite the reducing incidence of chronic hepatitis infections. Hepatocellular carcinoma (HCC) accounts for ∼90% of primary liver cancers, and in the last decade the development of this cancer has been linked to obesity-related metabolic syndrome which is on the rise world-wide (49).

To date, few studies have examined the role of KISS1/KISS1R in HCC and the findings have been contradictory. Ikeguchi et al. (33) *first* showed that *KISS1* and *KISS1R* mRNA were overexpressed (22 and 43%, respectively) in surgically resected HCC samples, compared to non-cancerous liver and this was positively associated with disease progression and poor survival. Since none of the patients received preoperative chemotherapy or radiation therapy, the increase in *KISS1*/*KISS1R* levels was likely the direct result of the disease (33). In agreement with these findings, Schmid et al. (36) reported that in HCC patients who underwent liver transplantation had a worsened clinical outcome that correlated with elevated KP-54 expression, as assessed by immunohistochemistry. In contrast to these studies, Shengbing et al. (37) reported that KISS1 protein expression is lost in HCC, thus suggesting tumor suppressive roles in HCC. The authors examined the expression of MMP-9, a key driver of metastasis and report a negative association of KISS1 with MMP-9 in HCC (37). It remains unclear why the discrepancy exists among these studies, highlighting the need for further interrogation.

METASTASIS SUPPRESSOR ROLES

Pancreatic Cancer

Pancreatic cancer remains a lethal disease since at the time of diagnosis most patients with pancreatic cancer have locally advanced tumors and/or metastases. While surgery represents the only curative treatment, just 10–15% of pancreatic cancer patients have resectable disease (50). Studies have shown that KISS1 and KISS1R are expressed in the pancreatic islets, in the endocrine alpha and beta cells, and regulates glucose stimulated insulin secretion (51, 52). Interestingly, in cancers such as pancreatic, ovarian, and colorectal cancer, a new facet in the relationship between KISS1/KISS1R and these cancers was uncovered. Namely, the expression of these proteins is high in the early stages of the disease but progressively become diminished with the advancement of the cancer. This observation is highlighted in some of the following studies but, as discussed, this requires further investigation.

Masui et al. (8) demonstrated that in pancreatic cancer patients, tumor *KISS1* levels were significantly higher compared to normal tissue ($n = 30$). The authors also compared *KISS1R* mRNA levels in cancer and matched normal tissues from each patient ($n = 5$) and found that the receptor expression was higher in cancer tissue compared to the adjacent normal in all paired samples. Nagai et al. made similar observations among 53 pancreatic ductal adenocarcinoma tissues, reporting strong immunostaining of KP-54 and KISS1R in tumors (38). They found that tumors that were negative for both KP-54 and KISS1R expression were significantly larger than tumors that were positive. Furthermore, they found that expression of KP-54 and KISS1R was associated with longer survival and recurrence was less frequent in patients who had KP-54-positive tumors compared with those who had KP-54-negative tumors. Plasma KP-54 levels were also measured in 23 patients; however, no significant difference in survival was found between the patients with high and low plasma levels. The lack of clinicopathological data for these patients as well as the absence of data on the KP-54 levels in healthy controls complicate the full interpretation of these findings.

Since KISS1 is expressed at reduced levels in advanced pancreatic cancer, McNally et al. (53) hypothesized that re-expression of *KISS1* would reduce metastases. Pancreatic cell lines such as BxPC-3, PANC-1 and SUIT-2 express low levels of endogenous *KISS1*, thus *KISS1* was overexpressed in a metastatic subclone of the SUIT-2 pancreatic adenocarcinoma cell line, S2VP10. SCID mice were implanted orthotopically with S2VP10L-KISS1 cells. Analyses of these mice revealed that mice bearing S2VP10L-KISS1 tumors developed fewer liver (98%) and lung (99%) metastases than control mice implanted with S2VP10L cells only expressing the empty KISS1 cloning vector. Based on the results, the authors concluded that KISS1 therapy might prove beneficial in suppressing the metastasis of pancreatic cancer.

It is important to note that the relationship between KISS1/KISS1R and pancreatic cancer requires further investigation as a study by Wang et al. (54) reported that the plasma KP-54 levels in pancreatic cancer patients were significantly higher when compared with healthy volunteers. However, a significant relationship was not found between KP-54 levels and clinicopathological factors such as tumor size, invasion, lymph node metastasis and distant metastasis.

Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer in Europe and the US, and 30% of patients have metastasis at diagnosis (55, 56). A decrease in this cancer mortality rate has been observed in countries where screening and improved treatments exist (57). Current treatments for metastatic colorectal cancer are considered palliative, but include combination of cytotoxic therapy as well as targeted therapy with anti-EGFR and anti-vascular endothelial growth factor. These treatments have significantly improved the progression-free survival of patients with metastatic CRC, which ranges from 22 to 29 months (58). However, many patients progress as tumors acquire resistance to therapies. Since CRC is a heterogeneous disease, there is a critical need for the discovery of novel biomarkers, to identify patients who will most likely benefit from treatment.

Okugawa et al. (5) conducted one of the first studies to determine the expression of *KISS1* mRNA and protein in 175 colorectal tumors and adjacent normal tissues from patients undergoing surgery, none of which received neo-adjuvant therapy. Kaplan-Meir survival studies indicated that patients with tumors with low *KISS1* mRNA expression had poorer prognosis and exhibited lymph node metastasis, in comparison to patients with tumors that had high *KISS1* levels. Using multivariate analysis, the authors concluded that decreased expression of *KISS1* is a significant independent prognostic marker. Although not quantified, immunodetection of KISS1 using an antibody raised against amino acids 46–146 revealed that KISS1 was highly expressed in primary CRC and in early stages of the disease and decreased in advanced stage-tumors. Canbay et al. (39) using a commercially available enzyme-immunoassay, measured plasma KP-54 levels in blood samples from 81 CRC patients and 59 age matched healthy controls. This study found plasma KP-54 levels were significantly higher in CRC patients (86.2 ± 20.5 ng/ml) than in controls (49 ± 12.7 ng/ml). They also found that KP-54 levels were significantly correlated with nodal involvement of CRC leading the authors to propose using plasma KP-54 as a predictive marker for lymph node metastases of CRC.

Ji et al. (6) examined *KISS1* and *KISS1R* transcripts in 94 samples from colorectal cancer tissues and 80 samples from normal tissue and found that the expression of *KISS1* had a negative correlation with Duke's staging, TMN staging, tumor size and lymph node metastasis. *KISS1R* was also reduced and low *KISS1R* expression was linked to poor prognosis in patients. Remarkably, patients who were undergoing chemo/radiotherapy showed a higher expression of *KISS1R* compared to the patients without radiotherapy. Mechanistically, KISS1 regulates CRC cell invasion by reducing the secretion and activity of MMP-9 in an ERK-dependent manner (6, 59). Chen et al. (7) also found that a decrease in *KISS1* expression and KP-54 protein expression correlated with an increased depth of invasion and lymph node metastasis in 126 CRC patients compared to 142 normal controls. To understand why *KISS1* expression was reduced, they investigated whether *KISS1* was inactivated by epigenetic mechanisms. By looking at the genomic methylation patterns of *KISS1*, they discovered hypermethylation of *KISS1* in 88.33% (105/126) of CRC samples; this was significantly higher than the rate observed in normal colorectal tissues (9/142). The DNA methyltransferase inhibitor azacitinde (5-Aza-2-deoxycytidine) was able to restore *KISS1* expression and the corresponding reduction of CRC cell invasion. These findings suggest that *KISS1* is epigenetically modified in CRC, however further studies are required to better understand the potential role of *KISS1* hypermethylation in the progression of colorectal cancer.

Bladder Cancer

Urinary bladder cancer is the ninth most common malignant disease and the 13 most common cause of cancer-related death in the world. In bladder cancer, *in situ* hybridization studies

revealed that *KISS1* expression was significantly decreased or lost in invasive bladder tumors ($n = 173$) compared with their respective normal urothelium ($n = 25$) (40). Patients ($n = 69$) with lower *KISS1* expression in bladder tumors showed a significant association with worse overall survival. *KISS1* expression was significantly lower in bladder tumors with vascular invasion compared with normal urothelium. Furthermore, in this study, the authors observed that all bladder tumors developing distant metastases showed a complete loss of *KISS1*.

As observed in CRC, *KISS1* was found to be hypermethylated in over 83% of 804 primary bladder tumors (2). This study also revealed that the epigenetic-induced loss of *KISS1* expression was associated with poor survival and suggested that *KISS1* levels have predictive value in identifying patients with poor outcome. Takeda et al. (3) examined KISS1 and KISS1R expression in 151 bladder cancer patients to determine their prognostic significance and reported that KISS1 immunoreactivity was significantly decreased in advanced stages of bladder cancer and inversely associated with tumor grade and stage. However, no association in KISS1R expression was found with disease progression. Moreover, this study demonstrated that KP-54 treatment significantly reduced the invasiveness of bladder cancer cells and lung metastasis in a metastasis animal model by inhibiting the expression and activity of MMP-9 via blockage of the nuclear translocation of NF-kB, a transcriptional regulator of MMP-9. Thus, this study provided pre-clinical evidence that KP-54 treatment may be an effective inhibitor of metastasis in urothelial carcinoma. Multivariate analysis revealed that KISS1 expression was an independent predictor of bladder cancer metastasis and overall patient survival. Thus, these studies strongly suggest that KISS1 expression in bladder tumors might be a biomarker of disease, especially for predicting the occurrence of metastases in highly aggressive urothelial carcinoma.

Ovarian Cancer

Ovarian cancer is a commonly diagnosed cancer worldwide and causes more deaths than any other cancer of the female reproductive system. It ranks among the top five deadliest cancers in most countries (48) and in the United States alone, each year about 20,000 women are diagnosed with ovarian cancer (60). KISS1 expression levels have also been linked to survival in ovarian cancer patients as reported in several studies. Hata et al. (61) observed that ovarian cancer patients exhibiting elevated levels of *KISS1/KISS1R* expression, as detected by quantitative PCR, had favorable prognosis using COX regression analysis in a cohort of 76 patients. Cao et al. (62) reported that *KISS1* expression was significantly higher in 40 pre-operative epithelial ovarian cancer (EOC) primary tumors compared to 20 uterine fibroids used as normal tissue. The presence of metastasis and tumor size was negatively associated with pre-operative *KISS1* expression; patients with low *KISS1* expression had shorter survival time than those with high expression. (62).

In addition to the mRNA expression studies described above, protein levels were also assessed in primary ovarian tumors. Interestingly, while the former study reported high *KISS1* levels in pre-operative EOC primary tumors, Yu et al. (42) found that KISS1 immunostaining was much lower in 207 primary EOC biopsies compared to control biopsies (60 benign tumors, serous- or mucinous-cystadenoma). Nevertheless, Kaplan-Meir survival data indicated that patients with EOC KISS1-negative tissues had a lower survival rate compared to KISS1-positive patients, and among these patients, KISS1 protein expression was inversely associated with tumor grade and stage. Similarly, in the largest study of ovarian biopsies done to date, Prentice et al. (4) conducted an immunohistochemical analysis of KISS1 and KISS1R on a tissue microarray consisting of 518 ovarian carcinomas and found that strong KISS1 and KISS1R immunoreactivity was significantly associated with favorable prognosis and overall survival.

To determine whether circulating levels of plasma kisspeptins are dependent on the stage of ovarian cancer, Jayasena et al. (43) measured the levels of plasma kisspeptin concentration in 31 patients with ovarian carcinoma (Stages I to IV) and 31 healthy volunteers, using an in-house radioimmunoassay. They found that the mean kisspeptin concentration in the patients with stage I was significantly higher, compared with the patients with ovarian carcinoma of stages 2 to 4 vs. controls: 25.1 $\pm$15.2) pmol/L (stage 1), 11.8 $\pm$10.3 pmol/L (stages 2 to 4), and 13.1 $\pm$6.92 pmol/L (controls). Thus, stage 1 patients had an increased plasma level compared to the control and stages 2–4. Larger studies are required to further examine the relationship between plasma kisspeptins and ovarian cancer. Based on the various ovarian cancer studies, the independent findings propose that KISS1/KISS1R protein levels could be used as prognostic biomarkers of disease progression in ovarian cancer.

Prostate Cancer

Prostate cancer is the second leading cause of cancer mortality in men of 40 years of age and older (63). Currently, chronically administered gonadotropin-releasing hormone receptor (GnRH-R) agonists, which induce androgen deprivation, represent the primary clinical tools used for treating prostate cancer (64). Kisspeptin is powerful trigger of GnRH secretion and thereby a key positive regulator of the hypothalamus-pituitary-gonadal (HPG) axis. Studies have shown that administration of potent and long-acting kisspeptin agonists decreased serum testosterone levels by suppressing the HPG axis in rats and humans (65–67). Thus, it would be interesting to see if treating patients with kisspeptin agonists and antagonsits results in regression of prostate tumors and better outcome.

To determine whether KISS1 also has direct anti-metastatic activity in prostate cancer cells, Wang et al. examined KISS1 protein expression in 253 prostate tissue samples (normal tissue and prostate cancer) and found that KISS1 expression correlated negatively with clinical staging (10). Interestingly, in another study it was observed that there was no significant difference in plasma kisspeptin levels in 92 prostate cancer patients compared to healthy subjects (68). In addition to the prostate tissue samples, Wang et al. also measured *KISS1* and *KISS1R* expression in metastatic human prostate cancer cell lines and observed that

decreased mRNA expression correlated with increased metastatic ability of these cancer cell lines (10).

To understand how kisspeptin might exert direct anti-metastatic effects in prostate cancer, KISS1 was re-expressed in PC3M cells that lack KISS1 and this resulted in an inhibition of cell migration and invasion and re-sensitization of cells to chemotherapeutics (10). Another study showed that KISS1R signaling induced the activation of eukaryotic translation initiation factor 2α kinase 2 (EIF2AK2) in prostate cancers and thereby inhibited cell growth and metastasis (69). Clearly, kisspeptin can act on prostate cancer cells both indirectly via the HPG axis and directly, thus its clinical value in treating prostate cancer is very promising and awaits further studies.

Lung Cancer

Lung cancer is the most commonly diagnosed cancer and the leading cause of death in men (57). Zheng et al. showed that the expression of *KISS1* was higher in stage I-II compared to stage III-IV and therefore showed an inverse relationship between *KISS1* expression and progression of non-small cell lung cancer (NSLCL). *KISS1* expression was also higher in the primary tumors compared to the second metastatic site, again showing that *KISS1* functions as a metastasis suppressor (70). In another study by Sun et al. the authors analyzed the expression of KISS1 and KISS1R in 28 patients and found that KISS1/KISS1R expression was higher in stage III compared to stage IV, again demonstrating an inverse relationship between KISS1/KISS1R expression and progression of NSCLC (71). Karapanagiotou et al. measured circulating levels of kisspeptin in 96 NSCLC patients (76 with metastatic disease and 21 with locally advanced disease) and detected no difference in plasma kisspeptin levels between NSCLC patients and healthy volunteers or between locally advanced and metastatic disease patients (72). The finding is reminiscent of that seen in prostate cancer where at the cellular level while KISS1 and KISS1R expression correlated with the disease, serum kisspeptin did not (10, 68).

CONCLUDING REMARKS AND PERSPECTIVE

KISS1 and KISS1R clearly play important roles in regulating the progression of cancers. However, the roles (that is, suppressor or promoter) these molecules play are cancer context-specific and may be further modulated by other factors such as the micro-environment. Thus, as highlighted in this review, the discrepancies reported among some studies analyzing the same cancer type is not entirely surprising. Discrepancies may also be the result of different experimental procedures and use of different reagents. In particular, the use of different antibodies might be a major culprit. It is well known that many of the commercially-available KISS1R antibodies in use are not highly specific and thus, if proper and detailed antibody control studies are not conducted, some results might be incorrectly interpreted. For example, antibody specificity should be verified using knock-out and/or knock-down approaches, as we have employed in our studies (20). Additionally, it is important to know whether a given antibody detects the full length KISS1 or smaller peptides. It remains possible that the full-length protein might have one relationship with the given cancer (for e.g., high KISS1 is associated with higher survival rates) while the peptides may have the opposite relationship. Thus, in addition to assessing protein levels, it is important to investigate changes in *KISS1*/*KISS1R* transcript levels as well. Positive correlation between mRNA and protein data strengthens a given study. The lack of clinicopathological parameters such as whether patients received neo-adjuvant chemotherapy or whether the primary tumor was surgically removed or left in place can also greatly complicate the interpretation of the results leading to discrepant findings and conclusions. Additionally, the size of clinical cohorts remains a great challenge and care must be placed on the over-interpretation of data based on small clinical populations. Nevertheless, even small cohorts have their utility in helping to design larger cohort studies.

To summarize, in the cancers where KISS1 is thought to function as a tumor suppressor, clinical analysis of KISS1/KISS1R expression patterns suggests two general scenarios. It is possible that there is an initial upregulation of KISS1/KISS1R expression to suppress tumor progression (for example, as seen in pancreatic and ovarian cancers). KISS1/KISS1R expression is high in early stages of the disease and patients with high gene expression had longer survival and better prognosis. In colorectal, bladder and renal cell carcinoma, KISS1 expression is decreased (likely due to hypermethylation), and this was associated with lower survival rates and increase in metastasis. In breast cancer, such as TNBC where ERα is lacking, KISS1/KISS1R expression is elevated in tumors, and this pathway appears to promote tumor growth and metastasis and is associated with poor patient outcome. However, several questions remain unanswered. For example, does circulating plasma kisspeptin modify cancerous tissue? Do tumors produce kisspeptin *in situ*? Is the expression of KISS1/KISS1R associated with an earlier or later development of cancer? Do normal cells produce kisspeptin to minimize metastasis? Further studies are warranted to study the KISS1/KISS1R signaling pathway in each cancer type.

In conclusion, despite the limitations of some clinical studies, it remains abundantly clear that KISS1 and KISS1R play important roles in regulating the progression of cancers and the molecules have the strong potential to be used in developing novel therapies and/or as prognostic markers in treating cancer, the second most deadly disease worldwide.

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MoB is the senior author on this manuscript. She helped with manuscript preparation and identification of the topics to cover. SG wrote the manuscript. SR, MuB, and AB assisted with manuscript preparation and provided critical feedback, especially with the clinical aspects.

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REVIEW
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Role of Kisspeptin and Neurokinin B in Puberty in Female Non-Human Primates

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In human patients, loss-of-function mutations in the genes encoding kisspeptin (*KISS1*) and neurokinin B (*NKB*) and their receptors (*KISS1R* and *NK3R*, respectively) result in an abnormal timing of puberty or the absence of puberty. To understand the neuroendocrine mechanism of puberty, we investigated the contribution of kisspeptin and NKB signaling to the pubertal increase in GnRH release using rhesus monkeys as a model. Direct measurements of GnRH and kisspeptin in the median eminence of the hypothalamus with infusion of agonists and antagonists for kisspeptin and NKB reveal that kisspeptin and NKB signaling stimulate GnRH release independently or collaboratively by forming kisspeptin and NKB neuronal networks depending on the developmental age. For example, while in prepubertal females, kisspeptin and NKB signaling independently stimulate GnRH release, in pubertal females, the formation of a collaborative kisspeptin and NKB network further accelerates the pubertal increase in GnRH release. It is speculated that the collaborative mechanism between kisspeptin and NKB signaling to GnRH neurons is necessary for the complex reproductive function in females.

Keywords: kisspeptin, neurokinin B, GnRH, puberty, nonhuman primate

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INTRODUCTION

Puberty is a transitional period between the sexually immature juvenile stage and adulthood, after which full reproductive function is attained. In the 1980s, the concept that an increase in GnRH release initiates puberty was established. Although from 1980 to 2000, it became clear that central inhibition over GnRH release during the prepubertal period needs to be removed or diminished in primates (1), the discovery that gene mutations in kisspeptin (*KISS1*) and its receptor (*KISS1R*) in human patients result in delayed puberty or no puberty (2, 3) has generated great progress in understanding the mechanism of puberty. Together, with the subsequent findings showing that mutations in neurokinin B (NKB) and its receptor (*NK3R*) in humans also result in delayed puberty or no puberty (4), this led us to study how kisspeptin and NKB signaling changes before and after puberty onset in female rhesus monkeys. This short review article summarizes our findings and perspectives regarding the role of kisspeptin and NKB signaling in puberty onset in females.

DEVELOPMENTAL CHANGES IN GONADOTROPIN SECRETION IN FEMALE RHESUS MONKEYS

Based on developmental changes in LH and FSH levels and external signs of puberty, we have defined the pubertal stages as follows: The "prepubertal stage" is when female monkeys do not exhibit any external signs of puberty and gonadotropin levels are low, generally before 20 months of age.

Prepubertal monkeys exhibit a low frequency and amplitude of LH pulses and there is little nocturnal increase in LH (5). The "early pubertal stage" is defined as the time between the appearance of the first external signs of puberty and menarche. The first external signs of female puberty, such as a slight increase in the nipple size and subsequent swelling of perineal sex-skin, usually occur at 20–25 months of age. These external signs of puberty are a consequence of increased levels of circulating gonadotropins and ovarian estrogens: The LH pulse amplitude starts to increase and a nocturnal elevation of gonadotropin levels becomes prominent (5). Subsequently, menarche occurs at 26–30 months of age. After menarche, females have irregular menstrual cycles without ovulation. Mean LH levels, LH pulse amplitude (not pulse frequency), and nocturnal LH further increase and at 36–45 months of age, monkeys start to ovulate. We have defined this developmental stage between menarche and first ovulation as the "midpubertal stage" [**Figure 1**; (5)].

RELEASE OF GnRH AND KISSPEPTIN INCREASES AT PUBERTY

GnRH Release

An increase in GnRH is a prerequisite for the initiation of puberty. This concept is based on an experiment showing that pulsatile infusion of GnRH in sexually immature female monkeys by infusion pump resulted in precocious puberty (6) and that an increase in GnRH release occurs at puberty onset in female rhesus monkeys (7). In the prepubertal female, GnRH release is pulsatile and characterized by low mean levels, low pulse frequency, low amplitude, and no nocturnal increases (7). In early pubertal females, mean GnRH levels, pulse frequency, and pulse amplitude are all increased, and nocturnal increases in GnRH release start to appear (7, 8). In midpubertal females, mean GnRH levels and pulse-amplitude, but not pulse frequency, further increase reaching the highest levels (7). Additionally, nocturnal GnRH increases become most prominent (7, 8). A similar pubertal increase in pulsatle GnRH release in rodents and sheep (9–11) has been shown by direct measurements, and in humans by indirect LH measurements (12–14). Because the pubertal increase in GnRH release is ovarian steroid independent (1), ovariectomized (OVX) females at the prepubertal stage exhibit a low mean, low pulse frequency, and low pulse amplitude GnRH release, similar to those in gonadally intact counterparts. In OVX females, at the early and midpubertal stages, mean GnRH levels and GnRH pulse amplitude are much higher than in ovarian intact females, but the pulse frequency stays similar, at ~1 pulse/h (8). A similar pubertal change in LH release in human gonadal dysgenesis patients with Turner's syndrome has also been reported (14, 15).

Kisspeptin Release

As we described for GnRH release, kisspeptin is released in the median eminence in a pulsatile manner (16). Additionally, kisspeptin release in females undergoes pubertal changes, parallel to those with GnRH release. The mean release, pulse frequency, and pulse amplitude of kisspeptin release in pubertal female monkeys are all higher than those in prepubertal females (17). Again, examination of the effects of OVX on kisspeptin release indicates that while OVX stimulates kisspeptin release in pubertal females, it does not change in prepubertal females (17). That is, kisspepetin release in prepubertal OVX females is characterized with low mean release, low pulse frequency, and low amplitude similar to those in ovarian intact prepubertal females, whereas kisspeptin release in pubertal OVX females consists of higher mean release and higher pulse amplitude, but not higher pulse frequency, when compared to ovarian intact pubertal females (17). Therefore, the pubertal increase in kisspeptin release in primates is ovarian steroid independent. Importantly, however, similar to GnRH release (18), treatment with estradiol suppresses elevated kisspeptin levels in pubertal females, whereas estradiol does not change kisspeptin levels in prepubertal females (17).

In humans, elevated levels of circulating kisspeptin in association with precocious puberty or premature thelarche have been reported (19–23). This is consistent with our results derived from direct kisspeptin measurements in the hypothalamus. Nevertheless, the validity of the finding in human studies is unclear, as circulating kisspeptin may not be of hypothalamic origin. In mammalian species, kisspeptin is synthesized not only in the various part of the brain (24) and placenta but also in peripheral tissues such as the adrenals, ovaries, testes, and kidney (25–28).

GnRH RESPONSE TO THE KISSPEPTIN RECEPTOR AGONIST, KISSPEPTIN-10, INCREASES AT PUBERTY

Since its discovery, kisspeptin has been identified as the most powerful secretagoue for GnRH release (29). GnRH neurons express kisspeptin receptors (Kiss1r) (30, 31), kisspeptin-10

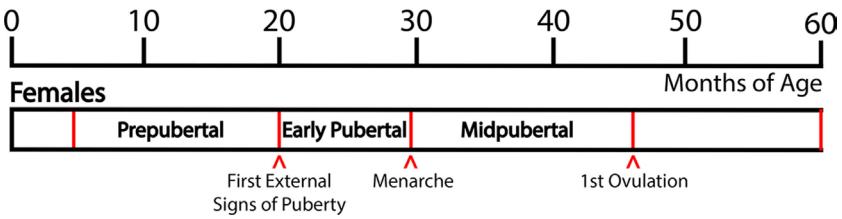

FIGURE 1 | Developmental stages of pubertal progression in female rhesus monkeys. Based on changes in physiological characteristics and in circulating hormone levels during the developmental course, prepubertal, early pubertal, and midpubertal stages are defined as shown in this figure. Actual age of the onset of puberty and subsequent progress vary among animals.

(hKP10) directly depolarizes GnRH neurons and sensitivity of GnRH response to kisspeptin undergoes pubertal changes in rodents (30). In humans and monkeys, contacts between GnRH and kisspeptin neuroterminals in the median eminence, which is indicative of a non-synaptic signaling mechanism, have been reported (32, 33).

To clarify the role of kisspeptin signaling in the pubertal increase in GnRH in female monkeys, we first assessed the manner in which the GnRH response to hKP10 changes throughout puberty. GnRH neurons in gonadally intact prepubertal and pubertal females respond to human hKP10 at 0.01 and 0.1 μM doses in a dose-responsive manner (34). Importantly, the GnRH response to hKP10 at the same dose in pubertal females is larger than that in prepubertal females [**Figure 2**; (34)]. This indicates that GnRH neurons in pubertal monkeys are more sensitive than in prepubertal monkeys.

Because circulating gonadal steroid levels between prepubertal and pubertal animals differ, higher sensitivity of GnRH neurons to kisspeptin signaling in pubertal females may be due to circulating steroids, namely estradiol. Accordingly, we examined the effect of OVX on the developmental changes in GnRH responses to hKP10 in female monkeys (34). While OVX in prepubertal animals does not alter GnRH response to hKP10, OVX completely abolished the hKP10-induced GnRH release in OVX pubertal females. Importantly, estradiol replacement in OVX pubertal females only partially restores the hKP10-induced GnRH release, suggesting that circulating estradiol is important for kisspeptin action on GnRH neurons in pubertal females (34). One can argue that the absence of GnRH response to hKP10 in OVX monkeys is due to the limitation of the maximized kisspeptin neurosecretory capacity after OVX. We believe this is not the case, because (1) two doses (10 and 100 nM) of hKP10 failed to stimulate GnRH release in pubertal OVX females, whereas in prepubertal females, the lower dose (10 nM) is sufficient to stimulate GnRH release (34), and (2) hKP10 (10 nM) can stimulate GnRH release in OVX pubertal females after priming with estradiol (34), although GnRH release in estradiol primed OVX pubertal females was much smaller than that in ovarian intact pubertal females (34). Therefore, it is likely that once KISS1R is exposed to estradiol after the onset of puberty, the properties of KISS1R are altered, such that normal KISS1R function requires the presence of circulating estradiol or, at least, a periodical exposure to estradiol. This speculation, however, needs to be experimentally confirmed by examining whether changes in the KISS1R properties occur in the presence or absence of estradiol and how developmental factors are involved in the mechanism of the estrogen-induced KISS1R property change.

Collectively, we can interpret our findings to mean that the contribution of kisspeptin signaling to the pubertal increase in GnRH release in female monkeys is twofold: first, after puberty onset, a larger amount of kisspeptin is available to stimulate GnRH release, and second, sensitivity of KISS1R on GnRH neurons is higher because of the pubertal increase in circulating estradiol.

GnRH RESPONSE TO THE NKB AGONIST, SENKTIDE, DOES NOT UNDERGO PUBERTAL CHANGE

Neurokinin B action is primarily mediated by NK3R encoded by the *TACR3* gene. Whether GnRH neurons express NK3R is somewhat controversial. While direct application of the NK3R agonist senktide on sliced brain preparation stimulates GnRH neuronal activity in mice (36) and the NK3R is described in close proximity to GnRH neuroterminals in rats and sheep (37, 38), only a small number or no GnRH neuronal cell bodies express NK3R in rat and mice (37, 39, 40). In mice and sheep, however, kisspeptin, NKB, and dynorphin (KNDy) neurons expressing NK3R in the arcuate nucleus (ARC) appear to mediate NKB action to GnRH neurons (39–41). Importantly, however, NKB neurons can signal to GnRH neurons directly at the median eminence, as similar to GnRH fibers, abundant NKB fibers project into the median

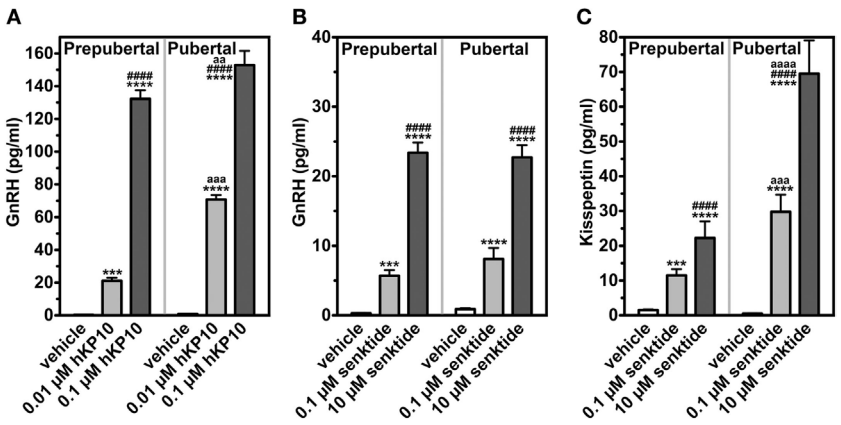

FIGURE 2 | Changes in release of GnRH and kisspeptin (area under the curve in response to challenge of secretagogues). GnRH in response to human kisspeptin-10 (hKP10) **(A)** and senktide **(B)** in female rhesus monkeys are shown. Kisspeptin response to senktide **(C)** is also shown. $^{***}p < 0.001$; $^{****}p < 0.0001$ vs. vehicle control (within a group). $^{####}p < 0.0001$ vs. lower dose (within a group). aa: $p < 0.01$; aaa: $p < 0.001$; aaaa: $p < 0.0001$ vs. prepubertal stage (between groups at the same dose of challenge). Modified from Ref. (35) with data from Ref. (34) with Copyright Permission.

eminence and GnRH neuroterminal fibers readily express NK3R in the median eminence of rats and humans (33, 37, 42, 43).

We examined the effects of the NKB agonist, senktide, on GnRH release in gonadally intact prepubertal and pubertal female monkeys. Senktide infusion into the median eminence at 0.1 and 10 μM stimulated GnRH release in a dose responsive manner within the same developmental stage [(35); **Figure 2**]. However, neither 0.1 nor 10 μM senktide results in developmental amplification. The results indicate that the NKB system appears not to be sensitive to the pubertal increases in steroid hormones. We have not conducted the parallel experiments in OVX monkeys.

Stimulatory effects of senktide on GnRH release in our study are consistent with those reported in juvenile orchidectomized male rhesus monkeys assessed by LH measurement (44). However, in rodents, both stimulatory and inhibitory effects of NKB on LH/GnRH release (depending on sex, gonadal status, and ages) have been reported (39, 40, 45–47).

KISSPEPTIN RESPONSE TO THE NKB AGONIST, SENKTIDE, UNDERGOES PUBERTAL CHANGE

Kisspeptin, NKB, and dynorphin (KNDy) are 100% co-localized in the ARC and express estrogen receptor alpha in sheep. Based on the anatomical characteristics along with the self-regulating stimulatory and inhibitory circuitry between NKB, kisspeptin, and dynorphin, Goodman and co-workers (48, 49) have proposed the hypothesis that KNDy neurons in the ARC are responsible for GnRH pulse-generation (50). Subsequently, this concept, including the 100% colocalization rate of three peptides in the ARC, and KNDy neurons as a driver of GnRH pulse-generation, has been confirmed in several species, including rats, mice, and goats (51–54). Nevertheless, we have hypothesized that kisspeptin, NKB, and dynorphin neurons in the hypothalamus of monkeys form a network as each independent unit. This hypothesis is based on the reports that (1) in the human hypothalamus the co-localization rate of kisspeptin, NKB, and dynorphin in the infundibular nucleus (aka ARC) is considerably lower than in other species (33, 55), (2) co-localization of kisspeptin and NKB fibers in the median eminence in humans is relatively rare (33) although this is not the case in male monkeys (56), and (3) unlike in rodents (57, 58), perikarya of kisspeptin neurons in monkeys and human and perikarya of NKB neurons in humans are seen in the median eminence (32, 33).

As the first step to test this hypothesis, we measured kisspeptin in the same samples collected from the median eminence, in which the effects of senktide on GnRH release were examined. The effects of senktide on kisspeptin release in females are strikingly parallel to its effects on GnRH (35). Kisspeptin responses to senktide at 0.1 and 10 μM in females are dose dependent within the developmental stage. However, senktide at both 0.1 and 10 μM doses yield an approximately twofold developmental amplification of kisspeptin release in females [(35); **Figure 2**]. We speculate that circulating estradiol is responsible for the developmental amplification of senktide-induced kisspeptin release, as the female kisspeptin system is highly sensitive to estradiol.

The important question here is why a larger release of kisspeptin induced by senktide in pubertal females than prepubertal females is not directly transduced to a larger GnRH release? We speculate that this is due to involvement of opioid input, as opioid tone increases after puberty onset. In fact, it has been shown that opioid tone increases along with the pubertal increase in estradiol/testosterone. For example, while administration of antagonists for opioid peptides, such as naloxone and naltrexone, in prepubertal children, chimpanzees, and rhesus monkeys failed to stimulate LH/GnRH release (59–64), these opioid antagonists consistently suppress pulsatile LH release in sexually mature humans and monkeys (65–68). Moreover, proopiomelanocortin mRNA expression increases along with progress of puberty in male monkeys (69) and β-endorphin release in the median eminence increases in association with puberty onset in female monkeys (70). A similar view has been reported in ewe (71). Perhaps, the pubertal increase in stimulatory kisspeptin and NKB signaling tones is counterbalanced by opioid peptides. Additional investigations are needed to confirm this view.

DEVELOPMENTAL CHANGES IN THE NEUROCIRCUITS INVOLVED IN THE PUBERTAL INCREASE IN GnRH RELEASE

As described above, both hKP10 and senktide stimulate GnRH release in a dose-responsive manner in prepubertal as well as pubertal female monkeys (34, 35). We also described that senktide greatly stimulates kisspeptin release in a dose-dependent manner in both prepubertal and pubertal females (35). However, these observations in females do not suggest any hierarchical relationship between NKB and kisspeptin signaling. Moreover, the network between kisspeptin and NKB signaling may undergo pubertal changes. Therefore, in the next series of studies, we have examined whether NKB signaling is mediated through kisspeptin neurons or kisspeptin signaling is mediated through NKB neurons using respective agonists and antagonists. The results indicate that the senktide-induced GnRH release is blocked in the presence of the KISS1R antagonist, peptide 234, in pubertal, but not prepubertal monkeys (35). Similarly, hKP10-induced GnRH release is blocked by the NK3R antagonist SB222200 in pubertal, but not prepubertal monkeys (35). These results suggest that while in prepubertal female monkeys, kisspeptin and NKB signaling influences GnRH release as independent units, in pubertal female monkeys, a reciprocal signaling network (i.e., NKB signaling through kisspeptin neurons and kisspeptin signaling through NKB neurons) is established (**Figure 3**). This cooperative mechanism by the kisspeptin and NKB networks appears to underlie the pubertal increase in GnRH release in female monkeys. We speculate that the cooperative mechanism between kisspeptin and NKB signaling to GnRH release would ensure the success of complex reproductive functions in females.

Our findings reported in this manuscript are obtained from dialysates collected from the median eminence, where agonists and antagonists for kisspeptin and NKB are directly infused. Because of our technical precision (72), interactions between GnRH and kisspeptin neurons, kisspeptin and NKB neurons, and

GnRH and NKB neurons are likely taking place at the median eminence and infundibular stalk (extended median eminence). As we discussed above, the primate median eminence appears to be equipped for this purpose. In the median eminence neuroterminal interactions between NKB, kissepeptin, and GnRH neurons are likely to occur through a non-synaptic mechanism, but the presence of kisspeptin, NKB, and GnRH neuronal cell bodies in the median eminence (32, 33, 73, 74) indicates possible synaptic interactions as well. Nevertheless, currently, we do not know the degree to which the median eminence kisspeptin-NKB system is influenced by the infundibular (ARC) kisspeptin-NKB system. It will be a major task to clarify the mechanisms of developmental changes in these signaling pathways that regulate GnRH release.

PULSATILITY OF GnRH RELEASE AND TIMING OF PUBERTY

Genetic findings in humans indicate that both kisspeptin and NKB signaling is critical for the mechanism governing puberty onset (2–4). Similar findings in kisspeptin and NKB knockout mice were also reported (75, 76). As we discussed above, kisspeptin signaling itself (77, 78) or the KNDy network (50) is indispensable for pulsatility of GnRH release and an increase in pulsatile GnRH release is required for puberty onset (1). Here, a critical question arises as to whether kisspeptin and NKB signaling determines the timing of puberty in primates. In other words, does an increased activity of kisspeptin signaling/KNDy network during development facilitate pulsatile GnRH release initiating puberty onset OR is an increased activity of kisspeptin signaling/KNDy network a consequence of reduction in "Central Inhibition"? The following is our view.

In primates, GnRH neurons in the hypothalamus are already active at birth and elevated GnRH neuronal activity induces a so-called "mini-puberty" during the neonatal period (79). However, activity of the GnRH neurosecretory system is suppressed by "Central Inhibition" and becomes dormant throughout the prepubertal period (80). Neuronal substrates that represent "Central Inhibition" are currently unclear. Our previous studies indicate that tonic inhibition by γ-aminobutyric acid (GABA) neurons may be one component (1, 81) and neuroestradiol (72) might be another component. It has also been postulated that MKRN3 protein may be responsible for suppression of GnRH release before puberty, as mutations of the *makorin RING finger protein 3* gene (*MKRN3*) result in precocious puberty in humans (82). More recently, based on the gene array comparison between castrated prepubertal and pubertal male monkey hypothalami, followed by physiological experiments, the transcriptional repressor protein, GATAD1, is postulated as a substrate responsible for prepubertal GnRH suppression (83). Nevertheless, the report that the kisspeptin antagonist, peptide 234, blocks the $GABA_A$ antagonist bicuculline-induced GnRH increase in prepubertal females (84) suggests that "Central Inhibition" by GABA is upstream of the kisspeptin signaling system. We speculate that GABA is also upstream of NKB signaling and the NKB antagonist SB222200 would block the $GABA_A$ antagonist bicuculline-induced GnRH increase in prepubertal females. Therefore, removal or reduction in "Central Inhibition" is a prerequisite for allowing the pubertal increase in activity of kisspeptin neurons or the KNDy network (**Figure 3**). Once kisspeptin/KNDy neurons become active, kisspeptin and NKB signaling ensures the pulsatile GnRH release, resulting in the onset of puberty.

The concept of "Central Inhibition" is well documented in humans (85) and rhesus monkeys (1, 86), but it remains controversial in non-primate species. In fact, there are several species differences in the mechanism of puberty onset: (1) As described above, while neonatal castration in primates induces elevated LH/FSH release only transiently (87), the same procedure in rats and sheep results in a sustained increase in gonadotropin release throughout life (11, 88); (2) while the GnRH neuroscretory system in prepubertal monkeys is insensitive to estradiol and sensitivity to estradiol negative feedback is acquired during the early pubertal stage (18), the GnRH neurosecretory system in rodents is highly sensitive to estradiol action during the entire juvenile period and sensitivity to estradiol decreases after first ovulation (88); and (3) while precocious puberty induced by infusion of pulsatile GnRH or *N*-methyl-D-aspartic acid (NMDA) in prepubertal monkeys is halted by the cessation of the infusion (6, 89), precocious puberty induced in rodents with a similar treatment, such as NMDA administration, leads to the maintenance of adult gonadal function after discontinuation of treatment, i.e., NMDA-induced precocious puberty in rats is followed by cyclic ovulation (90).

Despite these species differences, however, in rodents, there are some parallel findings consistent with the concept of the "Central Inhibition" described in primates. For example, in mice, Mkrn3 mRNA expression in the ARC is highest during the first 10–12 postnatal days (P), starts to decrease at P15, and becomes the

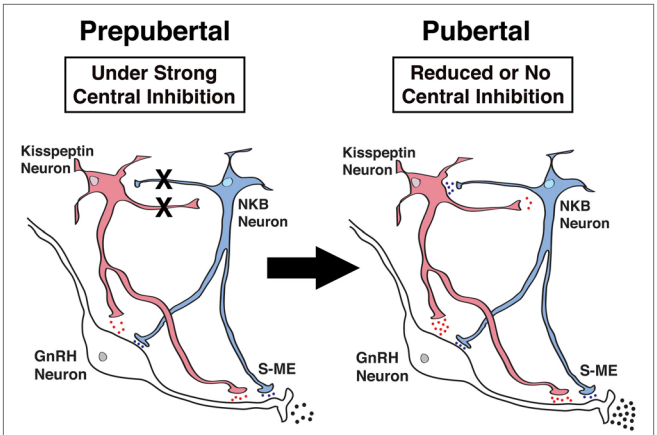

FIGURE 3 | Schematic diagram showing the developmental changes in kisspeptin (red) and neurokinin B (NKB) (blue) signaling to GnRH neurons in the stalk-median eminence (S-ME) in prepubertal (*left panel*) and pubertal (*right panel*) female monkeys. Note that X's between kisspeptin and NKB neurons indicate the absence of signaling pathways and the blue, red and black dots indicate relative amount of neuropeptide release. Kisspeptin signaling to NKB neurons is hypothetical, as in the present study, we did not measure the kisspeptin-induced NKB release. Modified from Ref. (35) with Copyright Permission.

lowest by P30, just prior to vaginal opening (82), and overexpression of human *GATAD1* gene by transfection in the mouse ARC results in delayed puberty, as postulated in prepubertal monkeys (83). Collectively, it appears that "Central Inhibition" is present in the rodent brain, but its functional significance may differ from that in primates.

CONCLUSION

We have shown that both kisspeptin signaling and NKB signaling appear to contribute to the pubertal increase in GnRH release independently or in concert in females. That is, while there is no interaction between kisspeptin and NKB signaling in sexually immature females, increases in kisspeptin signaling through NKB neurons and NKB signaling through kisspeptin neurons both augment the pubertal increase in GnRH release during the progress of puberty. The contribution of direct NKB signaling to GnRH release, however, may be secondary, as NKB signaling to GnRH release does not change across puberty, whereas NKB signaling to kisspeptin release greatly increases (**Figure 2**). Thus, in females, kisspeptin signaling appears to be the main force driving the pubertal GnRH release increases with their signaling intensity and an increased sensitivity of the receptor, KISS1R [(17, 34); **Figure 2**]. The role of NKB in the pubertal increase in GnRH release, however, requires further experiments, measuring developmental changes in NKB release in the presence or absence of kisspeptin agonists/antagonists.

We speculate that, in females, reciprocal signaling pathways between kisspeptin and NKB neurons would provide efficiency and flexibility for the stimulation of GnRH release, which ensures complex reproductive functions, such as cyclic ovulations and pregnancy. In summary, kisspeptin signaling and NKB signaling are both indispensable to facilitate the pubertal increase in GnRH after removal or diminution of "Central Inhibition." Further studies, such as measurements of NKB release in the hypothalamus and examination of the role of dynorphin would strengthen our views.

AUTHOR CONTRIBUTIONS

ET and JPG designed experiments. JPG and KLK conducted experiments. JPG analyzed the data, and ET, JPG, and SBS wrote the manuscript.

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REVIEW
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The Roles of Kisspeptin System in the Reproductive Physiology of Fish With Special Reference to Chub Mackerel Studies as Main Axis

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Kisspeptin, a novel neuropeptide product of the *Kiss1* gene, activates the G protein-coupled membrane receptor G protein-coupled receptor 54 (now termed Kiss1r). Over the last 15 years, the importance of the kisspeptin system has been the subject of much debate in the mammalian research field. At the heart of the debate is whether kisspeptin is an absolute upstream regulator of gonadotropin-releasing hormone secretion, as it has been proposed to be the master molecule in reproductive events and plays a special role not only during puberty but also in adulthood. The teleostean kisspeptin system was first documented in 2004. Although there have been a number of kisspeptin studies in various fish species, the role of kisspeptin in reproduction remains a subject of controversy and has not been widely recognized. There is an extensive literature on the physiological and endocrinological bases of gametogenesis in fish, largely derived from studying small, model fish species, and reports on non-model species are limited. The reason for this discrepancy is the technical difficulty inherent in developing rigorous experimental systems in many farmed fish species. We have already established methods for the full life-cycle breeding of a commercially important marine fish, the chub mackerel (cm), and are interested in understanding the reproductive function of kisspeptins from various perspectives. Based on a series of experiments clarifying the role of the brain–pituitary–gonad axis in modulating reproduction in cm, we theorize that the kisspeptin system plays an important role in the reproduction of this scombroid species. In this review article, we provide an overview of kisspeptin studies in cm, which substantially aids in elucidating the role of kisspeptins in fish reproduction.

Keywords: kisspeptin, puberty, brain–pituitary–gonad axis, marine teleost, perciform, aquaculture

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INTRODUCTION

Kisspeptin is an RFamide peptide product of the *Kiss1* gene and the natural ligand of the G protein-coupled receptor 54 (GPR54), now named Kiss1r (1, 2). Mature kisspeptins in mammals are cleaved into endogenous fragments: Kp54, Kp16, Kp14, Kp13, and Kp10 (3). The C-terminus decapeptide Kp10 (Kiss-10) region is the minimum active site and is highly conserved across vertebrates. Mutations in the *Kiss1R* gene are correlated with an absence of puberty onset and hypogonadotropic hypogonadism in humans (4, 5). These abnormalities are due to the disruption of the hierarchical reproductive network, especially the kisspeptin–gonadotropin-releasing hormone (Gnrh)–luteinizing

hormone (Lh) pathway. Kisspeptin fibers have been observed in the vicinity of Gnrh neuron cell bodies, and a large population of Gnrh neurons expresses *Kiss1r* mRNA, clearly indicating that kisspeptin neurons directly signal Gnrh neurons (6–8). Indeed, administration of Kp10 was found to elicit a robust increase in the circulating levels of Gnrh (9). Several studies in mammals have strongly demonstrated the absolute necessity of kisspeptin signaling for puberty onset and ovulation through the regulation of Gnrh secretion.

In 2004, the isolation of the complementary DNA (cDNA) of a piscine ortholog of the kisspeptin receptor (*kissr2*) in Nile tilapia (*Oreochromis niloticus*) was the first evidence for the existence of a kisspeptin system in fish (10). In mammals, only one gene (*Kiss1*) coding for the ligand and one for the receptor (*Kiss1r*) are present. Teleost fishes, known to have undergone a third genome duplication event, have two paralogous kisspeptin genes (*kiss1* and *kiss2*), and four genes encoding kisspeptin receptors have been reported, although most fish have only two receptors, *kissr2* and *kissr3* (also known as *gpr54-2b* and *gpr54-1b*) (11, 12).

At least two molecular forms of Gnrh are present in the brains of all vertebrate species, with some teleosts expressing three different forms (i.e., Gnrh1, Gnrh2, and Gnrh3) (13). Gnrh1 is considered the major hypophysiotropic hormone controlling the synthesis and release of Lh in all vertebrates. By contrast, Gnrh3 is a teleost-specific form that is expressed in neuronal populations in the olfactory bulb, the terminal nerve ganglion region, and the pre-optic area (POA). Gnrh3 axonal fibers project into different brain regions, suggesting a role in neuromodulation. In fish expressing two Gnrh forms, such as the Salmonidae and Cyprinidae, Gnrh3 not only functions as a neuromodulator but also regulates the secretion of pituitary gonadotropins (14, 15).

The major underlying question is whether fish kisspeptin is an important regulator of the reproductive brain–pituitary–gonad (BPG) axis. Several studies strongly suggest that, similar to its mammalian counterpart, the fish kisspeptin ortholog is a potent activator of the reproductive axis. Gnrh1 neurons express *kissr2* mRNA in cichlid fish (e.g., *O. niloticus* and *Astatotilapia burtoni*) (10, 16), and striped bass (*Morone saxatilis*) (17) is a notable example. In addition, several *in vivo* studies have shown that the injection of Kiss2 peptide promotes the secretion of Gnrh and gonadotropins (18, 19). Furthermore, kisspeptin antagonists were found to inhibit sperm production in striped bass (20). However, in the zebrafish (*Danio rerio*), *kiss-* or *kissr*-knockout mutants exhibited normal gonadal maturation, indicating that kisspeptin signaling is not indispensable for reproduction in this species (21). A few studies have indicated that Gnrh neurons do not express kisspeptin receptors in medaka (*Oryzias latipes*) (22) or European sea bass (*Dicentrarchus labrax*) (23). Hence, the true role of kisspeptin in fish reproduction remains open to debate.

The chub mackerel (cm) (*Scomber japonicus*) is a small marine pelagic fish, which belongs to the order Perciformes and the family Scombridae. This species is one of the top 10 principal food fish contributing to global capture fisheries production. In addition, cm is a suitable experimental model fish for reproductive endocrinological research in Perciformes, which is the most evolved and largest teleost fish group and includes many target aquaculture species. Our team recently developed standardized methods to support the full life cycle of this species in land-based small-scale aquaculture. This system aids in obtaining a series of captive cm samples throughout the entire reproductive cycle for the analysis of key hormones acting in the BPG axis and enables breeding experiments. Using these fish sampling facilities, our group has isolated the key molecular elements of the cm BPG axis in reproduction, namely Gnrhs (Gnrh1, 2, and 3) (24, 25), the Gnrh receptor (GnrhR1) (26), gonadotropic hormones (Gths) (27–30), and Gth receptors (31). Furthermore, steroid hormones involved in vitellogenesis and oocyte maturation have been demonstrated in this species (32). There is an extensive literature on the physiological and endocrinological bases of gametogenesis in fish; this literature is largely derived from small, model fish species, such as zebrafish and medaka. However, in many cases, data on the mechanisms of reproductive regulation in teleosts vary greatly among species. This may be due to the considerable length of their evolutionary process and the diversity of species, reproductive patterns, and habitats. To clarify the general mechanisms underlying the reproductive physiology of fish, studies in non-model species may be informative.

In recent years, we have focused on inducing the potency of kisspeptin peptides in gonadal development. Of particular interest is the period of pubertal transition and gonadal recrudescence, which is important for the establishment of an efficient aquaculture of any fish species. The aim of this article is to review our previous 11 papers on the kisspeptin system in cm reproduction. At the same time, we strongly adhere to a comparative approach involving other model and non-model teleost fish species to provide a comprehensive summary of the knowledge on fish kisspeptins to date.

CHARACTERISTICS OF KISSPEPTINS AND THEIR RECEPTORS

Ligands

The cm possesses two kisspeptin genes, *kiss1* and *kiss2* (33). These sequences were submitted to GenBank as follows: *kiss1*, GU731672 and *kiss2*, GU731673. *cmkiss1* and *cmkiss2* cDNAs encode 105 and 123 amino acids, respectively, and display very low sequence similarity (18%).

Kisspeptin coding sequences have been isolated in many fish species. All reported teleost species possess the *kiss2* gene, whereas the genomes of puffer fish and sticklebacks (*Gasterosteus aculeatus*) lack the *kiss1* gene and contain only the *kiss2* gene (18).

The C-terminus decapeptide Kp10 region is highly conserved within mammalian and non-mammalian vertebrates. In an initial study of fish kisspeptin, the deduced sequences for Kiss1-10 and Kiss2-10 were assumed to be the minimum functional core peptides. The modified fish KP44 peptide, which lacks the C-terminus KP10 region, has no bioactivity, suggesting that the KP10 region is essential for receptor binding, as is the case with mammalian kisspeptin (34). However, in teleost fish, the Kiss1 precursor contains a conserved dibasic 5-amino acid site upstream of the KP10 region, indicating that it produces a mature pentadecapeptide (Kiss1-15), which should have pyroglutamate at the N-terminus because the residue at the N-terminal end of

Kiss1-15 is glutamine in all reported fish species (35). Similarly, a conserved arginine residue at position 13 is present in all available Kiss2 sequences, indicating the presence of a putative cleavage site that produces a mature dodecapeptide (Kiss2-12) (35). Both the Kiss1-15 and Kiss2-12 regions are highly conserved across teleost fishes.

The cmKiss1-15 (QDMSSYNFNSFGLRY-NH_2) and cmKiss2-12 (SNFNFNPFGLRF-NH_2) peptides showed the highest potency for the activation of cognate receptors, stronger than their corresponding KP10 peptides in cm (36). The same results were reported in zebrafish (35) and European sea bass (37). These results suggest that amino acid sequences other than the KP10 region are also functionally important, perhaps due to factors such as the structure, hydrophobicity, or electric charge of fish kisspeptins. Choosing the right peptide form to test is therefore important for clarifying the bioactivity of fish kisspeptins.

The cmKiss1 precursor contains a putative cleavage site located seven amino acids upstream of the core sequence (33), indicating that it produces a mature hexadecapeptide (cmKiss1-16: HQDMSSYNFNSFGLRY-NH_2). This Kiss1-16 showed higher sensitivity for receptor activation than Kiss1-15 (38). Another example, a mature Kiss2 tridecapeptide (Kiss2-13) was isolated in masu salmon (*Oncorhynchus masou masou* and *Oncorhynchus nerka*) (39). This may also reflect differences in the basic site of the precursor protein.

Receptors

Chub mackerel possess two receptor subtypes (*kissr2* and *kissr3*), which have already been submitted to GenBank as *kissr2* (*gpr54-2*): JX982323 and *kissr3* (*gpr54-1*): JX982322 (36). The *kissr2* and *kissr3* cDNAs have open reading frames of 1,137 bp (378 amino acids) and 1,110 bp (369 amino acids), respectively. The amino acid sequences of the receptors share only 52% identity; however, high sequence identity was found between the transmembrane domains (73%). cmKissR2 exhibited >80% identity with all other teleosts and >93% identity with other Perciform fish KissR2 sequences. cmKissR3 showed high similarity to European sea bass (88%), followed by striped bass (87%); however, slightly lower homology with goldfish (65%) and zebrafish (62%) KissR3 sequences was observed.

Since its first discover in 2004, kisspeptin receptors have been cloned and sequenced in more than 50 different teleost fish species. Almost all these fish possess the piscine ortholog *kissr2*, whereas few species possess *kissr2* and *kissr3* sequences in their genomes. There are a few exceptions; for example, the European eel (*Anguilla anguilla*), coelacanth (*Latimeria chalumnae*), and spotted gar (*Lepisosteus oculatus*) possess three types of receptors (*kissr1*, *kissr2*, and *kissr3*), and coelacanth and the spotted gar further possess *kissr4* genes, as detailed in the phylogenetic tree (11, 40). It is important to note that the nomenclature of fish kisspeptin receptors has not yet been established, and different research teams use different abbreviations. In this article, the receptor names are adopted from syntenic studies by Pasquier et al. (11, 12). Elsewhere, *kissr2* is sometimes referred to as *kiss2r* or *gpr54-2*. Furthermore, other designations of *kissr3* include *kissr1*, *kiss1r*, and *gpr54-1*, because, in many cases, this receptor showed high sensitivity for the Kiss1 peptide. Our previous kisspeptin papers referred to the kisspeptin receptors as *kissr1* and *kissr2*.

Ligand Sensitivity

The ligand sensitivities of the two subtypes of cm kisspeptin receptors were examined by reporter gene assays using mammalian cell lines. The results revealed that cm kisspeptin receptor signals are preferentially transduced *via* the protein kinase C (PKC)/mitogen activated protein kinases rather than by the protein kinase A (PKA) pathway (36). Synthetic cm Kiss1-15 (or Kiss1-16) and Kiss2-12 peptides showed the highest potency for the activation of KissR3 and KissR2, respectively (**Figures 1A,B**) (36, 38). Thus, we concluded that KissR3 and KissR2 are the intrinsic receptors for the Kiss1 and Kiss2 peptides, respectively, and signals are mainly transduced *via* the PKC pathway in this species (36).

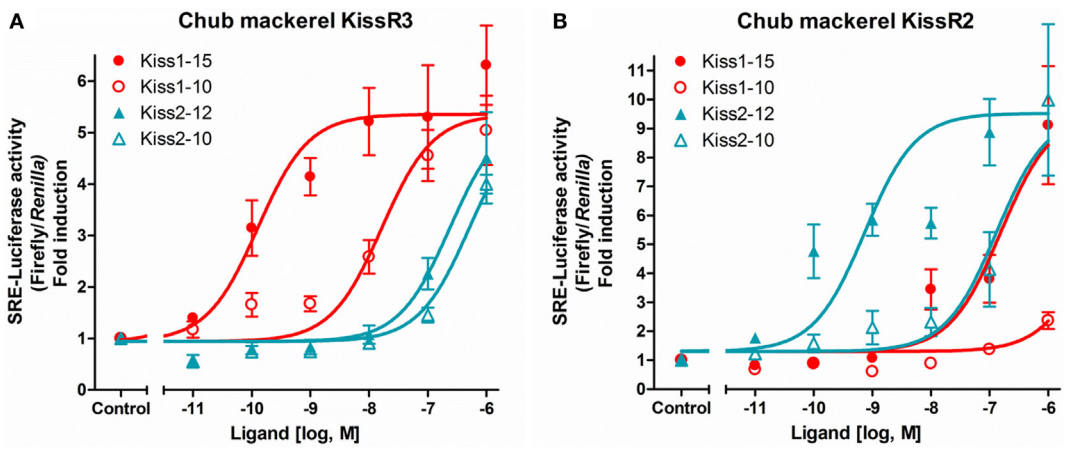

FIGURE 1 | Ligand selectivity of the chub mackerel (cm) kisspeptin receptors. KissR3 **(A)** and KissR2 **(B)**, each together with SRE-Luc. Transfected cells were treated with graded concentration of each peptides. The data are expressed as the ratio of changes in firefly luciferase activity over the control *Renilla* luciferase activity. Each point was determined in quadruplicate and is given as a mean ± SEM. Modified from Ref. (36), by permission of Elsevier.

Ligand sensitivity has been analyzed in duplicated kisspeptin systems in zebrafish (35, 41), goldfish (*Carassius auratus*) (42), Southern bluefin tuna (*Thunnus maccoyii*) (43), yellowtail kingfish (*Seriola lalandi*) (43), medaka (22), European sea bass (37), and striped bass (20) and, in one species, the orange-spotted grouper (*Epinephelus coioides*), with only the Kiss2/KissR2 pair (44). In all studies, intracellular signals were preferentially transduced *via* the PKC pathway; however, the PKA pathway was also activated by ligand stimulation in goldfish (42), medaka (22), and European sea bass (37). In many cases, KissR2 and KissR3 showed high sensitivity for the Kiss2 and Kiss1 peptides, respectively. Conversely, in goldfish, Kiss1-10 enhanced KissR2 activation, and Kiss2-10 exhibited a higher preference for KissR3 (42). It should also be noted that KissR2 showed equal sensitivity to both Kiss1 and Kiss2 peptides in the zebrafish (35), Southern bluefin tuna, and yellowtail kingfish (43); KissR3 showed the same binding potency for Kiss1 and Kiss2 peptides in striped bass (20). The high species specificity and the complexity of the ligand sensitivity or signaling pathways in duplicated kisspeptin systems in fish is noteworthy.

BIOACTIVITY

Acute Effects

We evaluated the biological potency of kisspeptin peptides to induce transcriptional changes in *gnrh1*, follicle-stimulating hormone *(fsh)b*, and *lhb* in gonadal recrudescent cm. Synthetic Kiss1-15 and Kiss2-12 were administered at a dose of 100 ng into the intracerebroventricular (i.c.v.) region, and brains were sampled at 6 and 12 h post-administration (**Figure 2A**). In both sexes, the transcription levels were quantified using quantitative real-time PCR (qRT-PCR). i.c.v. administration of Kiss2-12 but not Kiss1-15 significantly elevated pituitary *fshb* and *lhb* transcripts at 12 h post-administration in comparison to saline-injected controls (**Figures 2B,C**) (45). In addition, in females, the transcription levels of POA *gnrh1* were downregulated by both kisspeptin peptides at 12 h post-administration (45). It is clear that central administration of kisspeptin peptides influenced Gnrh and gonadotropin synthesis, suggesting that kisspeptin peptides may play a central role in the regulation of the reproductive BPG axis in cm.

The third ventricle was chosen as the site of administration due to its proximity to the brain centers controlling reproductive activity; it is the most accurate way to study the role of centrally acting peptides (45). Thus, delivering exogenous kisspeptins to the central nervous system is important, and our study was the first to demonstrate the effects of kisspeptin peptides on brain *gnrh* mRNA expression. In the same manner, Espigares et al. evaluated the bioactivity of kisspeptin peptides in immature adult European sea bass. i.c.v. administration of Kiss2-12 stimulated Gnrh1 release into the pituitary and increased serum Fsh, Lh, and sex steroids until 72 h post-treatment (19). These

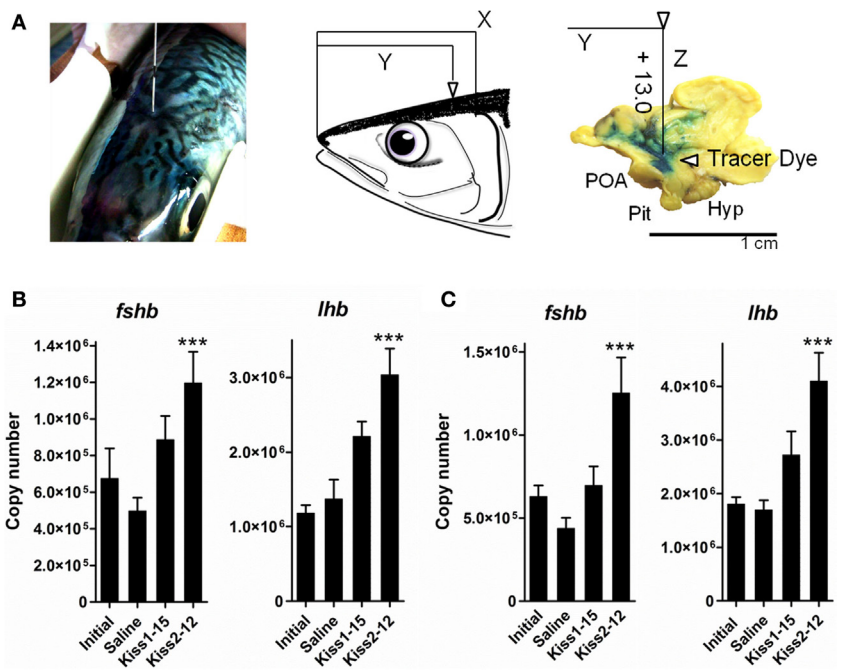

FIGURE 2 | Schematic illustration of coordinates for injection into the intracerebroventricular (i.c.v.) region of chub mackerel **(A)**. We determined that if head length (rostral end to upper end of gill) was assumed to be *X* and length of the rostral end to immediately above the third ventricle was assumed to be *Y*, the ratio of *X* to *Y* converged from 1.4 to 1.0. Tested peptide or PBS with blue dye was administered into the third ventricle to a depth of 13 mm below the tissue surface. Abbreviations: Hyp, hypothalamus; Pit, pituitary; POA, pre-optic area. **(B,C)** Analysis of the effect of i.c.v. administration of Kiss1-15 and Kiss2-12 peptides on *fshb* and *lhb* mRNA levels in the pituitary at 12 h post-administration, each together in adult male **(B)** or female **(C)** immature subjects. Transcription levels are the mean ± SEM of 9–16 independent determinations. ***$P < 0.001$, one-way ANOVA followed by a Tukey's multiple comparison test. Modified from Ref. (45), by permission of Elsevier.

results suggest that kisspeptin potently stimulates reproductive axis activity.

The biological effects of kisspeptin peptides through peripheral [intraperitoneal (i.p.) and intramuscular (i.m.)] administration have been studied in some fish species. In early to mid-pubertal fathead minnows (*Pimephales promelas*), i.p. administration of mammalian KP10 increased the expression of *gnrh3* in the brain after 10 h (46). In sexually mature female zebrafish, i.p. administration of Kiss2-10 upregulated *fshb* and *lhb* expression 12 h post-treatment (47). In goldfish, i.p. administration of Kiss1-10 stimulated Lh secretion until 6 h post-treatment (42). In sexually mature female orange-spotted grouper, i.p. administration of Kiss2-10 increased hypothalamic expression of *gnrh1* and pituitary *fshb* expression until 12 h post-treatment (44). Finally, in pubertal hybrid bass (*Morone* species), i.m. administration of Kiss2-12 upregulated plasma Lh levels until 24 h post-treatment and, during gonadal recrudescence, both Kiss1-15 and Kiss2-12 induced Lh secretion after 24 h (17). Taken together, these data conclusively demonstrate that kisspeptins induce Gnrh and gonadotropin release in many fish species.

Chronic Treatment *In Vivo*

We evaluated the potency of Kiss1 and Kiss2 in inducing gonadal development in sexually immature prepubertal and gonadal recrudescent cm. In pubertal fish, synthetic Kiss1-15 or Kiss2-12 peptides were mixed with molten cocoa butter (slow-releasing medium), and peripheral injection was repeated three times at an interval of 2 weeks. In adult fish, the same peptides were administered subcutaneously using Alzet mini-osmotic pumps (Model 2006). In both studies, peptide treatments were continued for a period of 6 weeks.

These results deserve explicit emphasis. In pubertal males, 66.7% of Kiss1-15-treated fish had spermatozoa (SPZ) in their testes, whereas no SPZ were observed in Kiss2-12- and saline-treated fish (48). In addition, the levels of sex steroids 11-ketotestosterone (11-KT) and estradiol-17β (E2) were significantly higher in Kiss1-15-treated fish (48). In pubertal females, Kiss1-15-treated fish displayed yolk vesicles in the growing oocytes, whereas Kiss2-12- and saline-treated fish did not. E2 levels were significantly higher in Kiss1-15-injected fish than in control fish (49). The effects on gonadal recrudescence were more critical. In recrudescent males, Kiss1-15-treated fish exhibited a significantly higher gonadosomatic index (GSI) than did all other treatment groups, and SPZ were present in the testes of Kiss1-15-treated fish at the end of the experiment (**Figures 3A–E,K,L**) (50). The GSI values of adult females did not differ among treatments; however, the mean oocyte diameters of Kiss1-15-treated fish, representing the early vitellogenic oocytes, were significantly higher than those of the saline- and Kiss2-12-treated fish (**Figures 3F–J,M**) (50). In both sexes, Kiss1-15-treated fish exhibited higher levels of circulating 11-KT and E2 (**Figures 3N,O**) (50). These results suggest that synthetic kisspeptin peptides can induce pubertal onset and gonadal recrudescence in cm.

There is ample evidence to indicate the importance of kisspeptin stimulation for gonadotropin secretion and gonadal development in fish. Beck et al. reported that subcutaneous biweekly administration of European sea bass Kiss1-10 but not Kiss2-10 for 7 weeks significantly increased the GSI and SPZ volume in prepubertal male white bass (*M. chrysops*) (51). Nocillado et al. utilized slow-release implants to chronically deliver synthetic kisspeptin to prepubertal male yellowtail kingfish and reported that fish treated with Kiss1-10 were 100% developed over 4 weeks and exhibited the most advanced stage of development, with testes containing mostly spermatids and SPZ (52). On the other hand, 8-week treatment with Kiss2-10 had a stronger stimulatory effect on testicular development during the non-breeding period (52). In male and female cinnamon clownfish (*Amphiprion melanopus*), treatment with human KP10 upregulated Gnrhs, Gths, Gth receptors, estrogen, and vitellogenin in the brain, pituitary, gonads, serum, and liver, respectively, and also promoted gonadal development over 6 weeks (53). In male European sea bass, Kiss2-12 treatment induced a significant increase in cumulative milt on days 3 and 7 after i.c.v. administration (19). Finally, in male and female Nile tilapia, cognate Kiss2-10 was administered i.p. twice weekly, and this increased the expression of *gnrh1*, *fshb* and *lhb* mRNA, and plasma levels of E2 and 11-KT (54). This study also showed accelerated testicular development after 4 weeks (with eight total administrations of kisspeptin). Overall, these studies suggest that chronic treatment with kisspeptin peptides modulates gonadotropin secretion and influences gonadal development in many fish species. However, the effects on ovarian development may be slower. For example, oocyte size increased, but there were no histological differences in prepubertal white bass after a 7-week period of Kiss1-10 or Kiss2-10 treatment (51), in prepubertal striped bass after 10 weeks of Kiss1-15 treatment (55), or in immature Nile tilapia after 4 weeks of Kiss2-10 treatment (54). The same trend was confirmed in our study using prepubertal female cm (49). Detailed and long-term research is needed to comprehensively examine both the basic science and commercial application of kisspeptin peptides.

GENE EXPRESSION

Expression in BPG Axis

Quantitative real-time PCR analysis revealed that kisspeptin system transcripts are distributed in different tissues of the BPG axis in adult male and female cm. The *kiss1* gene was expressed in the brain, pituitary, and gonad (33). The *kiss2* gene was expressed only in the brain (33). The *kissr2* gene was expressed in the brain, pituitary, and testes but not in the ovary (36). The *kissr3* gene was expressed in the brain and testes but not in the ovary (36).

Early Developmental Stages

We analyzed expression changes in these genes during early development [0–30 days post-hatching (dph)] and during the period of gonadal sex differentiation (37–60 dph) in cm using qRT-PCR assays. During early development, the expression of *kiss1*, *kiss2*, and *kissr2* in the whole head did not vary significantly; however, *kissr3* expression decreased significantly at 20 dph compared with expression levels just after hatch (56). Interestingly, *kiss2*, *kissr2*, and *kissr3* were significantly elevated at the start of gonadal sex differentiation in both males and females (56). These results

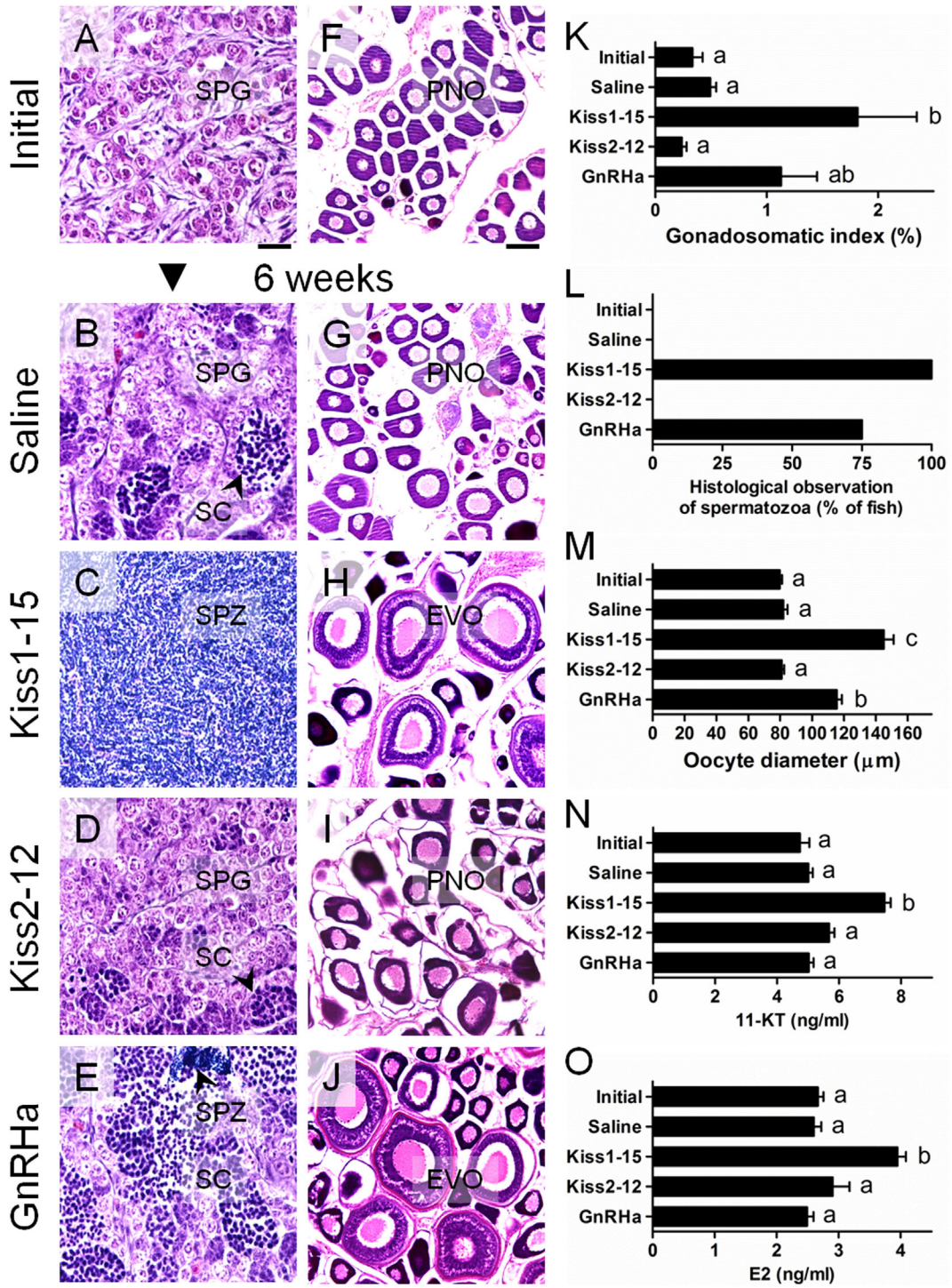

FIGURE 3 | Changes in the gonadal histology of male **(A–E)** and female **(F–J)** chub mackerel in different treatments. Abbreviations; SPG, spermatogonia; SC, spermatocytes; SPZ, spermatozoa; PNO, perinucleolar oocyte; EVO, early vitellogenic oocyte. Scale bars = 100 μm. **(K)** Changes in the gonadosomatic index (GSI) of male fish. **(L)** Percentage of fish by treatment showing histological presence of SPZ in the testes. **(M)** Changes the mean oocyte diameter of fish at different treatments, respectively. Changes in serum 11-ketotestosterone (11-KT) in male **(N)** and estradiol-17b (E2) in female **(O)**, respectively. Values are means ± SEM (n = 4–6 for each treatment). Different letters above the bars represent significant differences between treatments ($p < 0.05$, one-way ANOVA followed by a Tukey's multiple comparison test). GnRHa: GnRH analog (D-Ala^6, des-Gly^{10})-LHRH ethylamide. Modified from Ref. (50), by permission of Zoological Society of Japan.

suggest the potential involvement of the kisspeptin system during early development and gonadal sex differentiation in the cm.

There is strong evidence to suggest that the early kisspeptin system modulates the proliferation of the neuronal network. In zebrafish, Zhao et al. showed that early treatment with both Kiss1 and Kiss2 stimulated the proliferation of trigeminal Gnrh3 neurons located in the peripheral nervous system, and Kiss1, but not Kiss2, stimulated the proliferation of the terminal nerve and hypothalamic populations of Gnrh3 neurons in the central nervous system (57). In the same species, qRT-PCR revealed detectable levels of *kiss1* and *kiss2* mRNA by 1 day post-fertilization (dpf), which increased throughout embryonic and larval development (57). Furthermore, there are several other examples of kisspeptin expression at early developmental stages. In medaka, the expression of *kiss1*, *kiss2*, and *kissr3* was observed immediately 1 h post-fertilization, and both *kiss1* and *kiss2* levels peaked at 1 dpf (58). In cobia (*Rachycentron canadum*), *kissr2* expression was detected at 1 dph and peaked at 2 and 3 dph (59). In black rockfish (*Sebastes schlegelii*), both *kiss1* and *kissr2* expression displayed an increasing trend during early development (60).

Expression changes during gonadal sex differentiation were also confirmed in some reports. In cobia, *kissr2* expression increased in sexually differentiated males but not in females (59). In fathead minnow, *kissr2* expression increased in both males and females immediately after gonadal sex differentiation (46). In pejerrey (*Odontesthes bonariensis*), *kiss2* levels increased at 4 weeks after hatching, which is when gonadal differentiation occurs in this species (40). Finally, in tongue sole (*Cynoglossus semilaevis*), *kiss2* and *kissr2* transcripts rapidly increased during the early gonadal differentiation period (61). It is likely that an early kisspeptin system is important in brain development and involved in modulating gonadal sex differentiation in teleost fishes.

Pubertal Stage

We examined the temporal patterns of gene expression of the two kisspeptin subtypes and their receptors in the brain during the pubertal process in cm using qRT-PCR. In male fish, *kiss2* and *gnrh1* expression levels increased significantly just before the onset of meiosis in the testes (62). In female fish, *kiss1* and *kiss2* levels increased significantly, concomitant with increases in the *kissr2*, *kissr3*, and *gnrh1* levels, just before the onset of vitellogenesis in the oocytes (**Figure 4**) (62). Notably, upon pubertal onset, pituitary *gnrhr1*, *fshb*, and *lhb* began to gradually increase in both sexes (26, 63). These results suggest that, as is the case in

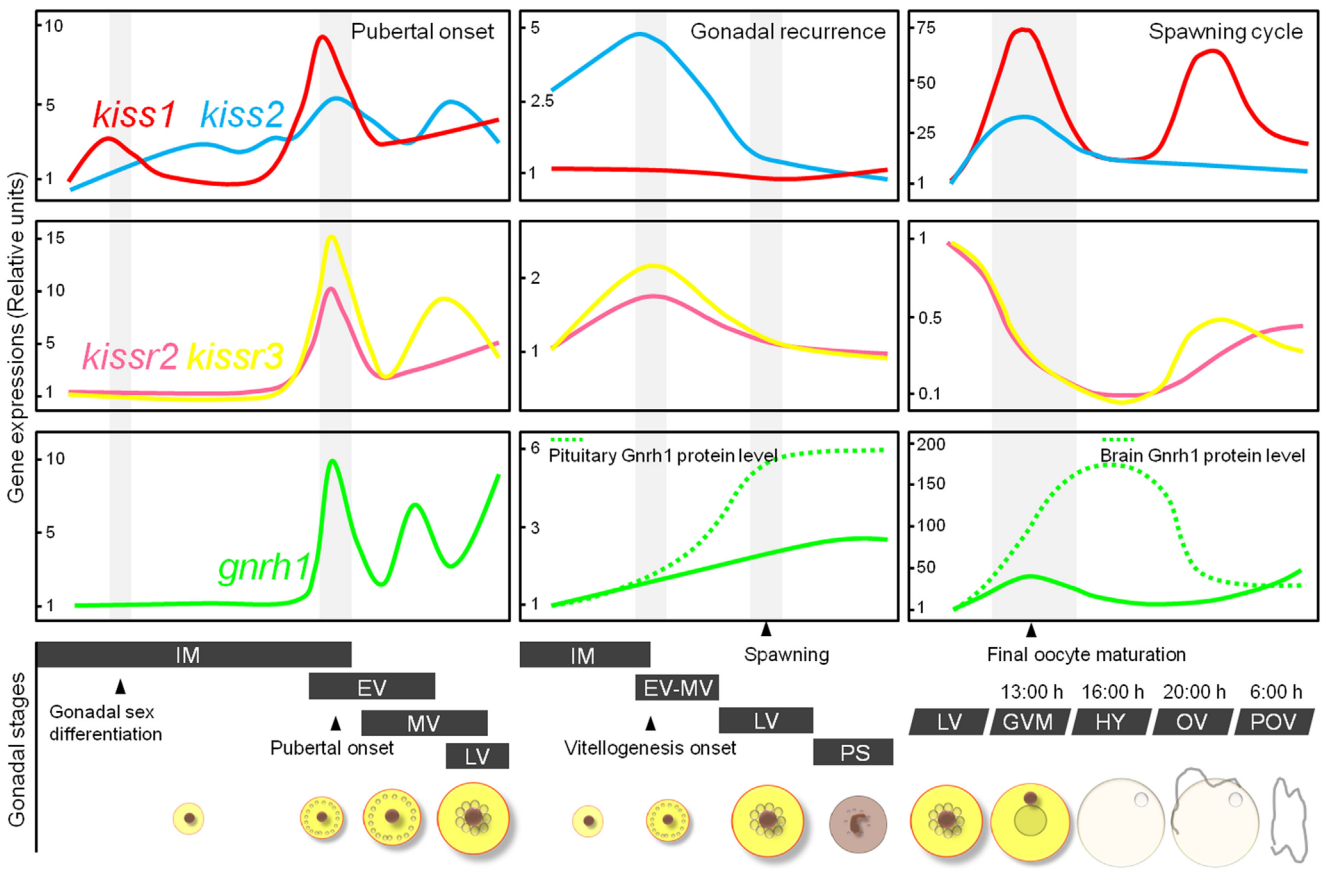

FIGURE 4 | Schematic representation showing relative changes in *kiss1*, *kiss2*, *kissr2*, *kissr3*, and *gnrh1* gene levels in the brain of female chub mackerel during the puberty, gonadal recurrence, and spawning cycle. Abbreviations: IM, immature; EV, early vitellogenesis; MV, mid-vitellogenesis; LV, late vitellogenesis; GVM, germinal vesicle migration; HY, hydration; OV, ovulation; POV, post-ovulation.

mammals, the positive involvement of the kisspeptin system in the pubertal process in cm.

For the moment, let us closely examine kisspeptin gene expression at puberty. The levels of *kissr2* mRNA in the brain were correlated with pubertal development in the cobia (59), grey mullet (*Mugil cephalus*) (64), zebrafish (41), fathead minnow (46), Nile tilapia (65), Senegalese sole (*Solea senegalensis*) (66, 67), Atlantic halibut (*Hippoglossus hippoglossus*) (68), and Atlantic salmon (*Salmo salar*) (69). In the brains of Nile tilapia, *kissr2* was expressed in a significantly higher percentage of Gnrh neurons in mature males than in immature males (10). Ligand expression during puberty was reported in female zebrafish, and *kiss1*, *kiss2* and *gnrh3* all showed increased gene expression at the onset of puberty (47). Similarly, brain *kiss2* expression levels increased significantly in flat fish, Senegalese sole (67, 70) and Japanese flounder (71). In Atlantic cod (*Gadus morhua*), acute *kiss2* elevation was observed in maturing individuals (72). In red sea bream (*Pagrus major*), the number of *kiss2*-expressing neurons in the hypothalamus was greater during the first spawning season in both males and females compared with fish in the post-spawning periods (73). In female striped bass, *kiss1* and *kiss2* and their receptors were dramatically elevated in mature females compared with prepubertal levels (17). These results clearly demonstrate that kisspeptin gene expression and pubertal timing are highly correlated in many teleost fish species. These results support the involvement of the kisspeptin system in pubertal onset in fish reproduction.

Annual Reproductive Stage

To elucidate the involvement of multiple kisspeptin systems in annual gonadal recurrence in cm, their relative gene expression profiles in the brain were analyzed at different gonadal stages using qRT-PCR. In males, *kiss1* and *kiss2* exhibited maximal expression levels between the immature and early spermiation periods and gradually decreased in the post-spawning period (33). In females, *kiss2* expression reached a maximal level at the start of the vitellogenic period, and two receptors also showed significantly high expression at this time (**Figure 4**) (33, 36). Our previous study demonstrated that the gene expression of Gnrh1 was closely related to seasonal ovarian development, and Gnrh1 peptide secretion increased at the start of gonadal recrudescence (25). Collectively, these findings suggest that the activation of kisspeptin systems during gonadal recrudescence may influence Gnrh1 release from the brain to the pituitary.

Similar studies are abundant. A study of the seasonal expression of kisspeptin genes during the seasonal gonadal cycle in the adult grass puffer (*Takifugu niphobles*) found that the *kiss2* and *kissr2* genes in the brain were significantly elevated during the prespawning and spawning periods in both sexes (74). The *kiss1*, *kiss2*, and *kissr2* expression levels in the whole brain in male European sea bass were significantly higher in mid- and late spermatogenesis compared with the post-spawn period (75). Alvarado et al. measured the expression of kisspeptin-related genes in the hypothalamus of adult male and female European sea bass and reported that there genes increased either before or during the advanced stage of oogenesis and decreased during the atretic stage (76). In the rohu (*Labeo rohita*), the brain *kiss1* expression levels were significantly elevated at the prespawning and spawning periods in males and females, respectively (77). Similarly, in golden mahseer (*Tor putitora*), brain *kiss1* and *kissr3* expression levels were comparatively higher during the initial stages of gonadal development than during spermiation or ovulation (78). In the sapphire devil (*Chrysiptera cyanea*), brain *kissr2* and *kissr3* levels increased during the late vitellogenic and post-spawning periods (79).

Overall, kisspeptin system genes were activated just before or during the advanced stage of gonadal growth in many fish species. These observations suggest that the kisspeptin system is important for reproduction but may also be involved in various functions other than reproduction.

Spawning Cycle

In the same manner, kisspeptin systems were found to be important during the spawning period in cm. We analyzed expression changes of kisspeptin-related genes in the brain during final oocyte maturation (FOM) and ovulation. Both *kiss1* and *kiss2* expression peaked during the FOM and ovulation stages (**Figure 4**) (80). Notably, the levels of Gnrh1 peptides also coincided with an increase in kisspeptin expression in the brain, and pituitary Lhb immunoreactivity was consistently high during FOM in the cm (27, 80). In contrast to other reproductive factors, the levels of kisspeptin receptors decreased during the FOM and ovulation phases (**Figure 4**) (81). In monkeys, continuous administration of human kisspeptin led to the desensitization of its receptor, Kiss1r (82, 83). Thus, desensitization of the kisspeptin receptors may have been involved in the decreased kisspeptin expression observed during the FOM and ovulation phases in cm.

Similarly, during their breeding seasons, medaka and goldfish displayed higher numbers of neurons expressing *kiss1* and *kiss2*, respectively, than they did during their non-breeding seasons (84, 85). In the grass puffer, both *kiss2* and *kissr2* showed clear diurnal and circadian variations in expression levels during the spawning season (86). In zebrafish, only males and females sampled at the time of spawning displayed strong *kiss2* expression in the periventricular hypothalamus (55).

In terms of breeding success, the estrogen feedback mechanism deserves a passing mention. Our previous study demonstrated that serum levels of E2 and its precursor, testosterone, showed characteristic variations during FOM and were significantly elevated when the largest and second largest oocytes were at the germinal vesicle migration stage and middle vitellogenesis stage of development, respectively (32). Interestingly, *kiss1* and *kiss2* expression levels peaked during FOM in the spawning cycle (80). Interactions between kisspeptins and steroid hormones require further investigation in cm. As in mammals, kisspeptin neurons express estrogen receptors and exhibit steroid sensitivity in medaka (84, 87), zebrafish (88), goldfish (85), and European sea bass (89).

PITUITARY KISSPEPTIN SYSTEM

To provide a basic understanding of the involvement of kisspeptins in reproduction, we analyzed their gene expression in the pituitary at different gonadal stages using qRT-PCR. In cm, only *kiss1* and *kissr2* were expressed in the pituitary and did not

show significant fluctuations during the annual reproductive cycle (33, 36). These results revealed that Kiss1 peptides exhibit lower affinity for KissR2 (36). The interactions between Kiss1 and KissR2 in the pituitary of cm remain unclear.

Functional analyses of the pituitary kisspeptin system have been reported in several species. *In vitro* studies on the actions of kisspeptin peptides have yielded conflicting results about their stimulatory effects on Lh release in goldfish (42, 90, 91). However, kisspeptin receptor expression was detected in immuno-identified gonadotrophs in the same species (90). An inhibitory effect of kisspeptin peptides on Lh secretion directly at the pituitary level was demonstrated in the European eel (92). In this species, *kissr1* and *kissr2* transcript levels were significantly downregulated in mature eels compared with in eels blocked at the prepubertal stage (11). In contrast, Kiss2-12, but not Kiss1-15, induced Fsh and Lh release from European sea bass pituitary cells, and Kiss2 cells also co-localized with gonadotropin-immunoreactive cells (93). In this species, *kiss2* and the two receptors increased either before or during the advanced stages of oogenesis in females (76). In a different study, in double transgenic *kiss2:mCherry/gnrh3:EGFP* zebrafish, both Kiss2 and Gnrh3 fibers extended to the pituitary *via* the pituitary stalk and were in direct contact with Gnrh3 fibers in the pars distalis (94). In addition, Zmora et al. reported that the Kiss1 and Kiss2 nucleus lateralis tuberis (NLT) populations probably act directly on pituitary gonadotrophs at the prespawning stage in male and female striped bass (55). The same study also reported that both Kiss1-15 and Kiss2-12 induced Fsh release, and Kiss2-12 induced Lh release from pituitary cells *in vitro*. The role of the pituitary kisspeptin system is still unknown. However, this system may be important in gonadotropin regulation in several teleost species, as these actions are independent of Gnrh signaling. More detailed studies should be performed in the near future to further clarify the role of the pituitary kisspeptin system in cm.

GONADAL KISSPEPTIN SYSTEM

It should also be noted that the kisspeptin system has an important role at the gonadal level. Our qRT-PCR analysis showed that *kiss1* and *kissr3* (cognate receptor of Kiss1) were expressed strongly in the testes and dramatically increased at the spermiation stage of the annual reproductive cycle in adult cm (33, 36). In particular, the *kissr3* transcript level was 10.8-fold higher at this stage than at the immature and post-spawning periods (36). The possibility that there are autocrine/paracrine effects of Kiss1 peptides on the testes cannot be excluded, but our expression and pharmacological analyses suggest a local role of the Kiss1 system in the testes of cm. Notably, ovarian *kiss1* expression significantly increased during vitellogenesis, but the ovaries did not express any kisspeptin receptors (33, 36). One possible explanation is that ovarian kisspeptin may exert effects *via* other RFamide receptors in this species. As a simple example, kisspeptins mediate physiological effects *via* the neuropeptide FF1 and FF2 receptors in mammals (95, 96).

At the gonadal level, a positive correlation between gonadal development and kisspeptin system expression has been reported in several species. In the grey mullet, ovarian *kissr2* expression showed an increasing trend during early development (64). In European sea bass, testicular *kissr2* expression exhibited a significant increase at the beginning of spermiation, and *kissr3* levels increased significantly in the full spermiation stage (37). In rohu, gonadal *kiss1* expression increased during the prespawning and spawning periods in male and female fish, respectively (77). In male golden mahseer, consistently high transcript levels of *kiss1* were observed during testicular development and, in female fish, *kiss1* and *kissr2* expression peaked in the late vitellogenic ovary (78). In male pejerrey, *kiss1* and *kissr3* increased during testicular development compared with their levels in immature testes, and female fish showed high levels of expression of receptors in the ovaries at final maturation (97).

The significance of kisspeptin expression in the fish gonad is still unknown but is a promising area of future research not only in cm but also in other fish species.

ANATOMY

Localization of Kisspeptin Neurons

The lack of anatomical evidence for a neuronal network is the central problem in elucidating the functions of kisspeptin in fish. We analyzed the localization of two kisspeptin neurons in the brains of adult cm using *in situ* hybridization (ISH). *Kiss1* mRNAs were detected in the anterior part of the POA, the ventral hypothalamus, including the NLT, and the dorsal hypothalamus, including the nucleus recessus lateralis (38). *Kiss2* mRNAs were detected in the anterior POA and NLT, and a large population was observed in the ventral hypothalamus, including the NRL (38). In our experiment, no sex differences were observed. The localization of neurons expressing the two kisspeptins has been reported in detail in five fish species: medaka (47, 84), zebrafish (88), striped bass (17), European sea bass (23, 89), and goldfish (85, 98). Furthermore, histological *kiss2* expression was examined in four species: red sea bream (73), Nile tilapia (99), masu salmon (39), and grass puffer (86). The distribution of cells expressing cm *kiss1* and *kiss2* roughly matched those of the four species mentioned above.

In fish, highly specific Kiss1 and Kiss2 antibodies were developed in zebrafish (88), and a Kiss2 antibody was developed in European sea bass (23). Immunohistochemistry (IHC) revealed that Kiss2 neurons are mainly located in the hypothalamus and project widely to the subpallium, the POA, the thalamus, the ventral and caudal hypothalamus, and the mesencephalon in zebrafish and European sea bass (23, 88). All these regions strongly expressed the *kissr2* mRNAs (see next section), indicating a very strong correlation with the wide distribution of Kiss2-positive fibers. On the other hand, a large population of Kiss2 neurons was localized in the POA region in goldfish and showed a strong sensitivity for estrogen feedback (85). This suggests that POA Kiss2 neurons play an important role in the regulation of reproduction in this species.

Kiss1 localization also varies among species. In zebrafish, medaka, and European sea bass, the main location of Kiss1

neurons and receptors is the habenular nucleus, and they may function by autocrine or paracrine action. Indeed, double-labeled ISH and *c-fos* expression after Kiss1 administration suggested the existence of autocrine regulation in zebrafish habenular nuclei (100). However, in our study, Kiss1 neurons were not expressed in the habenular nucleus, and the same result was reported in striped bass (17, 38). The physiological function or significance of the fish Kiss1 system remains largely unknown. Studies in goldfish found a dominant role of Kiss1 in the regulation of Lh secretion *in vivo* and *in vitro* (42, 90, 91). On the other hand, habenular Kiss1 modulates the serotonergic system and fear response in zebrafish (100, 101). In IHC studies in zebrafish, Kiss1 immunoreactive neurons were found in the ventromedial habenula, with axons contacting the interpeduncular and raphe nuclei, which express serotonergic neurons (88, 100). Clarifying the Kiss1 system in fish is an important and interesting future task; however, this peptide likely contributes to reproduction in cm and will be described later.

Localization of Kisspeptin Receptors

We also examined the localization of *kissr2*- and *kissr3*-expressing cells in the brains of cm using ISH. The *kissr2*- (cognate receptor of the Kiss2 peptide) expressing cells displayed a broader distribution in the anterior and posterior parts of the POA and were abundant in the ventral hypothalamus, including the ventral and lateral parts of the NLT; the ventral, lateral, and dorsal parts of the NRL; and the nucleus of the NRP (38). The *kissr3*- (cognate receptor of the Kiss1 peptide) expressing cells were present in the anterior POA and the dorsal and ventral parts of the habenular nucleus (38). No expression differences between the sexes were observed.

The localization of the two types of kisspeptin receptor-expressing cells has been reported in detail in zebrafish (88), striped bass (17), European sea bass (23), medaka (22), and in one species of African cichlid fish with only the *kissr2* transcript (16). The distributions of cells expressing kisspeptin receptors roughly matched and, overall, *kissr2* showed widespread, high expression levels, whereas *kissr3* was modestly expressed in limited regions of the brain.

Interactions With Functional Gnrh-Producing Cells

Central to the fish kisspeptin issue is the problem of interactions with Gnrh neurons. To investigate this, we conducted dual-labeled ISH and carefully assessed the signals using confocal laser-scanning microscopy. Our study clarified that Gnrh1 neurons co-expressed *kissr3* mRNA in the POA region in male and female cm (**Figures 5A–G**) (38). There is no doubt that the Kiss1 peptide is involved in reproduction, and some of its signals are inputs for Gnrh1 neurons. As noted earlier, Kiss1 treatment strongly induced gonadal development in cm (48, 50). In addition, *kissr2* was widely expressed in the POA; in many cases, it was expressed in close proximity to the Gnrh1 neurons, but they did not appear to be co-expressed (38).

The interactions between kisspeptin receptors and functional Gnrh neurons were first examined in a cichlid fish, Nile tilapia, using laser-captured single-cell PCR (10). The results clearly showed that all three types of Gnrh neuronal cells (Gnrh1, 2, and 3) expressed *kissr2* mRNA (10). This observation was supported by double-labeled ISH analysis by Grone et al., which showed that Gnrh1 and Gnrh3 neurons were co-expressed with *kissr2* transcripts in cichlid fish (16). Furthermore, *in vivo* treatment with Kiss2 modulated the reproductive axis and gonadal development in the same species, supporting the histological evidence (54). The same anatomical evidence was reported in a study in striped bass combining ISH and IHC, which showed that Gnrh1 cell bodies co-expressed *kissr2* mRNA, and *kissr3* was expressed in cells attached to Gnrh1 fibers (17). This may suggest dual modes of Gnrh1 regulation by the two kisspeptin

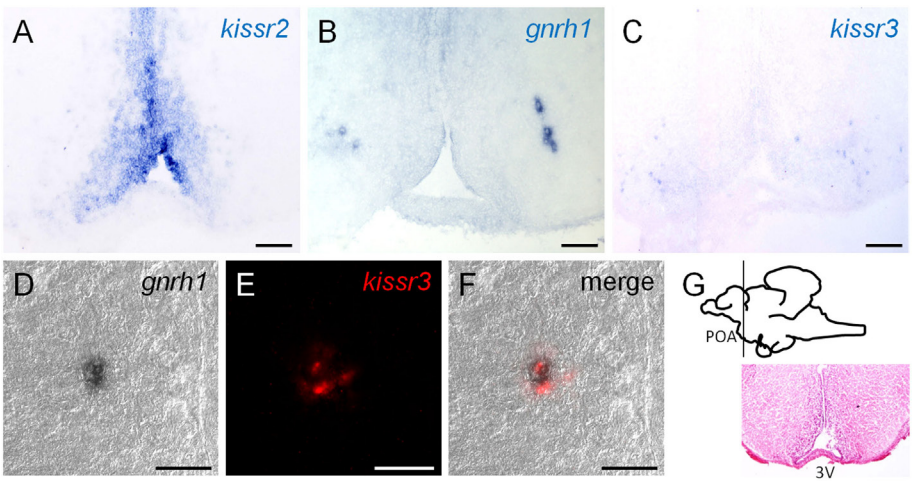

FIGURE 5 | **(A–C)** Localization of *kissr2* expressing cells, Gnrh1 neurons, and *kissr3*-expressing cells at pre-optic area (POA), respectively. Scale bars, 100 μm. **(D–F)** Double-label *in situ* hybridization at the POA region. Scale bars, 30 μm. **(D)** Differential interference contrast images of Gnrh1 neurons. **(E)** Red fluorescence indicate the *kissr3* mRNA. **(C)** Merged image of **(E,F)** and indicate that Gnrh1 neurons co-expressed *kissr3* mRNA. **(G)** Sagittal view of the chub mackerel brain. Planes of the ventral POA region are visualized by hematoxylin–eosin staining. Modified from Ref. (38), by permission of Oxford University Press.

peptides; indeed, both Kiss1 and Kiss2 enhanced Lh secretion in adult fish (17).

Alternatively, Gnrh neurons lack the expression of kisspeptin receptors in some species. An IHC study in zebrafish reported that some Gnrh3 neurons were contacted by Kiss2 fibers (88). Similarly, in double transgenic *kiss2:mCherry*/*gnrh3:EGFP* zebrafish, Kiss2 and Gnrh3 fibers were adjacent, and few contacts were observed in the telencephalon and hypothalamus (94). However, both studies similarly failed to find evidence of kisspeptin receptors in Gnrh3 neurons. In addition, morphological evidence from dual-labeled fluorescence ISH has shown that Gnrh1 neurons do not appear to express *kissr2* or *kissr3* mRNAs in medaka (22). In European sea bass, an antibody against preproGnrh1 was used for coupling with ISH for kisspeptin receptors. In this study, both *kissr2*- and *kissr3*-expressing cells in the ventral telencephalon and POA were often found in close proximity to Gnrh1 neurons; however, the authors did not detect a single case of co-expression (23). Nevertheless, administration of Kiss2 peptides upregulated the expression of the gonadotropin subunit genes as well as the secretion of gonadotropins in zebrafish and European sea bass, respectively (18, 19, 47). Based on the numerous studies of fish kisspeptins, it seems reasonable to suggest that kisspeptins affect gonadotropin regulation. However, the pathway of gonadotropin regulation may vary among species. The relationship between kisspeptin and Gnrh alone may not form the core of kisspeptin function in fish reproductive physiology.

Interactions With Other Factors

Finally, there is one other important factor for kisspeptin function in fish. Despite the lack of direct contact between Kiss2 and Gnrh1 neurons, i.c.v. administration of Kiss2 peptides strongly enhanced the transcription of gonadotropins and influenced *gnrh1* expression in cm (45). Our histological observations showed that *kissr2* was abundantly expressed in the vicinity of Gnrh1 neurons and hypothalamic regions (38). The transcript levels were quantified by qRT-PCR, and *kissr2* transcripts were expressed at far higher levels than were *kissr3* transcripts, indicating that the expression of *kiss2*/*kissr2* can be very dynamic, depending on maturational stage, as shown in many previous studies in cm (33, 36). One hypothesis is that Kiss2 indirectly regulates the reproductive axis *via* interneurons that are expressed in close proximity to Gnrh1 neurons.

In the earliest study, Grone et al. discovered that not only Gnrh1 neurons but also some unknown non-Gnrh cells in the vicinity of Gnrh1 neurons expressed *kissr2* mRNA in African cichlid fish (16). Recent studies have clarified that various types of neurons co-express kisspeptin receptors in fish. Escobar et al. reported that neuronal nitric oxide synthase (nNOS), neuropeptide Y (NPY), tyrosine hydroxylase, and somatostatin (SRIF) neurons co-expressed *kissr2* transcripts in the brains of European sea bass (23). In mice, Gnrh neurons are surrounded by nNOS neurons, which express *Kiss1r*, and nNOS may contribute to the direct modulation of Gnrh neuronal activity in a manner codependent with kisspeptin signaling (102, 103). Also, NPY was shown to induce Lh secretion in European sea bass (104). Gnrh1 neurons in European sea bass do not express *kissr2* mRNA; it is likely that Kiss2 regulates Gnrh and gonadotropin secretion *via* various neuronal networks.

Kisspeptin may also play an important role in reproductive behavior. Grone et al. reported that significantly more *kissr2* transcripts were found in Gnrh3 neurons than in Gnrh1 neurons in African cichlid fish (16). The same report also clarified that high-status territorial males have higher brain levels of *kissr2* mRNA than low-status non-territorial males. Some teleost fishes have three distinct populations of Gnrh neurons, and Gnrh3 neurons are considered to be particularly important for reproductive behaviors (105). On the other hand, Zhao and Wayne reported changes in Gnrh3 electrical activity after the application of Kiss1 through some interneurons in medaka (106). Furthermore, breeding medaka showed changes in hypothalamic *kiss1* and telencephalic *gnrh3* expression depending on differences in dominance hierarchy (107). In the same species, Kanda et al. showed that arginine vasotocin (Avt) and isotocin (It) neurons co-expressed *kissr2* mRNA in the brains (22). In teleost fish, Avt and It are mainly implicated in spawning reflex, courtship and mate-guarding behavior, and hierarchical status (108, 109). These results suggest that kisspeptin neurons directly regulate some sexual behavioral functions *via* Gnrh3 neurons or Avt and It neurons. The correlation between kisspeptin systems and behavioral factors were similarly reported in striped bass: Avt neurons expressed *kissr2* mRNA, and It neurons expressed *kissr3* mRNA (20).

In another case, pituitary growth hormone (Gh)-producing cells expressed *kiss1* and its receptor transcripts, and pituitary prolactin-producing cells also expressed *kissr2* mRNA in goldfish (90). In the same study, Kiss1-10 increased the basal release of Gh and prolactin from pituitary cells *in vitro*. Furthermore, administration of human KP10 promoted Gh, insulin-like growth factor 1 (IGF-I), and somatolactin secretion and pituitary gene expression in cinnamon clownfish (110). As mentioned earlier, in European sea bass, which is the same marine Perciform species, SRIF neurons co-expressed kisspeptin receptors (23). SRIF is a highly conserved peptide that also acts as an inhibitor of Gh secretion in teleosts (111, 112). Although seemingly contradictory, these results point to the potential function of kisspeptins in regulating somatic growth-related factors.

The kisspeptin/neurokinin B/dynorphin A (KNDy) neurons will play a key role in regulating pulsatile secretion of Gnrh in mammals (113), but very little is known regarding the relationship between neurokinins and kisspeptins in the context of reproduction in fish. In past study, i.p. administration of tachykinins cording peptides, neurokinin B (NKB) and neurokinin F (NKF: unique neurokinin form in fish) elicited significant Lh secretion in sexually mature female zebrafish (114). However, ISH showed no co-expression of tachykinins mRNA with kisspeptins mRNA (115). On the other hand, i.m. administration of NKB and NKF reduced *kiss1* and *kiss2* gene expression in the brain and pituitary content of Gnrh1 in spermiating striped bass (116). Furthermore, tachykinin (*tac3*) neurons in the hypothalamus strongly innervated proximal Kiss2 neurons in the dorsal and ventral NRL, which in turn express cognate receptor (*tac3r*) (116). Finally, we may note in passing that a recent deep-sequencing study suggested that novel neural systems, such as cholecystokinin

and neuropeptide B, may also be under the control of kisspeptin signals in medaka (117). The multiple and integral regulation of the reproductive axis or non-reproductive functions by fish kisspeptins are still largely unknown and in need of further consideration.

PERSPECTIVES

In this article, we reviewed the current insights on fish kisspeptin physiology based on our cm studies. A take-home message from this article is that the kisspeptin system plays a role in the reproductive success not only of cm but also of many other fish species *via* various known or unknown neuronal networks. It must be noted that the methods of involvement differ from species to species. The correlation between kisspeptins and Gnrh neurons is a good illustration of the high species specificity of the kisspeptin system. Namely, it may be erroneous to assume that fish kisspeptin is a central and absolute upstream regulator of the Gnrh–Gth pathway as is the case in mammals. Fish are known as the earliest vertebrates, and it may be that their hierarchical reproductive fine network has not yet been completed.

A recent genome editing study clearly showed that kisspeptin-related genes null mutant zebrafish or medaka showed normal gonadal development and maturation (21, 117). However, in fish, various neuropeptidergic, catecholaminergic, and aminoacidergic neurons form direct or indirect contacts with gonadotrophs in the pituitary gland, possibly constituting a multiple back-up system to maintain appropriate gonadotropin release. This does not affect the validity of the reproductive functions of fish kisspeptins.

The strong potency of gonadotropin release is relevant to our research objectives. With capture fishery production relatively static since the late 1980s, aquaculture has been responsible for the impressive growth in the supply of fish for human consumption (118). Hence, the development of efficient aquaculture methods and a model of reproduction are imperative tasks. The "maturation induction" potency of kisspeptin peptides may have the potential to rescue reproductive failure in cultured fish. Additional comparative kisspeptin research will further elucidate the pleiotropic role of kisspeptin in fish and may contribute to the development of not only a basic understanding of fish physiology but also of the applied science used in aquaculture and stock management.

AUTHOR CONTRIBUTIONS

HO wrote a manuscript. HO, SS, and MM contributed substantially to the conception and design of the work, reviewing, final approvals of the version submitted, and agreed to be accountable for accuracy and integrity of content.

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Eel Kisspeptins: Identification, Functional Activity, and Inhibition on both Pituitary LH and GnRH Receptor Expression

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The European eel (*Anguilla anguilla*) presents a blockade of sexual maturation at a pre-pubertal stage due to a deficient production of gonadotropins. We previously initiated, in the eel, the investigation of the kisspeptin system, one of the major gatekeepers of puberty in mammals, and we predicted the sequence of two *Kiss* genes. In the present study, we cloned and sequenced *Kiss1* and *Kiss2* cDNAs from the eel brain. The tissue distributions of *Kiss1* and *Kiss2* transcripts, as investigated by quantitative real-time PCR, showed that both genes are primarily expressed in the eel brain and pituitary. The two 10-residue long sequences characteristic of kisspeptin, eel Kp1(10) and Kp2(10), as well as two longer sequences, predicted as mature peptides, eel Kp1(15) and Kp2(12), were synthesized and functionally analyzed. Using rat Kiss1 receptor-transfected Chinese hamster ovary cells, we found that the four synthesized eel peptides were able to induce $[Ca^{2+}]_i$ responses, indicating their ability to bind mammalian KissR-1 and to activate second messenger pathways. In primary culture of eel pituitary cells, all four peptides were able to specifically and dose-dependently inhibit *lhβ* expression, without any effect on *fshβ*, confirming our previous data with heterologous kisspeptins. Furthermore, in this eel *in vitro* system, all four peptides inhibited the expression of the type 2 GnRH receptor (*gnrh-r2*). Our data revealed a dual inhibitory effect of homologous kisspeptins on both pituitary *lhβ* and *gnrh-r2* expression in the European eel.

Keywords: kisspeptins, tissue distribution, functional activity, pituitary cell culture, LH, GnRH receptor, European eel

INTRODUCTION

The European eel, *Anguilla anguilla*, exhibits a complex life cycle, with a blockade of sexual maturation at a prepubertal stage (silver stage) as long as its oceanic reproductive migration is prevented. This blockade is due to a deficient production of pituitary gonadotropins (1). The understanding of the mechanisms regulating the gonadotropic axis and controlling eel reproduction is of particular

interest, considering the drastic decline of wild populations (2) and the current lack of self-sustained aquaculture of this species. Furthermore, as the eel is a representative species of an early group of teleosts (the elopomorpha) (3), deciphering such mechanisms in this species may provide new insights on evolution and ancestral regulations of endocrine systems.

Kisspeptin (*Kiss1*) gene was first discovered as a metastasis suppressor gene in human melanoma (4). Soon after, an orphan receptor, GPR54, was cloned in the rat (5) and was subsequently identified as the cognate receptor of kisspeptins (Kiss), the natural peptides derived from the product of *Kiss1* (6–8). In 2003, a major breakthrough in reproductive endocrinology was achieved, as the GPR54 gene was shown to be essential for the onset of puberty. Mutations in *gpr54* caused hypogonadotropic hypogonadism in humans (9, 10) and mice (10, 11). Later, hypogonadotropic hypogonadism was also observed in *Kiss1* knockout mice (12), while precocious puberty onset occurred in humans with either *gpr54*-activating mutation leading to prolonged *in vitro* activation of intracellular signaling pathways in response to kisspeptin (13) or *Kiss1* mutations leading to higher *in vitro* kisspeptin resistance to degradation (14). Since these discoveries, the kisspeptin system has been considered as a major puberty gatekeeper and reproductive regulator, upstream of GnRH [for reviews see Ref. (15–17)]. Nevertheless, Tang and collaborators (18) recently showed that gametogenesis and reproductive capability are not impaired in zebrafish mutant lines for Kiss, as well as for kisspeptin receptors (KissR), suggesting that the Kiss/KissR systems may be dispensable for the reproduction of some non-mammalian vertebrates.

The kisspeptin system has been identified in a number of vertebrate species, leading to the discovery of multiple genes encoding kisspeptins (from *Kiss1* to *Kiss3*) as well as multiple genes encoding its receptors (from *KissR-1* to *KissR-4*) [for review see Ref. (19)]. To date, mature amidated kisspeptins have been purified only from human, *Xenopus*, turtle, and salmon. In human, multiple mature Kiss1 peptides (Kp1), including a mature peptide encompassing 54-aa [Kp1(54)] and shorter peptides [Kp1(14) and Kp1(13)], were isolated from placental extracts (6). Mature Kiss peptides, cleaved from the same precursor, share the same C-terminal 10-aa sequence, which is the minimal sequence required to specifically bind their cognate receptor, as first described for human Kiss1 by Kotani et al. (6). Although *Kiss2-like* gene has been identified in human, a Kiss2 peptide (Kp2) may not be produced as an endogenous ligand due to the lack of an amidation signal in the precursor polypeptide (20). In *Xenopus laevis*, a species presenting three *Kiss* genes (*Kiss1*, *2*, and *3*), only Kp2(12) has been isolated by HPLC (21). In the red-eared slider turtle (*Trachemys scripta*), the mature endogenous Kiss2 peptide is a 12-aa sequence, while in the masu salmon (*Oncorhynchus masou*), it is a 13-aa sequence (20).

In the eel, three Kiss receptor genes have been characterized, i.e., *KissR-1*, *KissR-2*, and *KissR-3* (19, 22–24). Their expressions mainly occur in the brain, pituitary, and gonads (19). Using heterologous Kiss peptides [human/lamprey Kp1(10); human Kp1(14); lamprey Kp1(13); and zebrafish Kp1(10), 2(10), 1(15), and 2(15)], we previously showed an unexpected and specific *in vitro* inhibitory effect on luteinizing hormone (*lh*β) expression by eel pituitary cells in primary culture (22). Three GnRH receptors were identified in the eel, two of type I (*Gnrh-r1a and 1b*) and one of type II (*Gnrh-r2*); all were shown to be expressed in the brain, pituitary, and gonads (25).

In the present study, we report the cloning of two eel Kiss transcripts (*Kiss1* and *Kiss2*), corresponding to the two previously defined ORFs (23). Using a specific quantitative real-time PCR (qPCR) approach, we investigated their distributions in various eel tissues. We synthesized the two minimal 10-aa peptides, eel Kp1(10) and Kp2(10), as well as the predicted mature peptides, eel Kp1(15) and Kp2(12). We tested all four peptides on rat KissR-1-transfected Chinese hamster ovary (CHO-K1) cells. We also studied their biological effects on the expression of gonadotropins (*lh*β; follicle-stimulating hormone, *fsh*β) and gonadotropin-releasing hormone receptors (*gnrh-r*) by eel pituitary cells in primary culture.

MATERIALS AND METHODS

Animals

European female eels were at the prepubertal "silver" stage, which corresponds to the last continental phase of the eel life cycle, preceding the oceanic reproductive migration. Eels were purchased from Gebr. Dil import-export BV (Akersloot, The Netherlands) and transferred to MNHN, France.

Animals were anesthetized by cold and then killed by decapitation under the supervision of authorized person (Karine Rousseau; No. R-75UPMC-F1-08) according to the protocol approved by Cuvier Ethic Committed (No. 68-027).

Cloning and Sequencing of European Eel *Kiss1* and *Kiss2* cDNAs

Total RNA from eel brain (pooled di-/mesencephalon) was extracted using Trizol reagent and reverse transcribed as previously described (22).

Predicted genomic sequences of eel *Kiss1* and *Kiss2* (23, 26) were used to design specific *Kiss1* and *Kiss2* primers, respectively (**Table 1**). Using the Advantage 2 PCR Kit (Clontech Laboratories Inc., PaloAlto, CA, USA), RACE PCRs with 5′-cDNA or 3′-cDNA as templates were performed as follows: an initial step of polymerase activation for 3 min at 94°C; then 10 cycles of 30 s at 94°C for denaturing, 30 s at 70°C for annealing, 90 s at 72°C for primer extension; and then 25 cycles of 30 s at 94°C for denaturing, 30 s at 68°C for annealing, 90 s at 72°C for primer extension, and a single final extension step of 5 min at 72°C. PCR products of appropriate estimated size were sequenced at GATC Biotech Ltd. (Konstanz, Germany).

The signal peptides of the Kiss precursors were predicted using SignalP tool (28). Cleavage and amidation sites, as well as mature peptides, were predicted from the Kiss precursor using NeuroPred tool (29).

Synthesis of Eel Kp1 and 2 Peptides

European eel Kp1(10), Kp2(10), Kp1(15), and Kp2(12) (**Table 2**) were synthesized (0.1-mmol scale) by the solid-phase

TABLE 1 | Primers used in the 3′- and 5′-RACE PCR and quantitative real-time PCR (qPCR) amplifications.

Primers	5′–3′ sequence (bp)	
Primers for 3′-RACE PCR		
Kiss1-F	CGCCACAAGCGGCCAAGAAG	
Kiss2-F	AGGGCCACATTTCCTGCCGACT	
Primers for 5′-RACE PCR		
Kiss1-R	CCCGCTTCTTGGCCGCTTGT	
Kiss2-R	CCGAACGGATTGCGGTTGAATTTG	
Primers for qPCR		
Kiss1-F	GGTCTCTTAGGTACACCCCGT	This study
Kiss1-R	ACAGCTCCTCGCTCATTTG	
Kiss2-F	ACGGACGACTCAGGTTCTCT	This study
Kiss2-R	GCCCTCGATTTCACTGTCTT	
actin-F	AGTATTTGCGCTCGGGTG	Aroua et al. (27)
actin-R	CAGCCTTCCTTCCTGGGT	
lhβ-F	TCACCTCCTTGTTTCTGCTG	Aroua et al. (27)
lhβ-R	TAGCTTGGGTCCTTGGTGATG	
fshβ-F	TCTCGCCAACATCTCCATC	Aroua et al. (27)
fshβ-R	AGAATCCTGGGTGAAGCACA	
gnrh-r1a-F	TGGTCATGAGTTGCTGCTACA	Penaranda et al. (25)
gnrh-r1a-R	AGACACCCCTCTCCGTCTTT	
gnrh-r1b-F	TCGTCACGCTCTACGTTGTC	Penaranda et al. (25)
gnrh-r1b-R	AGGCAGGACTCTCCACCTTT	
gnrh-r2-F	TCACCTTCTCCTGCCTCTTC	Penaranda et al. (25)
gnrh-r2-R	TTGGAAGATGCCTTCCCTTT	

TABLE 2 | Sequences of predicted eel kisspeptins.

Peptide	Symbol	Sequence	Length (aa)
Eel 1 kisspeptin-10	Kp1(10)	YNWNSFGLRY-NH2	10
Eel 1 kisspeptin-15	Kp1(15)	ENFSSYNWNSFGLRY-NH2	15
Eel 2 kisspeptin-10	Kp2(10)	FNRNPFGLRF-NH2	10
Eel 2 kisspeptin-12	Kp2(12)	SKFNRNPFGLRF-NH2	12

methodology on a Rink amide 4-methylbenzhydrylamine resin (Biochem, Meudon, France) using a 433A peptide synthesizer (Applied Biosystems, Courtaboeuf, France) and the standard procedure, as previously described (30). The synthetic peptides were purified by reversed-phase (RP) HPLC on a 2.2 cm × 25 cm Vydac 218TP1022 C_{18} column (Alltech, Templemars, France), using a linear gradient (20–40% over 60 min) of acetonitrile/TFA (99.9:0.1, v/v) in water, at a flow rate of 10 ml/min. Analytical RP-HPLC, performed on a 0.46 cm × 25 cm Vydac 218TP54 C_{18} column, showed that the purity of the peptide was greater than 99%. The molecular mass of the peptide was verified by mass spectrometry on a MALDI-TOF Voyager DE-PRO instrument (Applied Biosystems).

Functional Activity of Eel Kp1 and Kp2 Peptides in Rat KissR-1-Transfected CHO-K1 Cells

CHO-K1 cells were cultured to semiconfluence in 12-well plates using Ham's F12 medium supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution. Cells were transfected with different quantities of the recombinant plasmid, ranging from 3 to 5 µg, using Lipofectamine 2000 (Invitrogen, Cergy Pontoise, France) as previously described (30). Twenty-four hours after transfection, media were replaced by fresh F12 medium containing 1 mg/ml G418 (Geneticin; Life Technologies, Inc.). One week later, surviving cells were detached, diluted, and plated on 96-well plates at 0.7 cells/well. Monoclonal cell lines expressing rat KissR-1 (CHO-K1-rKissR-1) were followed daily by contrast phase microscopy. Seven independent monoclonal stable cell lines were obtained after a 3-week period of selection. By using qPCR, two cell lines were selected based on their expression levels being closest to physiological levels among all cell lines. Of these, one cell line was definitely selected based on its best performance in showing clear Ca^{2+}-mobilizing responses to treatment with rat Kp1(10).

TABLE 3 | Functional parameters of the response of KissR-1 CHO-K1 cells to treatment with eel kisspeptins as measured using FlexStation technology.

Peptide	E_{max} ± SEM (%)	EC_{50} ± SEM (nM)
Eel Kp1(10)	235.5 ± 8.4	8.5 ± 4.5
Eel Kp1(15)	276.6 ± 5.3	11.8 ± 2.0
Eel Kp2(10)	259.9 ± 9.5	56.6 ± 27.7
Eel Kp2(12)	255.9 ± 6.7	134.7 ± 18.9

To compare the KissR-1 agonistic activities of eel Kp1(10), Kp2(10), Kp1(15), and Kp2(12), the level of $[Ca^{2+}]_i$ after stimulation of CHO-K1 cells stably expressing KissR-1 by these peptides was monitored by spectrofluorometry as previously described (30, 31) with slight modifications. Briefly, after 24 h in culture, cells were incubated for 1 h in a humidified incubator (37°C, 5% CO_2) with 2 µM Fluo-4 acetoxymethyl ester (AM) calcium dye (Life Technologies, Saint Aubin, France) in Hank's Buffer Saline Solution (HBSS; Life Technologies) buffered with 5 mM HEPES and supplemented with 2.5 mM probenecid (Sigma-Aldrich, Saint-Quentin Fallavier, France). Cells were washed twice with HBSS/HEPES/probenecid to remove Fluo-4 AM from the incubation medium and incubated in 150 µl of the same medium at 37°C for 15 min. Fluorescence was recorded using a Flexstation 3 fluorescence plate reader system (Molecular Devices, Saint-Grégoire, France) during 180 s with an excitation wavelength of 480 nm, an emission wavelength of 525 nm, and a cutoff filter of 515 nm. After 15 s recording in basal conditions, 50 µl of graded concentration (10^{-12} to 10^{-6} M) of different peptides (four-fold final concentration) was added to the incubation medium with the built-in eight-channel pipettor at a rate of 62 µl/s to assess their agonistic activity. After subtraction of mean fluorescence background from control wells without Fluo-4 AM, baseline was normalized to 100%, and fluorescence peak values were determined for each concentration of peptide. Potency (EC_{50}) and efficacy (E_{max}) (**Table 3**) were determined with the Prism 6.0 software (GraphPad Software In., La Jolla, CA, USA) using a four-parameter logistic equation to fit peak fluorescence data.

Tissue Distribution of *Kiss* Transcripts in the European Eel

Various tissues were individually collected from eight freshwater female silver European eels to investigate the distribution of *Kiss1* and *Kiss2* expressions using qPCR. The following tissues were

sampled, stored in RNAlater (Ambion-Inc., Austin, TX, USA), and kept frozen at −20°C until RNA extraction: brain, pituitary, eye, liver, kidney, intestine, spleen, muscle, adipose tissue, and ovary. The brain was dissected into six parts olfactory bulb, telencephalon, mesencephalon, diencephalon, *cerebellum*, and *medulla oblongata*. In addition, testes from eight freshwater male silver European eels were also collected.

Primary Culture of Eel Pituitary Cells and *In Vitro* Treatments

Dispersion and Culture

Dispersion and primary culture of pituitary cells from 30 freshwater female silver eels were performed using an enzymatic and mechanical procedure as described by Ref. (33) and as previously used for the test of heterologous kisspeptins (22). Cultures were performed in serum-free culture medium (Medium 199 with Earle's salt, sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml fungizone) (Gibco, Thermo Fisher Scientific, Villebon sur Yvette, France) at 18°C under 3% CO_2 and saturated humidity.

In Vitro Treatments

Hormonal treatments were started 24 h after the beginning of culture to allow cell attachment (Day 0). Replicates of five wells for control and each treated group were used. Eel Kp1(10), Kp1(15), Kp2(10), and Kp2(12) were tested (see **Table 2**). Kisspeptin stock solutions (10^{-4} M) were prepared in ultrapure water and stored at −20°C. Stock solutions were diluted in culture medium just before addition to the culture wells. Culture medium was changed and kisspeptins were added to the cells on Day 0, Day 3, and Day 7. Control wells were treated with similar dilutions of ultrapure water. Cultures were stopped on Day 10, according to Ref. (22). The effects of treatments (10^{-7} to 10^{-11} M) were tested in at least three independent experiments performed on different cell preparations from different batches of fish.

RNA Extraction and cDNA Synthesis

Tissue samples were individually homogenized by sonication in Trizol, and total RNAs were extracted according to the manufacturer's instructions (Invitrogen). Following extraction, samples were treated with DNase I (Roche, Meylan, France), and the first strand of cDNA was synthesized from 400 ng of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers. The reaction was performed according to the following thermal conditions with an initial step at 25°C for 10 min followed by incubation at 50°C for 60 min and 70°C for 15 min. The samples obtained were stored at −20°C until qPCR. The extracted RNAs were the same as in Ref. (19).

For cell cultures, total RNA was directly extracted in wells using the Cell-to-cDNA II Kit (Ambion, Thermo Fisher Scientific) according to the manufacturer's recommendations. Cells were washed with sterile phosphate buffer (Gibco) and lysed with Cell Lysis II Buffer (80 µl/well). The lysates were digested with RNase-free DNase I (Roche). Four microliters of RNA solution of each sample was then reverse transcribed with a SuperScript III First Strand cDNA Synthesis Kit (Invitrogen). The samples obtained were stored at −20°C until qPCR.

Quantitative Real-time PCR

Eel *Kiss1*- and *Kiss2*-specific primers for qPCR (**Table 1**) were designed based on the European eel cDNA sequences cloned in this study and using Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, USA). To optimize the assays, different annealing temperatures were tested according to the melting temperature (T_m) of primers. To assess their specificity, amplification products were sequenced at GATC Biotech Ltd.

The qPCR primers for European eel β-*actin*, *lh*β, *fsh*β, *gnrh-r1a*, *gnrh-r1b*, and *gnrh-r2* were previously designed (25, 27) (**Table 1**). β-*actin* was used as reference gene. All primers were purchased from Eurofins (Ebersberg, Germany).

Quantitative assays of eel *Kiss1, Kiss2, lh*β, *fsh*β, *gnrh-r1a*, *gnrh-r1b*, *gnrh-r2*, and β-*actin* mRNAs were performed using the LightCycler® System (Roche) with SYBR Green I sequence-unspecific detection as previously described (19, 22, 23). The qPCRs were prepared with 4 µl of diluted cDNA template, 2 µl of PCR grade water, 2 µl of SYBR Green master mix, and 1 µl of each forward and reverse primers (0.5 pmol each at final concentration). The qPCRs were performed as follows: an initial step of polymerase activation for 10 min at 95°C; then 41 cycles of 15 s at 95°C for denaturing, 5 s at 60°C for annealing, 10 s at 72°C for primer extension (β-*actin*, *lh*β, *fsh*β) or 51 cycles of 15 s at 95°C for denaturing, 5 s at 61°C for annealing, 5 s at 72°C for primer extension, 5 s at 83°C to avoid measurement of non-specific annealing (*Kiss1, Kiss2*) or 42 cycles of 10 s at 95°C for denaturing, 7 s at 61°C for annealing, 4 s at 72°C for primer extension (*gnrh-r2*), or 42 cycles of 10 s at 95°C for denaturing, 10 s at 60°C for annealing, 7 s at 72°C for primer extension (*gnrh-r1a*, *gnrh-r1b*); and a single final extension step of 5 min at 72°C. Each qPCR run contained a non-template control (cDNA was substituted by water) for each primer pair. The efficiency of primers and the specificity of reaction were assessed as previously described (19). Serial dilutions of cDNA pool of brain and pituitary tissues were run in duplicate and used as a common standard curve and also included in each run as a calibrator.

Quantitative real-time PCR efficiencies for *Kiss1* and *Kiss2* primers (calculated by standard dilution curves) were as follows: *Kiss1* 89.84% and *Kiss2* 88.05%. Assay included a melting curve analysis for which all samples displayed a specific single peak.

Normalization of data was performed using total RNA content for the tissue distribution samples, and using β-*actin* mRNA level for the cell culture samples.

Statistical Analysis

Results are given as mean ± SEM. Non-parametric tests were performed. Mean values were compared by one-way ANOVA Tukey's multiple comparison test using Instat (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Identification of European Eel Kisspeptins

Cloning of European Eel *Kiss1* and *Kiss2* cDNAs

Using European eel-specific *Kiss1* primers designed on eel *Kiss1*-predicted genomic sequence (23, 26), RACE PCRs, performed on brain cDNAs, led to the cloning of a partial *Kiss1* transcript sequence (EMBL: LT962662) encompassing a partial coding sequence (CDS) of 314 bp and partial 3′-UTRs of 30 bp. Once translated, the cloned *Kiss1* CDS gave a partial 103-aa kisspeptin precursor exhibiting a 10-aa sequence (YNWNSFGLRY) characteristic of the kisspeptin family (**Figure 1A**).

Using European eel-specific *Kiss2* primers designed on eel *Kiss2*-predicted genomic sequence (23, 26), RACE PCRs, performed on brain cDNAs, led to the isolation of the complete *Kiss2* transcript sequence (EMBL: LT844561). The sequence encompassed 5′- and 3′-UTR of 114 and 495 bp, respectively, and a CDS of 402 bp. Once translated, the cloned *Kiss2* CDS gives a 134-aa kisspeptin precursor exhibiting a 10-aa sequence (FNRNPFGLRF) characteristic of the kisspeptin family (**Figure 1B**).

BLASTN analyses performed on the European eel draft genome, using the present eel *Kiss1* and *Kiss2* cloned sequences as queries, revealed that each transcript is encoded by two exons. Concerning *Kiss2*, the first exon encoded a 21-aa signal peptide. Both *Kiss1* and *Kiss2* exons-2 encoded the Kiss1 and Kiss2 mature peptides, respectively, including the characteristic 10-aa sequences of the kisspeptin family.

Prediction of European Eel Mature Kiss Peptides

The identification of the potential N-terminal and C-terminal cleavage sites of each precursor led to the prediction of two N-terminal extended putative mature peptides: Kp1(15) from *Kiss1* and Kp2(12) from *Kiss2* (**Figure 1**). Eel Kiss1 presented a conserved dibasic site (KR), 5 amino acids upstream the decapeptide, showing that a mature peptide of 15 amino acids [Kp1(15)] may be produced. Eel Kiss2 possessed a single basic amino acid (R) at position 13, indicating that the *Kiss2* cDNA encoded a putative peptide with 12 amino acids [Kp2(12)]. A 31-aa mature peptide for *Kiss1* and a 51-aa mature peptide for *Kiss2* could also be predicted (**Figure 1A**).

The sequence of eel Kp1(10) is identical to rat Kp1(10). In contrast, Kp2(10) is a newly identified sequence, which presented at its third position an arginine (R) that possessed different physicochemical properties from amino acids commonly found at this position. Kp1 and Kp2 sequences were followed at their C-terminal side by a proteolytic cleavage and/or an α-amidation motif, i.e., GK motif for *Kiss1* and GKR motif for *Kiss2* precursors, respectively (**Figure 1B**).

Tissue Distribution of European Eel *Kiss1* and *Kiss2* mRNAs

Specific qPCR protocols were developed for each eel *Kiss* and applied to the analysis of their respective tissue distribution (**Figure 2**).

Kiss1 mRNA Distribution

Kiss1 mRNAs were mainly expressed in the mesencephalon part of the brain. Its expression was lower in the diencephalon, close to the limit of detection in the telencephalon and *cerebellum* and under detection threshold in the olfactory bulb and *medulla oblongata*. *Kiss1* expression was abundant in the pituitary. In peripheral tissues, low *Kiss1* mRNA levels were measured in the eye and the testis. Its expression was at the limit of detection in muscle and under the detection threshold in the other investigated tissues (liver, kidney, intestine, spleen, adipose tissue, and ovary) (**Figure 2B**).

Kiss2 mRNA Distribution

Kiss2 mRNAs were mainly expressed in the diencephalon. *Kiss2* expression was lower in the mesencephalon and in the *medulla oblongata*, and under the detection threshold in the olfactory bulb, telencephalon, and *cerebellum*. *Kiss2* expression was also observed in the pituitary. In peripheral tissues, a low expression of *Kiss2* was detected in the testis. The expression level was under the limit of detection in the other tissues (eye, liver, kidney, intestine, spleen, adipose tissue, and ovary) (**Figure 2C**).

Functional Properties of Eel Kiss Peptides

The two 10-aa peptides, Kp1(10) and Kp2(10), and the two predicted mature peptides, Kp1(15) and Kp2(12), were synthesized for functional assays (**Table 2**). The functional activity of the four synthesized eel kisspeptins was assessed on the kinetics of $[Ca^{2+}]_i$ in CHO-K1 cells stably transfected with the rat KissR-1, by using a multimode FlexStation III system. All four kisspeptins were able to activate the rat Kiss1R, i.e., to trigger intracellular pathways leading to $[Ca^{2+}]_i$ increase. As shown in **Figure 3** and **Table 3**, eel/rat Kp1(10) and the other eel Kiss peptides, Kp1(15), Kp2(10), and Kp2(12), displayed the following EC_{50} values: 8.5 ± 4.5, 11.8 ± 2.0, 56.6 ± 27.7, and 134.7 ± 18.9 nM, respectively.

Effects of Eel Kiss Peptides on Primary Culture of Eel Pituitary Cells

We previously demonstrated that heterologous kisspeptins [human/lamprey Kp1(10); human Kp1(14); lamprey Kp1(13); zebrafish Kp1(10), Kp2(10), Kp1(15), and Kp2(15)] can inhibit *lh*β expression by eel pituitary cells in primary culture, with no effect on *fsh*β (22). In the present study, we tested the effects of concentrations ranging from 10^{-11} to 10^{-7} M of the homologous eel kisspeptins over 10 days in the same culture system.

Effects of Eel Kiss Peptides on Gonadotropin Expression

All four eel synthesized kisspeptins [Kp1(10), Kp1(15), Kp2(10), and Kp2(12)] significantly inhibited *lh*β expression at 10^{-7} M [×0.63 with Kp1(10), $P < 0.001$; ×0.59 with Kp2(10), $P < 0.001$; × 0.60 with Kp1(15), $P < 0.001$; ×0.62 with Kp2(12), $P < 0.001$] (**Figure 4A**). Inhibition by Kp2(10) and Kp2(12) was also significant at 10^{-9} M (×0.71, $P < 0.05$ and ×0.70, $P < 0.01$, respectively). In contrast, the eel Kiss peptides had no significant effect on *fsh*β expression at any dose tested (**Figure 4B**).

Effects of Eel Kiss Peptides on GnRH Receptor Expression

Among the three eel GnRH receptors, *gnrh-r2* was the only one with detectable specific expression in both control and treated

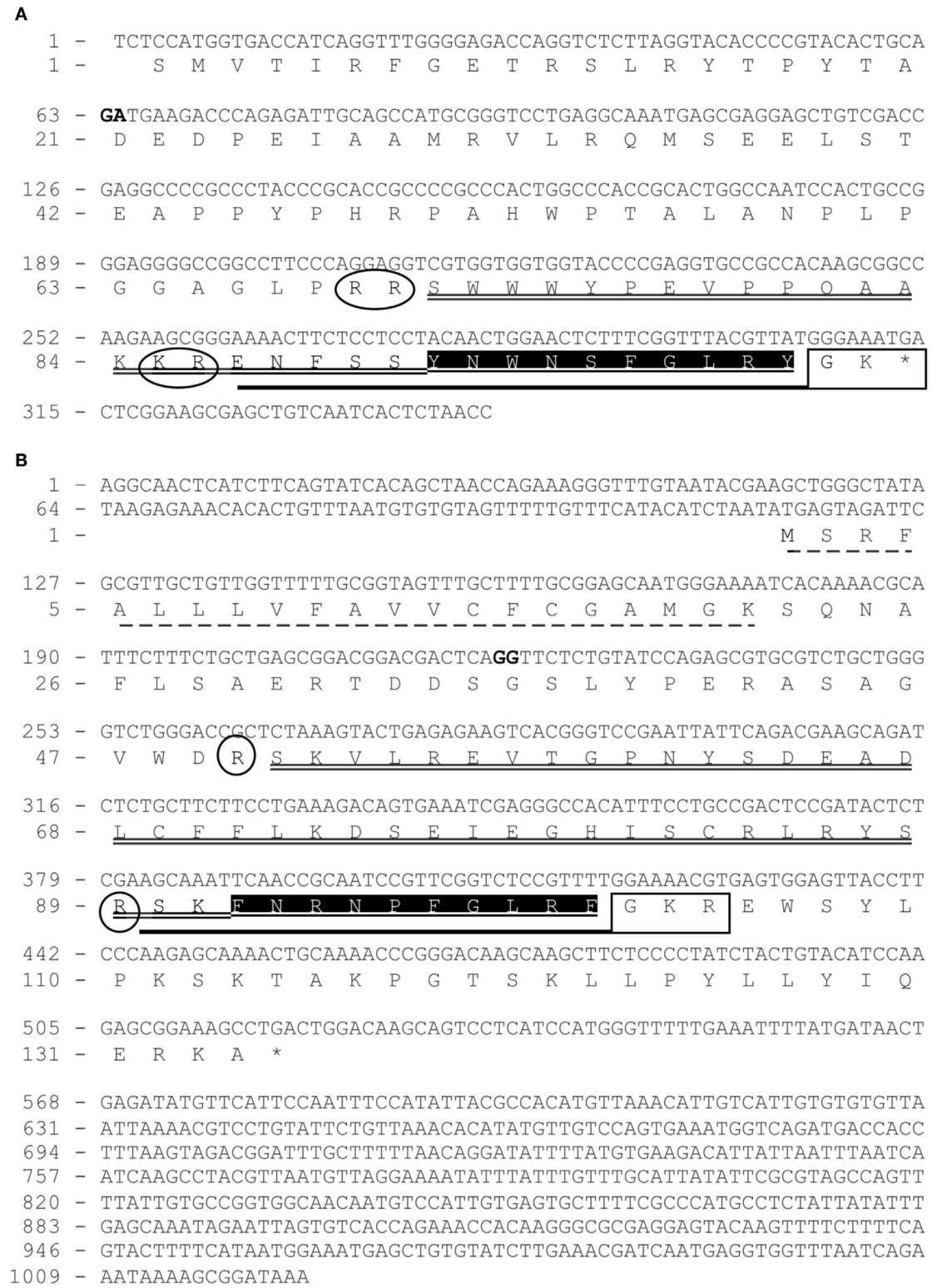

FIGURE 1 | Cloning of eel partial *Kiss1* mRNA **(A)** and complete *Kiss2* mRNA **(B)**. Nucleotides (upper line) are numbered from 5′ to 3′. The exon–intron junctions are indicated by two nucleotides in bold. The deduced amino acids (bottom line) are numbered beginning with the first methionine residue (M) in the ORF (for Kiss2) or with the first codon of the known sequence (for Kiss1 partial mRNA). The asterisk (*) indicates the stop codon, and signal peptide sequence is underlined by a dashed line. Kp1(10) and Kp2(10) are shaded in black. Kp1(15) and Kp2(12) are underlined by a bold line. Kp1(31) and Kp2(51) are underlined by a double line. C-Terminal proteolytic α-amidation sites are boxed in a square and predicted N-terminal cleavage sites are circled.

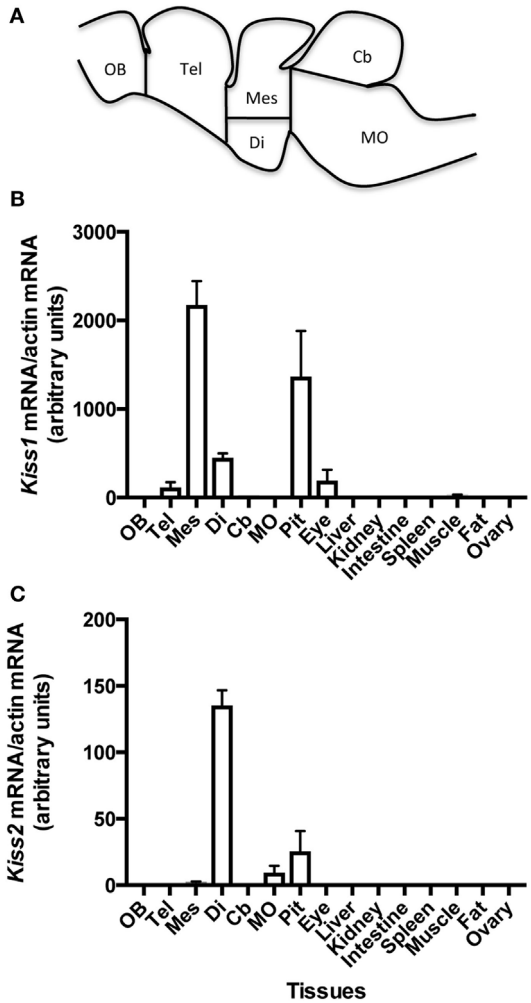

FIGURE 2 | Schematic representation of eel brain with dissection cut sites indicated adapted from Ref. (32) **(A)**, and tissue distribution of the expression of eel *Kiss1* **(B)** and *Kiss2* **(C)** mRNAs. Olfactory bulb (OB), telencephalon (Tel), diencephalon (Di), mesencephalon (Mes), *cerebellum* (Cb), *medulla oblongata* (MO), pituitary (Pit), eye, liver, kidney, spleen, muscle, adipose tissue (Fat), and ovary were dissected from female silver eels. The relative expression of each *Kiss* mRNA was normalized to actin mRNA. Each bar represents mean ± SEM from eight individual samples.

pituitary cells in primary culture; with *gnrh-r1a* and *gnrh-r1b* expressions at the limit of detection, primer dimer peaks (melting curve) were obtained. An inhibitory effect of all four synthesized eel Kiss peptides was observed on the expression of *gnrh-r2*. This inhibitory effect was dose-dependent, the highest inhibition being observed at 10^{-7} M [×0.35 for Kp1(10), $P < 0.001$; ×0.34 for Kp2(10), $P < 0.001$; ×0.33 for Kp1(15), $P < 0.001$; and ×0.44 for Kp2(12), $P < 0.001$] (**Figure 4C**).

DISCUSSION

Eel Kisspeptins

In order to assess the predicted *Kiss1* and *Kiss2* sequences and further investigate the gene exon–intron structures, we performed specific RACE PCR on *Kiss1* and *Kiss2* transcripts. The sequencing of the RACE PCR products and the comparison of their sequences to the European eel genome provided a partial *Kiss1* mRNA encompassing, at least, two exons and a complete *Kiss2* mRNA made of two exons. This structure exhibiting two exons is typical of the conserved structure of *Kiss* genes (34).

Once translated, both *Kiss1* and *Kiss2* transcripts encoded two proteins presenting the characteristics of the kisspeptin precursors. Among those hallmarks, Kiss1 and Kiss2 precursors presented the conserved kisspeptin-10 sequences, i.e., YNWNSFGLRY for Kiss1 and FNRNPFGLRF for Kiss2. Moreover, Kp1(10) and Kp2(10) were followed at their C-terminal extremity by a cleavage and α-amidation signature, i.e., GK and GKR motifs, respectively. For many neuropeptides, α-amidation is essential for biological activity (35, 36). Both eel kisspeptin-10 sequences were encoded by the second exon of each gene transcript in accordance with what has been observed in other species (34).

The eel Kp1(10) sequence was identical to the rat Kp1(10) but, to our knowledge, the only teleost sequence possessing a tryptophan (W) at its third position; other teleosts showed a leucine (L) except chub mackerel with a phenylalanine (F). The eel Kp2(10) sequence was rather unique due to the presence of an arginine (R) at its third position. The occurrence of an arginine at this position has only been reported so far in the Kp1(10) of the musk shrew (*Suncus murinus*) (37). Kp2(10) sequences appeared more conserved among vertebrates than Kp1(10) ones, differing by one amino acid versus three (Figure S1 in Supplementary Material).

Although kisspeptin-10 is considered as the minimal sequence capable of activating KissR (6, 7), *in silico* analysis did not provide any evidence for the existence of mature Kp1(10) or Kp2(10) in any vertebrate including the European eel. Using the NeuroPred tool, we were able to predict the N-terminal cleavage site for each kisspeptin precursor. The predictions of these N-terminal cleavage sites in addition to the C-terminal α-amidation motifs have delineated potential mature peptides that could be directly cleaved from Kiss1 and Kiss2 precursors. Thus, the two likely mature peptides in the European eel were Kp1(15) and Kp2(12). In addition, two potentially longer mature peptides could be predicted: a 31-aa mature peptide for *Kiss1* and a 51-aa mature peptide for *Kiss2*.

The presence of cleavage sites delineating a mature Kp1(15) appears to be conserved among teleosts (Figure S1 in Supplementary Material), except in goldfish *Carassius auratus* (38) and in chub mackerel *Scomber japonicus* (39) in which a Kp1(16) is predicted. In contrast, the presence of a glutamic acid (E) at the first position of the eel mature Kp1(15) seems to be a unique feature among teleosts. Interestingly, the presence of an E residue at the first position of a mature Kp1 is observed in Kp1(16) in the sarcopterygians, including mammals and the coelacanth (*Latimeria chalumnae*), and a non-teleost representative of actinopterygians, a holostean, the spotted gar (*Lepisosteus oculatus*) (23). This feature could thus represent a common characteristic of sarcopterygians and actinopterygians, which has been conserved in the eel and lost in some other teleosts.

The presence of a basic cleavage site leading to a mature Kp2(12) seems to be also conserved among teleosts such as in the European eel (Figure S1 in Supplementary Material). One

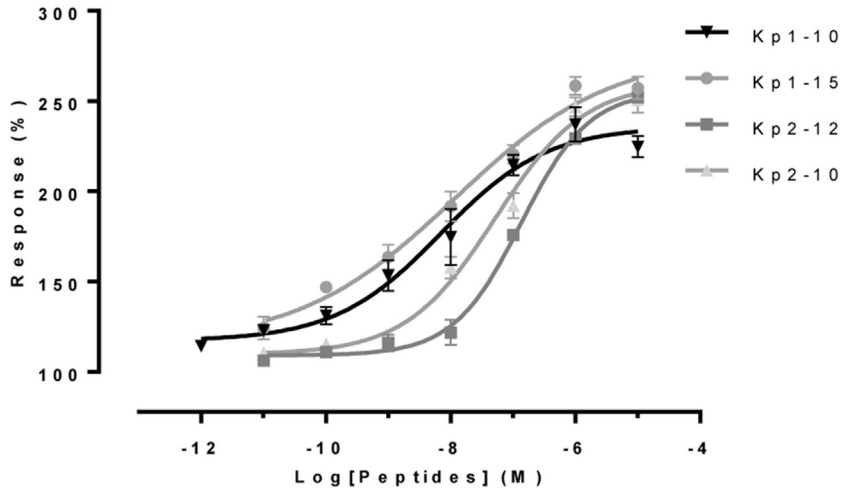

FIGURE 3 | Functional assays of eel Kiss peptides. Levels of $[Ca^{2+}]_i$ in CHO-K1 cells transfected with rat KissR-1 and stimulated with different doses of eel Kp1(10), Kp2(10), Kp1(15), and Kp2(12) were assessed by using FlexStation technology. After subtraction of mean fluorescence background from control wells without Fluo-4 acetoxymethyl ester, baseline was normalized to 100%, and fluorescence peak values were determined for each concentration of peptide. Values represent mean ± SEM from two independent experiments, and average dose–response curves are shown.

exception is observed in salmonids (masu salmon and kokanee salmon, *Oncorhynchus nerka*) in which the mature endogenous Kiss2 peptide is a 13-aa sequence (20). The existence of a mature Kp2(12) has been proven in *Xenopus* (21) and in the red-eared slider turtle (20).

Brain *Kiss1* and *Kiss2* Expression in the European Eel

Both eel *Kiss1* and *Kiss2* mRNAs were mainly expressed in the brain, as shown by specific qPCR, *Kiss1* being more abundant in the mesencephalon and *Kiss2* in the diencephalon. Their expressions were also observed in other parts of the central nervous system (i.e., in the telencephalon and *cerebellum* for *Kiss1* and in the *medulla oblongata* for *Kiss2*), although at lower levels. These distributions, which were obtained in the present study, in females at silver stage may differ in males or in females at a different stage.

In other teleosts presenting two *Kiss* genes, both *Kiss1* and *Kiss2* mRNAs are also expressed in the central nervous system [RT-PCR and qPCR, zebrafish (40–42); RT-PCR, goldfish (43); RT-PCR, sea bass (*Dicentrarchus labrax*), medaka (*Oryzias latipes*) (42); RT-PCR and qPCR, chub mackerel (44); qPCR, rohu, *Labeo rohita* (45); qPCR, *Catla catla* (46, 47); qPCR, pejerrey, *Odontesthes bonariensis* (48)], suggesting that kisspeptins exert important functions in the teleost brain. Anatomical studies, using ISH and laser-capture microdissection coupled with qPCR, have demonstrated that each transcript is located in different brain nuclei: *Kiss1* mRNA is observed in the ventral *habenula*, while *Kiss2* mRNA is found in the preoptic region and in the hypothalamus [for review see Ref. (49); European sea bass (50)]. Nevertheless, in striped bass *Morone saxatilis* (51) and in chub mackerel (39), no *Kiss1* expression was reported in the habenular nucleus. In zebrafish, generation of specific antibodies for each kisspeptin type made it possible to evidence that only Kiss2 neurons send projections to GnRH neurons and pituitary, suggesting a prominent involvement of Kiss2 rather than Kiss1 in the regulation of the gonadotropic activity of this species (52). This is probably also the case in the eel, as *Kiss2* is more actively expressed than *Kiss 1* in the diencephalon, which is the main neuroendocrine region of the brain.

In a previous study, we showed the expression of the three eel Kiss receptors in the brain (19). These receptors are differentially expressed in various brain regions. *KissR-1* mRNAs is widely expressed in all parts of the eel brain. This receptor is the unique receptor present in placental (eutherian) mammals; the European eel is the only extant teleost shown so far to possess KissR-1, which seems to have been lost in other teleosts (19, 23, 24). Eel *KissR-2* mRNAs is mainly expressed in the telencephalon and the di-/mesencephalon, while *KissR-3* expression is primarily observed in the di-/mesencephalon. These data suggest potential multiple actions of kisspeptins in the eel brain that need further investigations.

Pituitary *Kiss1* and *Kiss2* Expression in the European Eel

Both *Kiss1* and *Kiss2* transcripts were expressed in the eel pituitary. In other teleost species possessing two kisspeptin genes, different observations have been reported. Only *Kiss2* expression is detected in zebrafish (52) and pejerrey (48) pituitaries, while only *Kiss1* is expressed in the chub mackerel pituitary (44). In the medaka, none of the two kisspeptin genes is expressed in the pituitary (53), while in the sea bass, both genes are expressed (54), as in the eel.

We previously showed that *KissR-1* and *KissR-2* are the two receptors expressed in the eel pituitary (19). These data suggest that, in the eel, the pituitary could be a target for the neuroendocrine action of both cerebral and locally produced pituitary

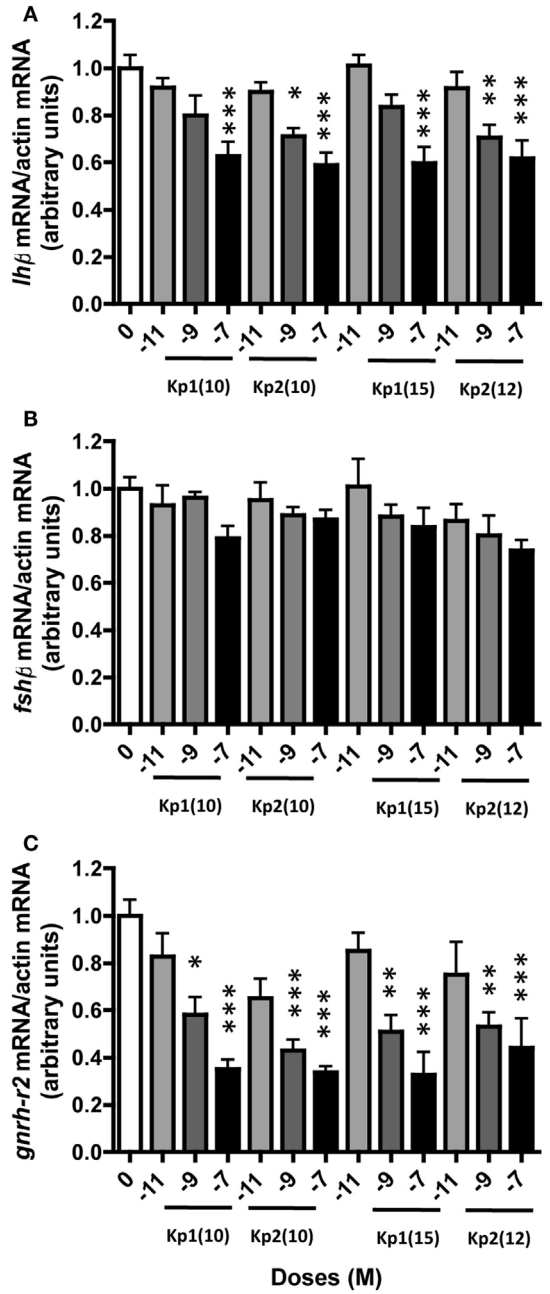

FIGURE 4 | Dose-dependent effect of eel Kiss peptides on pituitary *lh*β **(A)**, *fsh*β **(B)**, and *gnrh-r2* **(C)** expression by eel pituitary cells in primary culture. Pituitary cells were treated with various concentrations (10^{-11}, 10^{-9}, and 10^{-7} M) of eel Kp1(10), Kp2(10), Kp1(15), and Kp2(12) for 10 days. Pituitary mRNA levels were quantified by quantitative real-time PCR. Data were normalized against β-actin. This figure displays the results of a representative experiment. Each point represents mean ± SEM from five-well replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *versus* controls, ANOVA.

kisspeptins. Similarly, pituitary expression of *Kiss* and *KissR* mRNA, as well as the presence of their respective proteins, has been observed in mammals and amphibians [human (6, 7); rat (55–57); ovine (58); and amphibians (21, 59)]. The occurrence of kisspeptin and its receptor in the pituitary supports the notion that the peptide may exert paracrine and/or autocrine actions in this tissue.

Peripheral *Kiss1* and *Kiss2* Expression in the European Eel

In the eel, *Kiss1* and *Kiss2* were weakly expressed in the testis (data not shown) and not in the ovary at the silver stage (prepubertal blockade), which cannot predict a higher expression at a more advanced sexual stage. In other teleosts possessing two kisspeptin genes, different situations have been reported. In some species, both kisspeptins are detected in the gonads [medaka and zebrafish (42, 53); sea bass (60); rohu (45); and *Catla catla* (46, 47)]. In contrast, in the chub mackerel, *Kiss1* is expressed in the gonads of both sexes, while *Kiss2* expression is not detected (44), whereas in the pejerrey, expression of both genes is very low in the gonads (48). We previously showed a strong expression of *KissR-1* in both the ovary and testis of the European eel (19), which supports a potential endocrine action of *Kiss1* and/or *Kiss2* on European eel gonads.

In other peripheral organs, which do not belong to the brain–pituitary–gonad axis (muscle, liver, fat, kidney, intestine, spleen, and eye), eel *Kiss1* and *Kiss2* expression levels were under the detection limit except in the eye, where *Kiss1* mRNA, but not *Kiss2*, was clearly expressed. We previously showed the expression of *KissR-1* in the eye, suggesting the potential involvement of the kisspeptin system in the local regulation of visual functions. *Kiss1* and *Kiss2* expression also occurs in the eye of other teleost species [zebrafish and medaka (42); rohu (45); and pejerrey (48)]. Kisspeptin receptor mRNAs are also observed in the eye of zebrafish [*KissR-3* only (61)] and pejerrey [*KissR-2* and *KissR-3* (48)]. Interestingly, in the medaka, eye development is interrupted after zygotic knockdown of *Kiss1* (62), implying a possible function of the kisspeptin system during retina ontogenesis. Few data are available in other vertebrates. In X*enopus*, expression of *Kiss-1a* and *Kiss-1b* is observed in the eye, while none of the three kisspeptin receptors and neither *Kiss-2* are expressed in this organ (59). In mammals, one study supports a role of kisspeptin as metastasis suppressor gene in the eye, as the expression of both *Kiss1* and its receptor is detected in human uveal melanoma cell lines and correlates with survival rate (63).

Functional Properties of Eel Kiss Peptides

We demonstrated that all four synthesized eel Kiss peptides were able to bind kisspeptin receptor in heterologous system (CHO-K1 cells stably transfected with rat KissR-1), inducing a rise in intracellular calcium. Eel Kp1(15) exhibited about the same potency to activate rat KissR-1 than eel Kp1(10), which is identical to rat Kp1(10). Interestingly, eel Kiss2-derived peptides were also able to activate rat KissR-1, as zebrafish (59) and sea bass (60) peptides did with human KissR-1.

Zebrafish Kiss peptides are also highly efficient for activating mammalian (human) KissR-1 in COS-7 cells [zebrafish Kp1(10) (41)] and in CV-1 cells [zebrafish Kp1(15), Kp2(10), and Kp2(12) (59)]. Similarly, in CHO cells, sea bass Kiss peptides [sea bass Kp1(10), Kp2(10), Kp1(15), and Kp2(12)] can efficiently activate

human KissR-1, while, in the case of mouse KissR-1, sea bass Kp2(10) is not able to induce any activation (60).

Teleost models, other than eel, possess only two receptors, homologous to eel KissR-2 and eel KissR-3, respectively, as they have lost their KissR-1 paralog, homologous to eel and human KissR-1 (19). For clarity, the nomenclature of Pasquier et al (19, 24) for KissR will be used in the following paragraphs. In teleost models with two receptors, various studies have shown differential affinities of Kiss peptides toward homologous receptors. In zebrafish [CV-1 cells (59)] and sea bass [CHO cells (60)], Kp2(12) exhibits higher potency for activating KissR-3, while Kp1(15) exhibits a preference for KissR-2. These data are in agreement with the neuroanatomical distribution of Kiss and KissR-expressing neurons in these two species [zebrafish (52); sea bass (50, 64)]. However, contradictory results have been obtained by other authors in zebrafish, KissR-2 being activated by both Kp1(10) and Kp2(10), while KissR-3 is preferentially activated by Kp1(10) [COS-7, CHO-K1, and HEK293 cells (61)]. In striped bass, Kp1(15) and Kp2(12) activate KissR-3 with the same potency, while KissR-2 is more efficiently activated by Kp2(12) [COS-7 cells (65)]. In the chub mackerel, the predicted mature peptide Kp1(16) is more active than the shorter Kp1(15) on KissR-3 (39).

Furthermore, in these homologous receptor and peptide systems, distinct intracellular signal transduction pathways can be activated. KissR-2 and KissR-3 signals can be transduced *via* both PKA and PKC pathways in zebrafish (41), medaka (66), chub mackerel (67), and sea bass (60). In goldfish, the PKA pathway is activated by Kiss1/KissR-3, while PKC pathway is induced by Kiss2/KissR-2 (43). In the southern Bluefin tuna (*Thunnus maccoyii*) and in the yellowtail kingfish (*Seriola lalandi*), KissR-2 (the only receptor present in these species) shows stronger transduction *via* the PKC than the PKA pathway (68), while in the orange-spotted grouper, *Epinephelus coioides*, no PKA could be activated (69). In mammals (6, 7) and in the bullfrog *Rana catesbeiana* (21), KissR-1 conveys its signal *via* the PKC pathway and not the PKA pathway.

As, in the eel, three kisspeptin receptors, KissR-1, KissR-2, and KissR-3, are present, future studies should aim at investigating the potency of homologous kiss peptides to activate each eel KissR and study their signal transduction pathways.

Biological *In Vitro* Activities of Eel Kiss Peptides on Eel Pituitary Cells

All four synthetic eel kisspeptins specifically and dose-dependently inhibited *lh*β expression by eel pituitary cells in culture, while they had no effect on *fsh*β transcripts. These data confirm, using homologous peptides, the specific inhibitory effect of kisspeptin on *lh*β in the European eel that we previously reported with heterologous Kiss peptides (22).

This paradoxical inhibitory effect contrasts with the general action of kisspeptin as an activator of puberty and reproduction, mostly at the brain level (70), but also directly on the pituitary [for reviews see Ref. (71, 72)]. Our results in the European eel suggest that kisspeptins encoded by both *Kiss1* and *Kiss2* have an inhibitory *in vitro* effect on *lh*β expression. Another study, in the striped bass, reported that Kp1(15) had an inhibitory effect on *lh* expression, while Kp2(12) stimulated LH release (65). In contrast, in other studied teleosts, the action of kisspeptin is generally either stimulatory or absent as in mammals. For instance, in goldfish, homologous Kp1(10) and Kp2(10) are inactive on LH release by pituitary cells from sexually mature females (43), whereas homologous Kp1(10) increases the release and expression of LH by pituitary cells from mixed sexes at late stages of sexual regression (38). In the sea bass, Kp2(12) induces both *lh* expression and LH release by pituitary cells obtained from mature males, while Kp1(15) has no effect (73). In (striped and sea) basses, the different actions of Kiss1 and Kiss2 on LH regulation observed *in vitro* have also been reported *in vivo*. In the sea bass, Kp2(10) is significantly more potent than Kp1(10) in inducing LH secretion after intramuscular (im) injection to both prepubertal and adult fish (42). In the striped bass, im injection of Kp1(15) and Kp2(12) induces plasma LH levels in a stage-dependent manner: Kp1(15) has no effect on LH at pubertal stage, while both peptides could increase LH levels at gonadal recrudescence (51). Our results in the European eel suggest that kisspeptins encoded by both *Kiss1* and *Kiss2* have no *in vitro* effect on *fsh*β expression. In a few studies, a stimulatory effect of kisspeptins has been reported on FSH. In the striped bass, Kp1(15) and Kp2(12) stimulate FSH at both the gene transcription and peptide secretion levels (65). In the sea bass, Kp2(12), but not Kp1(15), can induce *in vitro* FSH release (73). Recently, it was reported that, in amphioxus (*Branchiostoma japonicum*), injection of amphioxus kisspeptin-like could upregulate the expression of *gpb5*, a paralog of glycoprotein vertebrate-like β subunit (74). Our results on primary cultures of eel pituitary cells indicate that Kiss peptides may act directly at the pituitary cell levels in the eel. The action of Kiss peptides may be exerted directly on pituitary gonadotrophs or *via* other pituitary cells. We have already shown that heterologous Kiss peptides did not change the expression of *gp*α, *gh*, *fsh*β, and *tsh*β in the same *in vitro* system (22), but indirect action on LH cells may occur *via* some other factors produced by pituitary cells other than LH cells. Our previous studies revealed that Kiss receptors (KissR-1 and KissR-2) are expressed in the eel pituitary (19). Future *in situ hybridization* studies would be necessary to decipher whether KissR and which type(s) are expressed by LH cells. Future experiments may also aim at investigating the *in vitro* effects of specific antagonists for KissR on *lh*β and *gnrh-r2* expression. In mammals, including humans, Kiss peptides may act not only *via* their cognate receptor (Kiss-R = GPR54) but also *via* other RF-amide receptors that show less specificity such as NPFF receptors 1 and 2 [for instance see Ref. (75)]. This opens further research avenues aiming at characterizing the full complement of RF-amide receptors in the eel.

Besides the inhibitory action on *lh*β expression of eel kisspeptins, the present study reveals a dose-dependent inhibitory effect on the expression of *gnrh-r2* by primary culture of eel pituitary cells. This receptor is the one increased during experimental maturation in both female and male eels (25). To the best of our knowledge, only a single other study investigated the effect of kisspeptin on GnRH receptor in teleost. In female striped bass at dummy run phase (ovarian development is initiated but not completed), chronic administration *in vivo* of both Kp1(15) and

Kp2(12) induces a decrease of pituitary *gnrh-r* mRNAs (76). In other vertebrates, the few studies available have been mainly performed in mammals and demonstrate a stimulatory or an absence of effect of kisspeptins on the expression of GnRH receptor. Using the mouse pituitary gonadotroph LβT2 cell line, Witham and collaborators (77) have found that kisspeptin treatment cannot activate GnRH receptor promoter, but, in contrast, Mijiddorj et al. (78) have recently demonstrated that kisspeptin increases the expression of GnRH receptor. While the presence of Kiss/KissR is under question in birds, repeated injections of human Kp1(10) upregulated pituitary expression of type II (but not type I) *gnrh-r* in the juvenile female Japanese quail (*Coturnix japonica*) (79). In the anuran amphibian *Pelophylax esculentus*, Kp10 treatment of testes in culture upregulated the expression of the three *gnrh-r* before and during the reproductive periods, and this effect was completely abolished/counteracted by the antagonist Kp-234 (80).

Our finding suggests that, in the eel, kisspeptins decreased *lh*β expression directly at the pituitary level and also decreased pituitary sensitivity to GnRH by downregulating GnRH receptor expression, leading to a double inhibitory control. The kisspeptin system may thus contribute to the strong inhibitory control of puberty observed in the European eel. This inhibition in the eel, which contrasts with the stimulatory role of kisspeptin in the reproduction of other vertebrates, reveals evolutionary change in the reproductive role of kisspeptin.

AUTHOR CONTRIBUTIONS

JP, A-GL, and FD: cloning—qPCR (tissue distribution). JP and KR: primary cultures. BL and JL: synthesis of kiss peptides. CD, AM-H, and JL: binding studies. JP, HV, JL, SD, and KR: design—writing. All authors: final approval.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fendo.2017.00353/full#supplementary-material.

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REVIEW
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The Role of *Kiss1* Neurons As Integrators of Endocrine, Metabolic, and Environmental Factors in the Hypothalamic–Pituitary–Gonadal Axis

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Kisspeptin–GPR54 signaling in the hypothalamus is required for reproduction and fertility in mammals. *Kiss1* neurons are key regulators of gonadotropin-releasing hormone (GnRH) release and modulation of the hypothalamic–pituitary–gonadal (HPG) axis. Arcuate *Kiss1* neurons project to GnRH nerve terminals in the median eminence, orchestrating the pulsatile secretion of luteinizing hormone (LH) through the intricate interaction between GnRH pulse frequency and the pituitary gonadotrophs. Arcuate *Kiss1* neurons, also known as KNDy neurons in rodents and ruminants because of their co-expression of neurokinin B and dynorphin represent an ideal hub to receive afferent inputs from other brain regions in response to physiological and environmental changes, which can regulate the HPG axis. This review will focus on studies performed primarily in rodent and ruminant species to explore potential afferent inputs to *Kiss1* neurons with emphasis on the arcuate region but also considering the rostral periventricular region of the third ventricle (RP3V). Specifically, we will discuss how these inputs can be modulated by hormonal, metabolic, and environmental factors to control gonadotropin secretion and fertility. We also summarize the methods and techniques that can be used to study functional inputs into *Kiss1* neurons.

Keywords: *Kiss1*, hypothalamus, arcuate nucleus, gonadotropin-releasing hormone, neural afferents, luteinizing hormone

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INTRODUCTION

Kisspeptin, encoded by the *Kiss1* gene, was initially proposed as a suppressor of metastasis, but its precise role in this process remains elusive (1, 2). Expression of the *KISS1* gene and the gene encoding its cognate receptor, the G-protein coupled receptor 54 (*GPR54*, also called *KISS1R*) has been detected in the brain and peripheral tissues, including the pancreas, liver, small intestine, pituitary, and placenta (1, 3, 4). Two seminal studies in 2003 defined a physiological role for kisspeptin by showing that inactivating mutations of the *GPR54* gene are associated with hypogonadotropic hypogonadism in humans and mice (5, 6). Kisspeptin signaling was thus identified as one of the critical regulators for both puberty onset and maintenance of normal reproductive functions in mammals (7, 8). Kisspeptin exerts its effects on the hypothalamic–pituitary–gonadal (HPG) axis, by acting as a neuropeptide essential for stimulation of gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus (9–13). Subsequently, kisspeptin signaling has also been implicated in regulating glucose homeostasis and body weight control (14, 15).

This review focuses mainly on how *Kiss1* neurons in the arcuate region of the hypothalamus ($Kiss1^{ARC}$) act as sensors to relay information about hormonal and nutritional status and environmental changes to GnRH neurons to regulate the secretion of the gonadotropins critical to sustain fertility (**Table 1**). It is important to decipher the upstream networks that connect to $Kiss1^{ARC}$

TABLE 1 | Summary of neuropeptides or hormones that interact with *Kiss1* neurons.

Candidates	Anatomical evidence to ARC or *Kiss1* neurons	ICV or electrophysiology studies	Receptors expressed in *Kiss1* neurons	Physiological relevance
Agouti-related peptide/neuropeptide Y (AgRP/NPY)	AgRP neurons formed synaptic connections with *Kiss1*^{ARC} neurons (mice) (135)	Channel rhodopsin-assisted mapping indicated AgRP neurons formed inhibitory synaptic connections with *Kiss1*^{ARC} neurons (mice) (135)	*Npy1r, Npy2r* and *Npy5r* (82)	Chemogenetic activation of AgRP neurons inhibited fertility *in vivo* (135)
Arginine vasopressin (AVP)	AVP-immunoreactive fibers in close apposition with *Kiss1*^{RP3V} neurons (hamsters) (32)	AVP-stimulated *Kiss1*^{RP3V} neurons (105)	*Avp1r* in *Kiss1*^{RP3V} neurons (32, 105)	Circadian AVP signaling on *Kiss1*^{RP3V} neurons is facilitated by estrogen during LH surge (32, 105)
Corticotropin-releasing hormone (CRH)	Close appositions of CRH-immunoreactive fibers and *Kiss1* neurons (125)	1. Central administration of CRH suppressed LH secretion in rats (117). 2. CRH stimulated LH and increased GnRH pulse amplitude in sheep (118, 119)	CRHR (125)	Possible regulator of GnRH secretion in stress-induced reproductive disorders
Dynorphin (DYN)	DYN-immunoreactive fibers in close apposition with *Kiss1*^{ARC} neurons (rats and sheep) (171, 172)	ICV injection of dynorphin in adult male rats suppressed LH secretion (71)	*Oprk1* or KOR (39, 59, 73)	Inhibitory
Ghrelin		1. ICV administration inhibited LH secretion (rats and sheep) (143, 144). 2. Ghrelin acts on a subset of *Kiss1*^{ARC} neurons and estradiol increases the sensitivity of these neurons to ghrelin signals (mice) (147)	*Ghsr or* GHSR1A (147)	Inhibitory
Leptin		ICV leptin treatment to fasted adult rats increased LH pulse frequency, amplitude, and mean levels (140)	*Lepr* (83, 141)	*Kiss1* neurons are indirect target of leptin during puberty onset (142)
Neurokinin B (NKB)	NKB-immunoreactive fibers in close apposition with *Kiss1*^{ARC} neurons (rats and sheep) (65, 172)	NKB increased action potential firing of *Kiss1*^{ARC} neurons *via* activation of NK3R (57–59)	NK1 tachykinin receptor (NK1R), NK2R and NK3R (58)	Stimulatory
Pituitary adenylate cyclase-activating peptide (PACAP)	Channel rhodopsin-assisted mapping indicated PACAP neurons from the PMV synapses onto *Kiss1* neurons (mice) (81)	1. ICV administered PACAP depressed plasma LH amplitude and pulse frequency in gonadectomized ewes (75). 2. PACAP elevated the plasma LH levels in male rats (76). 3. PMV PACAP neurons formed stimulatory synapses with *Kiss1* neurons (mice) (81)	*Adcyap1r1* (81, 82)	Critical for ovulatory cycling and fertility in females, acts as permissive role for leptin or nutritional regulation of reproductive function (81)
Proopiomelanocortin/ Cocaine- and amphetamine-regulated transcript	Appositions between α-MSH fibers and *Kiss1*^{ARC} neurons (rats and sheep) (131, 134)	Central alpha-MSH can stimulate or inhibit LH secretion in rats (129, 130)	MC4R (134)	*Kiss1*^{ARC} neurons relay the stimulatory effects of melanocortin signaling onto the reproductive axis during puberty (134)
Relaxin-3	Dense relaxin-3-immunoreactive fibers projecting to the ARC (mice) (103)	ICV administration of human relaxin-3 in adult male rats stimulated LH secretion (102)	*Rxfp 1* (82)	Stimulatory
RFamide-related peptide-3 (RFRP-3)	RFRP-3-immunoreactive fibers contacted *Kiss1* neurons (mice and rats) (33, 90)	1. ICV RFRP-3 injection inhibits LH secretion in rats (91, 92), whereas it had no effects (97) in ewes. 2. Stimulates LH secretion in male hamsters (94, 95), but inhibits LH release in females (87, 93)	*Gpr147* or *Npffr1* (33, 90)	Primary central target for the inhibitory action of melatonin signal on reproductive function (98)
Somatostatin (SST)	Appositions between SST-immunoreactive fibers and *Kiss1* neurons (sheep) (85)	Inhibits episodic LH secretion during anestrus in sheep (84).	*Sstr1, 2, 3,* and *5* (82)	Inhibitory
Substance P (SP)	SP-immunoreactive fibers in close apposition with *Kiss1*^{ARC} neurons (monkey) (70)	1. ICV administration of SP showed increased LH release (67). 2. SP activates *Kiss1*^{ARC} neurons (58)	*Tacr1* or NK1R (68)	Stimulatory; critical in sustaining reproductive capabilities in female mice (66)

Italic fonts indicated receptor gene expression identified by in situ hybridization or single cell reverse-transcription PCR. Receptors detected by immunostaining or pharmacological blockade were written in capital letters. ICV, intracerebroventricular; LH, luteinizing hormone; PMV, ventral premamillary nucleus of the hypothalamus; NK1, 2, and 3R, neurokinin-1-3 receptor; Sstr 1, 2, 3, and 5, somatostatin receptor 1, 2, 3, and 5; Gpr147, G protein-coupled receptor 147; Rxfp 1, relaxin/insulin like family peptide receptor 1; MC4R, melanocortin 4 receptor; Lepr, leptin receptor; Adcyap1r1, adenylate-cyclase-activating polypeptide 1 receptor 1; CRHR, corticotropin releasing hormone receptor; Npy1r, Npy2r, and Npy5r, neuropeptide Y receptor Y1, 2, and 5; Ghsr or GHSR1A, growth hormone secretagogue receptor type 1a; Npffr1, neuropeptide FF receptor 1; Oprk1 or KOR, opioid receptor, kappa 1; Tacr1, tachykinin receptor 1.

neurons, as this information will improve our understanding of how different cues can be integrated into the HPG axis, complementing the homeostatic regulation of reproductive function and maintaining fertility. Infertility is a global health issue affecting a significant proportion of humanity and is estimated to affect 8–12% of couples worldwide (16, 17). Given that kisspeptin has been successfully used in patients with hypothalamic amenorrhea (18, 19) as well as those with an absence of neurokinin B (NKB) signaling (20), insights into these central pathways will aid in the manipulation of kisspeptin signaling that may be used in the treatment of infertility and reproductive disorders.

THE ROLE OF *Kiss1* NEURONS IN THE INTEGRATION OF ENDOCRINE RESPONSES

In mammals, there are two main populations of neurons in the hypothalamus that synthesize kisspeptin. The first is located in the preoptic area (POA) of sheep and monkeys (21–23), or the rostral periventricular region of the third ventricle (RP3V) in rodents (24, 25), hereafter, termed $Kiss1^{RP3V}$. $Kiss1^{RP3V}$ neurons show a clear sexual dimorphism with greater numbers present in females (26) where they control the GnRH/LH surge that triggers ovulation (27). The $Kiss1^{RP3V}$ neurons express both estrogen receptor alpha (ERα) and progesterone receptor (27). Activation of $Kiss1^{RP3V}$ neurons by estradiol is essential for the positive feedback action of estrogen on the HPG axis and is associated with increased *Kiss1* mRNA expression (28). This is dependent on ERα because the increased firing of $Kiss1^{RP3V}$ neurons in response to estradiol is absent in ERα knockout mice (29–31). Peptides such as arginine vasopressin (AVP) (32) and gonadotropin-inhibitory hormone (GnIH) (33) also regulate $Kiss1^{RP3V}$ neurons. $Kiss1^{RP3V}$ neurons have been shown to co-express dopamine and galanin (34, 35). Neuroanatomical tracing using a recombinant adenovirus encoding farnesylated enhanced green fluorescent protein (EGFP) to facilitate the labeling of *Kiss1* neural axons revealed projection of $Kiss1^{RP3V}$ neurons to GnRH neuronal soma and proximal dendrites within the POA (36). The $Kiss1^{RP3V}$ neurons also project to the arcuate nucleus (ARC) and to the distal dendrites of GnRH neurons, suggesting that $Kiss1^{RP3V}$ and $Kiss1^{ARC}$ communicate with each other to synchronize or coordinate LH secretion (36).

A second population of *Kiss1* neurons, which are less well characterized, is found in the ARC region of the brain (also called the infundibular nucleus in humans and primates) (10, 21, 37, 38). In rodents, sheep, and goats, these neurons have been shown to co-express other neuropeptides such as NKB and dynorphin (DYN), and this has led to $Kiss1^{ARC}$ neurons being termed KNDy neurons (39–41). In KNDy neurons, estrogen suppresses *Kiss1* expression *via* ERα (28), and embryonic deletion of *Esr1* specifically in *Kiss1* neurons, advances puberty onset in association with significantly elevated LH levels (26, 42). $Kiss1^{ARC}$ neurons innervate the distal dendrons (a term describing a single projection structure that functions simultaneously as an axon and dendrite) (43) of GnRH neurons. Changes in $Kiss1^{ARC}$ neuron number, morphology, and connectivity with GnRH neurons have been detected across developmental stages (44–48).

THE ROLE OF $Kiss1^{ARC}$ NEURONS IN NEUROPEPTIDE INTEGRATION

Optogenetic stimulation of $Kiss1^{ARC}$ neurons *in vivo* has shown an important role for these neurons in orchestrating pulsatile GnRH/LH secretion (49). Since then, the mechanism by which $Kiss1^{ARC}$ neurons contribute to the GnRH pulse generator has been studied. Navarro and colleagues proposed a model where $Kiss1^{ARC}$ neurons are interconnected and use the co-expressed neuropeptides NKB and DYN (39) to form a synchronized network sending a rhythmic stimulatory signal to the GnRH neurons, thus generating pulsatile gonadotropin secretion. Interestingly, optogenetic inhibition of $Kiss1^{ARC}$ neurons revealed that the middle or caudal $Kiss1^{ARC}$ neurons are responsible for pulsatile LH secretion whereas inhibition of rostral ARC failed to suppress LH release (50). The ARC population has been revealed to have higher number of *Kiss1* cells compared to the RP3V region (51, 52), and they may be heterogeneous in terms of firing pattern or ion channel distribution, and possibly receptor expression (53, 54). All in all, we are beginning to appreciate the complexity of the $Kiss1^{ARC}$ neurons in terms of their morphology, functional heterogeneity, different projection areas, and afferent inputs.

Central administration of various classical neurotransmitters and neuropeptides has been found to alter LH secretion through a GnRH neuron-dependent pathway (55). Given that $Kiss1^{ARC}$ neurons are upstream regulator of GnRH neurons, this indicates that $Kiss1^{ARC}$ neurons may possibly receive signals from a variety of neuropeptides and neuromodulators, which modulate GnRH/LH secretion by interacting with receptors located on kisspeptin neuron cell bodies, dendrites, and terminals. Another way to evaluate upstream signals to *Kiss1* neurons is to use electrophysiological recordings after neuropeptide stimulation in brain slice preparations and selective pharmacological inhibition of these responses. This approach was taken to show that *Kiss1* neurons can respond directly to NKB, DYN (56–58), and substance P (SP) (58). Once an initial firing response is identified, the next step is to undertake intracerebral injection or intracerebroventricular (ICV) infusion of the neuropeptide or antagonist *in vivo* to assess the corresponding physiological effects.

There is good evidence regarding the effects of the tachykinin NKB on $Kiss1^{ARC}$ neurons and subsequent LH release. $Kiss1^{ARC}$ neurons are depolarized and increase action potential firing upon activation of NK3R, the membrane receptor for NKB (57–59). In rodents, the full excitatory effect of NKB on $Kiss1^{ARC}$ neuron firing requires the activation of all three tachykinin receptor subtypes [NK1 tachykinin receptor (NK1R), NK2R and NK3R], which may all be expressed in these neurons (58). Activation of NK3R, with the agonist senktide, has been used in several studies to probe the effects of NKB on LH release in rodents (39, 60–62). The mechanisms of $Kiss1^{ARC}$ NKB signaling may vary between species as pulsatile LH release is sensitive to NK3R blockade alone in sheep (63), while in rodents, it requires blockade of all three tachykinin receptors with no effect with blockade of NK3R alone (64). The theory of a synchronized KNDy network as the pulse generator may be plausible; however, it is not clear whether these reciprocal KNDy–KNDy connections (65) derive

from axon collaterals within a single neuron or connections from neighboring KNDy cells, or inputs from a segregated subset of KNDy neurons.

Substance P (SP) encoded by the *Tac1* gene, is another tachykinin, which can also influence reproduction. Female *Tac1* knockout mice display delayed puberty (66). Early studies with ovariectomized and estrogen-primed rats treated with intravenous or ICV administration of SP showed increased LH release (67). SP acts *via* the NK1R, and gonadotropin stimulation is blocked in the absence of kisspeptin (68). Moreover, SP activates $Kiss1^{ARC}$ neurons (58) and approximately half of the $Kiss1^{ARC}$ neurons express gene encoding the SP receptor (NK1R) (68). These findings support a role for SP acting *via* $Kiss1^{ARC}$ neurons to stimulate GnRH release and its critical role in sustaining reproductive capabilities in female mice. In rodents, populations of substance P cells were found in the ventromedial nucleus of the hypothalamus (VMH) (68, 69). One study reported SP fibers projection to the ARC, and they were in close apposition with $Kiss1^{ARC}$ neurons in male juvenile monkey (70). Majority of the SP cells were found in the premammillary nucleus, sparse SP cells were also observed in the ARC of the monkey.

Another substance that has been identified as an inhibitor of gonadotropin secretion is DYN (71), which belongs to the family of endogenous opioid peptides and is considered to mediate the negative feedback effects of progesterone on LH secretion (72). It has been reported that DYN exerts its inhibitory effects through *Kiss1* neurons (41) and the theory of DYN's inhibitory effect on the GnRH pulse generator has emerged. More than 90% of $Kiss1^{ARC}$ neurons in the ewe express kappa opioid receptor (KOR) (73); whereas a lower percentage of KOR was revealed by *in situ* hybridization (39, 74) and single-cell reverse transcription-PCR studies (59). It is hypothesized that DYN may inhibit GnRH pulse frequency by binding to postsynaptic KOR in $Kiss1^{ARC}$ neurons. Further elucidation of the mechanism underlying DYN/KOR-dependent GnRH pulse generator suppression is yet to be proven.

The role of pituitary adenylate cyclase-activating peptide (PACAP) in puberty has been shown in several recent studies. In ovariectomized ewes, ICV administration of PACAP depressed plasma LH amplitude and pulse frequency (75). In contrast, intravenous infusion of PACAP elevated the plasma LH levels in male rats (76). Knockout of the PACAP gene (*Adcyap1)* is partially lethal (C57Bl/6J genetic background) as the majority of PACAP-deficient mice died at around 3 weeks of age (77) from dysfunction of lipid and carbohydrate metabolism (78). Surviving PACAP-deficient female mice exhibited reduced fertility, with no obvious defects in the length of estrus cycles but their mating frequency was significantly reduced (79). Abundant expression of *Adcyap1* is found in the ventral premammillary nucleus of the hypothalamus (PMV) and the VMH, both regions known to be involved in leptin-related control of puberty and fertility (80, 81). Despite the presence of leptin receptor (LepR) in $Kiss1^{ARC}$ neurons (82), the main site of leptin's action to regulate reproduction is through cells in the PMV (83). Recently, channel rhodopsin-assisted circuit mapping revealed that the PMV PACAP neurons form direct monosynaptic contact with both $Kiss1^{ARC}$ and $Kiss1^{RP3V}$ neurons, which express the PACAP receptor (81). Furthermore, calcium-imaging experiments provided intriguing insights that PACAP exerts direct stimulatory effect exclusively on caudal $Kiss1^{ARC}$ neurons.

The peptide hormone somatostatin (SST), acting through the SSTR2 receptor, inhibits episodic LH secretion possibly *via* the mediobasal hypothalamus (MBH) during anestrus in sheep (84). Recently, Dufourny and Lomet discovered reciprocal connections between *Kiss1* and SST neurons in ewes (85), with most $Kiss1^{ARC}$ neurons showing SST-immunoreactive fiber appositions. The expression of *Sstr2* by *Kiss1* neurons is not yet proven but *Sstr2, 3,* and *4* are expressed in GnRH neurons in mice (86). SST neurons can be found in the periventricular area of the POA, in the ARC and in the ventrolateral area of the VMH. The functional relevance of *Kiss1* appositions on SST neurons remains to be verified since GPR54 has not yet been identified in SST neurons.

The RFamide-related peptide-3 (RFRP-3) is a mammalian analog of avian GnIH, found primarily in the dorsomedial nucleus of the hypothalamus (DMH) and adjacent structures (87, 88). This peptide inhibits LH secretion by suppressing GnRH secretion (89). Anatomical studies showed that about 20% of $Kiss1^{RP3V}$ neurons from proestrus female mice were contacted by RFRP-3 fibers, and only a small fraction of the $Kiss1^{RP3V}$ neurons expressed *Gpr147*, one of the receptors for RFRP-3 (33). Similarly, 35% of $Kiss1^{ARC}$ neurons receive RFRP fiber contacts, with approximately 25% expressed *Gpr147* (90). Even though RFRP-3 is considered an inhibitor of gonadotropin secretion in rats (91, 92) and female hamsters (87, 93), it is able to stimulate the gonadotropic axis in male Syrian and Siberian hamsters. RFRP-3′s stimulatory effects on gonadotropin and testosterone production were observed in male hamsters (94, 95). However, in the ewes, the initial inhibitory effects on LH secretion (96) was contradicted by a study performed by Decourt and colleagues (97), suggesting that RFRP-3 had no direct effect on LH release. Recently, RFRP-3 expressing neurons emerged as the primary central target for the inhibitory action of the melatonin signal after melatonin was reported have little or no effect on *Kiss1* neurons (98). RF-amide peptides are highly regulated by the melatonin-driven thyroid-stimulating hormone (TSH), which are critical for the control of seasonal breeding (99).

The neuropeptide oxytocin, which is involved in social bonding and sexual reproduction may also interact with *Kiss1* neurons. Central kisspeptin excitation of oxytocin neurons occurs in late pregnancy, and this excitation is likely to be mediated by a subpopulation of $Kiss1^{RP3V}$ projecting to the supraoptic nucleus (100). In contrast, the effect of oxytocin on $Kiss1^{ARC}$ neurons is unclear. One study revealed that intranasally applied oxytocin reaches the brain and oxytocin treatment at its highest dose increased the level of *Kiss1* and NKB mRNA in the anterior hypothalamus of female rats by approximately 400%, but *Kiss1* expression was unaffected by the lower doses of oxytocin. Also, elevated *Gnrh* mRNA expression following intranasally applied oxytocin was observed but the plasma LH concentration remained normal (101). *Kiss1* neurons have not been shown to express the oxytocin receptor; hence, the direct impact of oxytocin on *Kiss1* neurons within the anterior hypothalamus has yet to be confirmed.

McGowan and colleagues discovered a novel role for the neuropeptide relaxin-3 in the stimulation of the HPG axis *via* hypothalamic GnRH neurons (102), suggesting that this peptide may

play a role in coordinating feeding and reproductive responses in response to alterations in energy balance. ICV administration of human relaxin-3 (H3) in adult male rats significantly increased plasma LH and the effect was blocked by pretreatment with a peripheral GnRH antagonist. H3 stimulated the release of GnRH from hypothalamic explants and GT1-7 cells, which express relaxin/insulin like family peptide receptor 1 and 3 (RXFP1 and RXFP3). Immunohistochemical labeling of relaxin-3-expressing neurons in male rats indicated dense relaxin-3-immunoreactive fibers projecting to the ARC. These relaxin-3-expressing cells were derived from the nucleus incertus, pontine raphe nucleus, periaqueductal gray, and the dorsal area to the substantia nigra detected by *in situ* hybridization (103). Further studies are required to define the physiological importance of relaxin-3 in the regulation of reproduction, and the possible interaction with $Kiss1^{ARC}$ neurons.

Kiss1 NEURONS AND THE INTEGRATION OF ENVIRONMENTAL CUES

Many of the studies investigating $Kiss1^{RP3V}$ regulation have focused on factors that coordinate the LH surge with environmental cues, such as circadian inputs. Vasopressin (AVP) neurons from the suprachiasmatic nucleus (SCN) are the key SCN neurons regulating $Kiss1^{RP3V}$ *via* the V1a receptor and AVP-immunoreactive fibers project to $Kiss1^{RP3V}$ neurons (32). Vasopressin signaling is critically dependent on estrogen, in the presence of which vasopressin exerts a potent and direct stimulatory influence upon most $Kiss1^{RP3V}$ neurons (104, 105). Interestingly, estrogen permits circadian AVP signaling at $Kiss1^{RP3V}$ neurons without changes of AVP receptor signaling throughout the estrus cycle (105).

Seasonal breeders use photoperiod or day length as the primary environmental cue to time reproduction (106), ensuring birth occurs when environmental conditions favor the energetic demands of lactation and survival of offspring. The circulating levels of melatonin synchronize reproduction with photoperiod in these animals (98). These seasonal variations in reproduction are the direct result of changes in the neural network, specifically in (i) upstream neurons sensing the melatonin secretion from the pineal gland in response to photoperiod; (ii) neurons in the hypothalamus regulating GnRH and LH secretion (107). Ewes are short day breeders and remain in anestrus in long day conditions. Conversely, hamsters are long-day breeders (98). Both steroid-dependent and steroid-independent inhibition of gonadotropin secretion corresponding to photoperiod was demonstrated in sheep, Syrian and Siberian hamsters (108, 109). While $Kiss1^{ARC}$ neurons are the central site for the negative steroid feedback occurring in the breeding season, *Kiss1* expression is also inhibited by the short day melatonin signal (94, 110, 111). In the ewe, long days activate glutamatergic neurons that innervate A15 dopaminergic neurons in the retrochiasmatic area (112). Dopamine released from these neurons inhibits $Kiss1^{ARC}$ neuron activity, thus inhibiting GnRH/LH secretion and inducing infertility (113). In Syrian hamsters, long days stimulate the release of RFRP-3-expressing neurons in the DMH (114), which increases the activity of $Kiss1^{ARC}$ neurons. It is hypothesized that the increase in kisspeptin then stimulates GnRH secretion, causing testicular recrudescence and the resulting testosterone elevation stimulates $Kiss1^{RP3V}$ neurons (94). Conversely, kisspeptin appears to play no role in mediating the effects of photoperiod in male Siberian hamsters (115).

The hypothalamic paraventricular nucleus (PVN) contains a prominent population of corticotrophin-releasing factor (CRF) neurons, which regulate the hypothalamic–pituitary–adrenal axis (116). The role of PVN CRF in the control of LH secretion, however, is controversial even though it is known that stress responses can affect fertility. Central administration of CRH suppressed LH secretion in rats (117), whereas this is not the case in sheep (118, 119). Evidence of synaptic connections between CRF and GnRH neurons in the medial POA of rats (120) and the MBH of humans (121) indicate direct functional connection between CRF and GnRH neurons. Nevertheless, tract-tracing studies failed to find any CRF neurons projecting from the PVN to the POA where most GnRH neural soma are found in the rat (122). Stress-induced elevated CRF mRNA expression within the PVN did not correlate to LH pulse suppression (123) and finally, PVN lesions failed to interfere with the inhibitory effect of stress on LH release in rats (124). This suggests that CRF may act on one of the regulators of GnRH neurons rather than directly on GnRH neurons. Indeed, double-labeling immunohistochemistry revealed that most $Kiss1^{RP3V}$ and $Kiss1^{ARC}$ neurons in the female rat hypothalamus expressed the CRF receptor, CRHR. Close appositions of CRH-immunoreactive fibers on some of the $Kiss^{RP3V}$ and $Kiss1^{ARC}$ *n*eurons have been reported (125).

METABOLIC INTEGRATION *VIA* $Kiss1^{ARC}$ NEURONS

Reproduction is normally coordinated with nutritional status to ensure that pregnancy, parturition, and lactation occur during periods of ample food to maximize the survival of the individual and their offspring (126). One way in which this is coordinated is *via* the anorexigenic and orexigenic actions of proopiomelanocortin (POMC) and agouti-related peptide (AgRP) neurons, respectively within the ARC on $Kiss1^{ARC}$ neurons. POMC and AgRP neurons are the first-order sensors of peripheral metabolic signals, such as the leptin and insulin to maintain energy homeostasis. The adipose tissue-derived hormone, leptin and stomach-derived ghrelin have also been implicated to exert effects on $Kiss1^{ARC}$ neurons.

Alpha-Melanocyte-Stimulating (α-MSH) Hormone From POMC Neurons During Puberty

The role of α-MSH in the control of gonadotropin secretion in adults has been tested pharmacologically (127) and direct effects of α-MSH on GnRH neurons has been shown in adult mice (128). Central alpha-MSH can stimulate or inhibit LH secretion in rats depending on the hormonal milieu (129, 130). *Pomc* and *Kiss1* neurons appear to make mutual contacts in the adult ovine brain, α-MSH enhances *Kiss1* mRNA levels in the POA of sheep and decreases *Kiss1* expression in the ARC. Kisspeptin has been shown

to inhibit *Pomc* gene expression in the ARC of the sheep (131), while ARC POMC neurons displayed increased firing after kisspeptin stimulation in mice (132). The cocaine- and amphetamine-regulated transcript (CART), another neuropeptide co-expressed in most POMC neurons can directly stimulate $Kiss1^{ARC}$ neurons in female mice (133), implying a possible functional connectivity between POMC and $Kiss1^{ARC}$ neurons. Manfredi-Lozano and teammates (134) performed a comprehensive pharmacogenetic and optogenetic approaches where using expression analyses, electrophysiological recordings, and a chemogenetic approach to pinpoint the physiological role of leptin acting *via* an α-MSH-kisspeptin pathway in the metabolic regulation of puberty. $Kiss1^{ARC}$ neurons appear to transmit the stimulatory effects of melanocortin signaling onto the reproductive axis during puberty based on these data: (1) presence of appositions between α-MSH fibers and $Kiss1^{ARC}$ neuronal cell bodies of pubertal female rats; (2) reduction of *Kiss1* mRNA expression in the ARC of pubertal females subjected to chronic inhibition of melanocortin 3 and 4 receptors (MC3/4R); (3) significant attenuation of LH responses to α-MSH in mice with congenital inactivation of *Gpr54*; and, importantly, (4) reduced LH responses to α-MSH following chemogenetic inhibition of $Kiss1^{ARC}$ neurons.

Gamma-Aminobutyric Acid (GABA) From AgRP Neurons During Metabolic Deficiency

A seminal study by Padilla and colleagues indicated that AgRP-expressing neurons are activated during starvation and are involved in leptin-associated infertility during negative energy state (135). Using AgRP-neuron ablation and optogenetic strategies, they discovered inhibitory synaptic connections of AgRP neurons with neighboring $Kiss1^{ARC}$ neurons and rostral $Kiss1^{RP3V}$ neurons. The activated AgRP neurons release GABA, which has direct inhibitory actions on $Kiss1^{ARC}$ neurons. In agreement with this, $Kiss1^{ARC}$ neurons received less pre-synaptic inhibition in the absence of AgRP neurons after neonatal toxin-induced ablation. Chemogenetic activation of AgRP neurons as means of enhancing the activity of AgRP neurons over a sustained period is sufficient to perturb fertility *in vivo*. As a result, the animals exhibited delayed estrus cycles and decreased fertility (135). Interestingly, a direct, GABA-mediated connection between AgRP and GnRH neurons was not observed in this particular study despite the evidence that GnRH neurons are sensitive to a melanocortin agonist (136) and expressed NPY Y1 and Y5 (NPY1R and NPY5R*)* receptors (137). These findings confirmed that AgRP signaling contributes to infertility by inhibiting *Kiss1* during metabolic deficiency.

Leptin and Ghrelin

Leptin deficiency is associated with suppressed *Kiss1* expression in rodents and sheep, while leptin administration has been shown to increase *Kiss1* expression (131, 138, 139) as well as elevating LH pulse frequency, amplitude, and mean levels (140). Although $Kiss1^{ARC}$ neurons express the LepR, only a small fraction of $Kiss1^{ARC}$ neurons are responsive to leptin (141) and deletion of LepRs from *Kiss1* neurons resulted in no puberty or fertility deficits (83). To eliminate the possibility of developmental adaptations and system redundancies, the LepR was selectively re-expressed in *Kiss1* neurons of *Lepr*-null mice. These mice showed no pubertal development and no improvement of the metabolic phenotype: they remained obese, diabetic, and infertile (142). These findings clearly demonstrate that *Kiss1* neurons are not the direct target of leptin during puberty onset. Cravo and colleagues also confirmed that leptin signaling in *Kiss1* neurons occurs only after completion of sexual development (142).

In addition to its undisputed role in the regulation of metabolism and energy balance, increasing evidence shows that ghrelin can influence fertility. Studies conducted in several species, including rats, sheep, and humans, indicate that ghrelin administration suppresses gonadotropin secretion (143–145). The ghrelin receptor, GHSR1a (growth hormone secretagogue receptor) is present in several hypothalamic regions, including those known to be involved in the control of the reproductive function, indicating that this hormone can interact directly with hypothalamic neurons (146). Work by Frazao and colleagues (147) confirmed that ghrelin interacts directly with a subpopulation of $Kiss1^{ARC}$ neurons to modulate their activity and that exposure to estradiol increases the sensitivity of these neurons to ghrelin signals. The effects of ghrelin varies according to the estrogen milieu, as it exerts a more pronounced orexigenic effect in ovariectomized female rats and diestrus females when estrogen levels are low. Males with estradiol treatment are resistant to the stimulatory effects of ghrelin on food intake (148). The physiological relevance of ghrelin effects on $Kiss1^{ARC}$ neurons in food intake and metabolic regulation requires further investigation.

EXPERIMENTAL APPROACHES FOR INVESTIGATING FUNCTIONAL INPUTS TO $Kiss1^{ARC}$ NEURONS

Recently, methodological advances have allowed us to gain significant insights into upstream signals that converge on *Kiss1* neurons to modulate the reproductive axis. One approach has been to use of single cell RNA sequencing (scRNA-seq) to identify the gene expression profile of *Kiss1* neurons and thereby identify the repertoire of surface receptor that may mediate physiological responses (82, 149). This approach provides the most direct and unbiased method to define a cell type based on its transcriptional profile, which can provide additional insights into connectivity and function (150, 151). Two groups recently carried out single cell analysis of neurons from the hypothalamus or the ARC that included $Kiss1^{ARC}$ neurons (82, 149). Results from these studies provide extensive information about the neuropeptides, neurotransmitters, and receptors co-expressed in $Kiss1^{ARC}$ neurons, facilitating the assessment of crosstalk among different neuropeptide signals within the same cell.

Both scRNA-seq studies revealed high expression of *Slc17a6*, which encodes a vesicular glutamate transporter and *Tac2* (NKB) in $Kiss1^{ARC}$ cells. In parallel, with previous data from electrophysiology, *in situ* hybridization and immunohistochemistry studies, $Kiss1^{ARC}$ neurons have been shown to express the NK3R (*Tacr3)*, estrogen receptor 1 (*Esr1*), receptors for progesterone, prolactin, ghrelin, and the neuropeptide FF receptor 1 (27, 28, 74, 147, 152,

153). In addition, the nociceptin receptor (*Oprl1),* melanocortin receptors *(Mch3r and Mch4r),* NPY receptors *(Npy1r, Npy2r* and *Npy5r)*, thyrotropin-releasing hormone receptor (*Trhr*), and insulin receptor substrate 4 (*Irs4*); are among the receptors that were identified in the *Kiss1*^{ARC} single cells at low levels (149). These data suggest that *Kiss1*^{ARC} neurons may receive afferent inputs from neurons involved in nociception, energy homeostasis, insulin signaling, and TSH secretion. Interestingly, given that *Kiss1*^{ARC} single cells express four SST receptor subtypes (*Sstr1, 2, 3,* and *5*), the PACAP (*Adcyap1r1*), oxytocin (*Oxtr*), and RXFP1 (*Rxfp 1)* receptors (149), this information suggests a possible link between functional relevance of SST, PACAP, oxytocin, relaxin, and reproduction regulation *via Kiss1*^{ARC} neurons, at least in females.

One limitation of the scRNA-seq studies is that the experiments are often designed to generate gene expression profiles without appropriate considerations of neuroendocrine criteria. Cell samples may be pooled from several animals, combining males and females, and for female samples; the stage of the estrus cycle may not be considered. Nevertheless, these data are still valid as preliminary information about the range of receptors expressed in the *Kiss1*^{ARC} neural population. Specific scRNA-seq studies are necessary to further characterize the heterogeneity of the *Kiss1*^{ARC} neurons.

Another approach to investigate functional inputs to *Kiss1*^{ARC} neurons is to use powerful genetic methods in transgenic mice. The development of transgenic mouse lines with deletion of specific receptors in *Kiss1* neurons is a great tool in addressing the physiological relevance of these receptors. For example, mice have been generated in which the *Esr1* gene has been ablated in *Kiss1* neurons *via* a CRE-mediated recombination event (51), and these mice have defined the importance of sex steroid signaling in *Kiss1* neurons. Acute ablation of a gene from the earliest developmental time point, however, can sometimes be associated with compensatory changes in gene expression that can mask the effect of the gene disruption (154). If this occurs, a better alternative is to create a mouse model with inducible gene disruption to delineate the time windows in which gene inactivation is critical for the functional manifestation of a particular effect (155). Furthermore, varying the onset of gene manipulation at different time points and combining genetic manipulation with pharmacological or behavioral interventions will help to clarify gene–environment interactions that are crucial for the development or maintenance of reproductive phenotypes. The CRISPR/Cas9-based genome-editing tool implemented in mammalian cells has revolutionized gene-editing techniques (156). While this technique has generated huge impact on *in vitro* studies, progress is being made to also apply it *in vivo*. The combination of adeno-associated virus (AAV) and CRISPR/Cas9 system may be particularly useful in the future for editing reproductive-associated genes given that encouraging results have been obtained with the next-generation synthetic AAV capsids in several transgenic mouse models (157, 158).

A main objective in deciphering the neural circuitry is to define the synaptic inputs and outputs of specific neuronal subpopulations in different regions. Mapping the network of *Kiss1* neuronal inputs and outputs using a combination of molecular genetics and viral tract tracing techniques to provide both anatomical and functional circuit information is crucial. Until recently, the input–output relationships have been mapped using neuroanatomical tracers to reveal connections between regions (159). Classical tracers, such as biotin-dextran amine, fluorescent latex microspheres, fluorescent cholera toxin conjugates, or phaseolus vulgaris-leucoagglutinin, have provided very useful information to trace fibers in anterograde and retrograde directions, depending on the type of tracer applied (65, 160, 161), but they reveal only the axonal projections, not synaptic connections, and can be difficult to genetically target to specific neuronal types. Trans-synaptic tracing using retrograde viruses such as pseudorabies virus (PRV) is useful in revealing the brain regions forming synaptic connectivity with *Kiss1*^{ARC} neurons (162), but this technique is only limited to rodents. The PRV is contagious to domestic mammals as it causes Aujeszsky disease in cattle and swines. However, the major drawback of this technique is that the PRV crosses multiple synapses, making it difficult to distinguish the first order and higher order synaptic inputs unless the PRV is combined with other neuronal tracers. To overcome the limitations of PRV tracing, monosynaptic tracing using glycoprotein (G)-deleted rabies virus is now a widely adopted method to delineate brain-wide monosynaptic connectivity (163).

Intensive efforts are being made to delineate the complete wiring diagram or connectome of the mammalian brain as a means to better understand how neural circuits control behavior. High-throughput electron microscopy has been used to delineate microscale connectivity (164), while tracing strategies utilizing viral tracers encoded with fluorophores have allowed for milliscale circuit mapping (165). These studies have elegantly dissected a number of complex circuits. However, these methodologies are not designed to provide molecular information about the pre-synaptic neural populations. These shortcomings warrant the identification of marker genes for neurons within the circuits to enable the testing of their functional role.

While neuroanatomical methods enable high-resolution mapping of neural circuitry, these approaches do not allow molecular profiling of neurons based on their connectivity. An advanced approach for translational profiling of neurons based on connectivity using viral translating ribosome affinity purification (vTRAP) has been reported recently (166). In this approach, CRE-dependent AAV or other retrograde viruses (rabies or canine adenovirus) are engineered to express an EGFP-tagged ribosome protein enabling isolation of mRNA that is being translated from a discrete CRE-expressing neural population. Projection-specific translational profiling is achieved by selectively precipitating neuronal ribosomes based on connectivity. Quantitative PCR is then used for selected target genes or high-throughput RNA sequencing to determine the neuronal identity without the need for detailed anatomical or electrophysiological investigation (166). The drawback of this technique is that high-throughput RNA sequencing on the immunoprecipitated RNA is critical and a substantial amount of validations is required prior to selection of appropriate marker genes for the projecting neurons.

An elegant study performed by Nectow and colleagues also used the vTRAP method to delineate the dorsal raphe nucleus (DRN) circuit in regulating feeding (167). First, they used an unbiased approach to map sites of neural activation in response

to fasting, re-feeding, and hormonal cues, where subsets of DRN neurons were activated. Following this, comprehensive pharmacogenetic and optogenetic approaches were applied to carefully dissect the roles of DRN GABAergic neurons (DRN^{Vgat}) and glutamatergic neurons (DRN^{VGLUT3}) in modulating food intake. Transmembrane receptors that were enriched in both DRN^{Vgat} and DRN^{VGLUT3} neurons using the vTRAP method can be used for pharmacological screening of the ligands of these receptors. Finally, the effects of appropriate ligands for each receptor associated with the predicted response on feeding were tested using electrophysiological studies. This approach could also be used to molecularly characterize how the transcriptional profile of $Kiss1^{ARC}$ neurons changes in response to physiological stimuli.

Another powerful method to identify functional inputs into *Kiss1* neurons is to use optogenetic or chemogenetic approaches. These involve *a priori* identification of the specific pre-synaptic neural population as these methods required the use of neuron-specific CRE-expressing animal model. Both approaches involve the expression of non-native proteins that can function as channels (channel rhodopsin), pumps, or receptors in neurons. These novel proteins are sensitive only to exogenous non-native stimuli such as light or the compound clozapine-N-oxide (CNO). In this way, both approaches provide an exclusive selectivity for neuronal manipulations, enabling causal analyses of the roles of neural circuits in defined functions (168). Optogenetics allows millisecond-scale temporal accuracy in manipulating neuronal activity, which is critical for assessing circuit or behavioral functions that emphasizes on the rate or timing of neural activity. However, light activation of channel rhodopsin expressing neurons mainly release fast neurotransmitters such as glutamate and GABA; whereas neuropeptides require higher frequency and prolonged stimulations to be released. These factors may have explained the apparent lack of success for optogenetic release of neuropeptides (169). Alternatively, designer receptors exclusively activated by designer drugs that are sensitive to CNO and can be stimulated by a simple systemic CNO administration can be used. The main limitation of this approach is that their action is slow, in the order of minutes, rendering limited application in analyzing neural processes that rely on rate or timing of neural activity. But, this is ideal for examining the effects of chronic stimulation of neuronal populations.

To conclude, the plasticity or dynamics of the underlying kisspeptin–GnRH network in different physiological conditions is important. Relatively little is known about the role of kisspeptin under various physiological conditions such as negative energy state, lactation, and reproductive senescence. An excellent way to investigate the dynamics of the neural circuits connecting to $Kiss1^{ARC}$ neurons under these physiological conditions is to implement vTRAP on $Kiss1^{ARC}$ neurons followed by extensive chemogenetic and optogenetic strategies for causal analyses of the roles of specific neural circuits in defined behavioral responses or reproductive phenotypes.

CONCLUDING REMARKS

Precise control of gonadotropin release by the HPG axis is essential for sustaining fertility in all mammals (170). Therefore, the HPG axis must be able to respond to changes in endocrine, metabolic, or environmental cues to regulate GnRH/LH release. The $Kiss1^{ARC}$ neurons are positioned as an ideal hub receiving afferent inputs from other brain regions in response to the internal homeostatic and external signal. Our understanding of the neural networks connecting with $Kiss1^{ARC}$ neurons is limited, however. To precisely identify and functionally characterize specific synaptic inputs to $Kiss1^{ARC}$ neurons poses a challenge, given that: (1) $Kiss1^{ARC}$ neurons are heterogeneous; (2) estrogen may have an organizational effect on the inputs; (3) there could be an interplay between the $Kiss1^{RP3V}$ and $Kiss1^{ARC}$ neurons in fine-tuning pulsatile GnRH/LH release. The search for afferent inputs into $Kiss1^{ARC}$ neurons is continuing using new technologies to decipher the neural network associated with the *Kiss1*-GnRH system. Techniques such as trans-synaptic viral tracing, single cell RNA sequencing combined with optogenetics and chemogenetics to allow functional analyses are providing significant knowledge about the regulation of $Kiss1^{ARC}$ neurons. The precise mechanisms delineating how neuropeptides/neuromodulators regulate $Kiss1^{ARC}$ neurons and fine-tune GnRH/LH secretion requires further characterization and validation. Also, careful considerations need to be implemented to distinguish direct actions of the neuropeptides on GnRH neurons or effects mediated through $Kiss1^{ARC}$ neurons.

AUTHOR CONTRIBUTIONS

S-HY wrote the review with input from WC.

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REVIEW
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Metabolic Impact on the Hypothalamic Kisspeptin-Kiss1r Signaling Pathway

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A large body of data has established the hypothalamic kisspeptin (KP) and its receptor, KISS1R, as major players in the activation of the neuroendocrine reproductive axis at the time of puberty and maintenance of reproductive capacity in the adult. Due to its strategic location, this ligand-receptor pair acts as an integrator of cues from gonadal steroids as well as of circadian and seasonal variation-related information on the reproductive axis. Besides these cues, the activity of the hypothalamic KP signaling is very sensitive to the current metabolic status of the body. In conditions of energy imbalance, either positive or negative, a number of alterations in the hypothalamic KP signaling pathway have been documented in different mammalian models including nonhuman primates and human. Deficiency of metabolic fuels during fasting causes a marked reduction of *Kiss1* gene transcript levels in the hypothalamus and, hence, decreases the output of KP-containing neurons. Food intake or exogenous supply of metabolic cues, such as leptin, reverses metabolic insufficiency-related changes in the hypothalamic KP signaling. Likewise, alterations in Kiss1 expression have also been reported in other situations of energy imbalance like diabetes and obesity. Information related to the body's current metabolic status reaches to KP neurons both directly as well as indirectly *via* a complex network of other neurons. In this review article, we have provided an updated summary of the available literature on the regulation of the hypothalamic KP-Kiss1r signaling by metabolic cues. In particular, the potential mechanisms of metabolic impact on the hypothalamic KP-Kiss1r signaling, in light of available evidence, are discussed.

Keywords: ***Kiss1,*** **kisspeptin, Kiss1r, metabolism, reproduction, metabolic hormones, proopiomelanocortin, AgRP**

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INTRODUCTION

Kisspeptin (KP), a hypothalamic neuropeptide, and KISS1R/Kiss1r, the KP receptor, are the main components of an important hypothalamic signaling pathway (1, 2). KP and KISS1R are encoded by *KISS1* and *KISS1R* genes, respectively (3, 4). A large body of data has established an important role for the KP-Kiss1r signaling in the initiation of puberty in both non-primate and primate vertebrates

Abbreviations: KP, kisspeptin; GnRH, gonadotropin-releasing hormone; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; NPY, neuropeptide Y; AgRP, agouti-related protein; POMC, proopiomelanocortin; CART, cocaine- and amphetamine-related transcript; LepR, leptin receptor; IR, insulin receptor; GHSR, growth hormone secretagogue receptor.

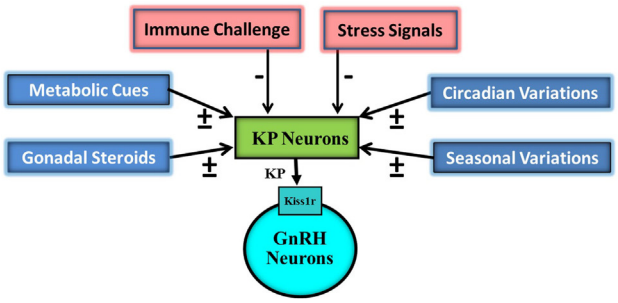

FIGURE 1 | Schematic representation of impact of various external and internal signals on the hypothalamic Kisspeptin (KP) system. KP neurons are targeted by gonadal steroids, metabolic, circadian, seasonal, immune, and stress signals. Some of gonadal steroids, metabolic, circadian, and seasonal signals result in upregulation (+) of KP expressions while others in downregulation. Immune and stress signals cause down regulation (–) of KP expression. KP neurons then on the basis of this information modulate pulsatile discharge of gonadotropin-releasing hormone (GnRH) from GnRH neurons.

TABLE 1 | Effect of different metabolic hormones and neuropeptides on the hypothalamic Kisspeptin (KP) system under different experimental setup in rodents and primates.

Hormone/ neuropeptide	Effect on KP	Experimental setup	Experimental model	Reference
Adiponectin	↓	*In vivo* and *in vitro*	Mouse	(58)
Leptin	↑	*In vivo*	Mouse and rat	(30, 31, 33)
Ghrelin	↓	*In vivo*	Mouse and rat	(80, 81)
Insulin	↑ =	*In vivo* and *in vitro*	Mouse and sheep	(30, 89)
Melanocortin	↑	*In vivo*	Mouse	(126)
Glucagon-like peptide 1	↑ =	*In vitro* and *in vivo*	Mouse	(128)

Increase (↑), Decrease (↓), no effect (=).

(5–8). Loss of function mutations in human *KISS1* or *KISS1R* genes causes absence of or delayed puberty (8–11), whereas a gain of function mutation in *KISS1R* gene results in precocious puberty (12). Likewise, administration of KP in immature rats elicits an early onset of puberty, whereas KP antagonist infusion leads to a delay in the achievement of pubertal hallmarks (5, 13, 14).

Kisspeptin signaling also plays an important role in the maintenance of the reproductive capacity in the adult (1, 15–17). Administration of KP, peripherally as well as centrally, has been reported to markedly increase systemic levels of reproductive hormones both in normal as well as subjects with reproductive insufficiency phenotype (7, 15, 18, 19). Due to their strategic position in the hypothalamus, the KP-containing neurons also act as a conduit for transferring information related to a number of different intrinsic and extrinsic cues to the gonadotropin-releasing hormone (GnRH) neurons (**Figure 1**). These neurons are involved in circadian and seasonal regulation of reproduction (20, 21). Moreover, this ligand-receptor pair acts as an integrator of the action of gonadal steroids and metabolic cues on the reproductive axis (22–26).

Proper functioning of the hypothalamic KP signaling is very sensitive to the current metabolic status of the body (23, 25) (**Table 1**). Conditions of energy imbalance, either positive or negative, induce a number of alterations in the hypothalamic KP signaling pathway in different mammalian experimental animal models (22, 26, 27). Deficiency of metabolic fuels during fasting causes a clear reduction of the *Kiss1* gene transcript levels in the hypothalamus and hence decreases the output of KP-containing neurons (5, 28). Food intake or exogenous supply of metabolic cues, such as leptin, overcomes metabolic insufficiency-related changes in the hypothalamic KP signaling (29, 30). Likewise, alterations in *Kiss1* expression have also been reported in other situations of energy imbalance like diabetes and obesity (30, 31). All these findings indicate a high sensitivity of KP signaling to alterations in the body's energy homeostasis. In this review, we summarize and discuss the presently available pieces of evidence indicating an impact of metabolic status-related cues on the hypothalamic KP-Kiss1r signaling in conditions of energy imbalance. We also discuss potential mechanisms of the transmission of the metabolic information on the hypothalamic KP system and ultimately reproduction.

SENSITIVITY OF THE HYPOTHALAMIC KP-Kiss1r SIGNALING PATHWAY TO METABOLIC ALTERATIONS IN CONDITIONS OF ALTERED ENERGY HOMEOSTASIS

The hypothalamic KP-Kiss1r system is highly sensitive to alterations in the metabolic cues levels in the systemic circulation. All sorts of metabolic perturbances exert negative impact on the *Kiss1* expressing neurons (**Figure 2**) (22, 23, 26, 27, 32). It is well established that reduction of metabolic fuels in food-deprived conditions causes a decrease in *Kiss1* transcript levels in the arcuate nucleus (ARC) (5, 28). In some conditions of energy imbalance, such as diabetes and obesity, very high energy reserves are present in the body, but due to the body's inability to properly utilize them, an attenuation of *Kiss1* mRNA expression was observed (30, 31, 33).

Both direct and indirect evidence suggests that deficiency in metabolic fuels severely affects the KP neuronal network in the hypothalamus. Short-term fasting-associated metabolic cues alterations lead to a marked reduction of hypothalamic *Kiss1* expression in prepubertal as well as adult rats (5, 29, 34–36). Castellano et al. (5) carried out a first comprehensive analysis of the short-term fasting impact on the hypothalamic Kiss1 system in prepubertal rats. In fasted rats, delayed puberty, as monitored by vaginal opening, was associated with a reduction of whole hypothalamic *Kiss1* gene transcript levels. However, *Kiss1r* mRNA expression was increased in these rats as compared to normally fed control animals. A possible explanation, as provided by authors (5), for this opposite change in Kiss1 and Kiss1r is that a major reduction in ligand (KP encoding gene) expression might cause a compensatory increase in the expression of its receptor gene, leading to a situation of sensitization to the effects of KP. Importantly, exogenous administration of KP

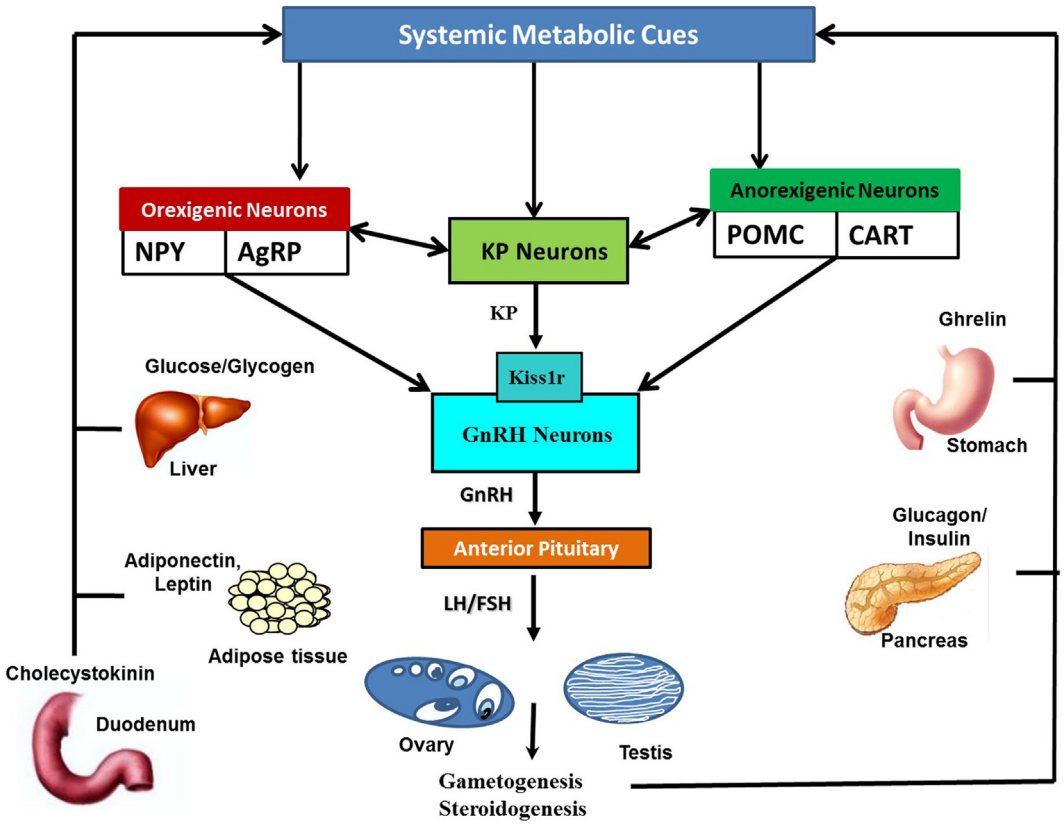

FIGURE 2 | Schematic representation of the interaction of systemic metabolic cues with Kisspeptin (KP), orexigenic, and anorexigenic neurons: metabolic cues are secreted by metabolic organs in responses to alterations in metabolic status. Metabolic cues include insulin and glucagon from pancreas, leptin, adiponectin and leptin from adipose tissues, ghrelin from stomach, glucose, fatty acid, cholecystokinin, glucocorticoids, and thyroid hormones, among many others. Alterations in metabolic cues, either directly or indirectly *via* anorexigenic and orexigenic neurons, modulate KP neuronal activities. KP neurons in turn transfer this information to the HPG axis *via* gonadotropin-releasing hormone (GnRH) neuronal network. Likewise, orexigenic and anorexigenic neurons can also directly convey current metabolic status related information to GnRH neurons.

not only rescues the suppression of the reproductive axis in these rats but also overcomes the negative energy balance-induced pubertal delay (5). This finding indicates that a proper reserve of energy is critical for the achievement of reproductive capacity at the time of puberty. The energy reserve related cues, in turn, communicate with the neuroendocrine center for the regulation of reproduction through the hypothalamic neural circuitry of KP neurons (37). Subsequent studies analyzed the impact of food restriction on the distinct hypothalamic KP neuron subpopulations in ARC and anteroventral periventricular nucleus (AVPV). In adult ovariectomized female rats, fasting decreased AVPV *Kiss1* mRNA levels, but not *Kiss1* mRNA expression in the ARC (34). In the intact adult female rats, food deprivation resulted in a prolongation of the reproductive cycle *via* a reduction in ARC *Kiss1* mRNA expression (34). However, these researchers did not observe any changes in AVPV *Kiss1* mRNA expression. Likewise, chronic food deprivation in pubertal female rats diminished expression of *Kiss1* in ARC, but not in the AVPV (38). In mice and rhesus macaques, in contrast to rats, the hypothalamic transcript levels of both *Kiss1* and *Kiss1r* are reduced by a 48-h fast (28, 30).

In addition to the aforementioned expression data, KP administration data also indirectly pinpointed a high sensitivity of the hypothalamic KP system to fasting-induced negative energy balance (5, 39). Administration of exogenous KP has been documented to overcome the negative energy balance-induced suppression of the reproductive axis, further advocating the idea that the endogenous KP system is negatively affected by fasting (5, 39).

Besides the condition of fasting, experimental data from other paradigms of energy imbalance such as diabetes, obesity, and lactation also indicate an impact of metabolic perturbations on the KP neurons output (30, 31, 40). The hypothalamic expression of *Kiss1* gene is significantly reduced not only in the rat model of diabetes but also in obesity rodent models (30, 31, 40). In both congenital leptin deficiency and high-fat-diet-induced models of obesity, Kiss1-expressing neurons output is greatly reduced (33, 40). Likewise, a reduction in *Kiss1* expression has also been reported in lactating female rats (41, 42). Moreover, exogenous administration of KP has been noted to rescue the energy imbalance impact on the reproductive axis (41, 42).

Taken together, the evidence summarized above strongly suggests a very high sensitivity of KP-containing neurons to metabolic alterations in the body.

MECHANISM OF METABOLIC IMPACT ON THE HYPOTHALAMIC KP SYSTEM

The exact mechanism by which changes in metabolic cues alter the hypothalamic KP system is still not fully clear. Available data suggests both direct and indirect mechanisms. Hypothalamic KPergic neurons can most likely sense metabolic cues directly because receptors for a number of peripheral metabolic hormones have been shown to be expressed by these neurons (22, 23, 25, 26, 32). Indirect sensing of metabolic status-related information is also possible because KP neurons receive information from various neuronal networks by direct cell-cell-communication, and neurons capable of sensing systemic metabolic cues are part of these networks (23, 25, 32, 43, 44). In this section, we summarize available data on both direct and indirect impact of metabolic cues on the hypothalamic KPergic neurons.

Direct Impact of Peripheral Metabolic Factors on KP Secreting Neurons in the Hypothalamus

Adiponectin

Adiponectin, a white adipocyte-secreted adipocytokine, was first documented in 1995 independently by various groups (45–48). It is a 244 amino acid protein hormone encoded by the *APMI* gene. It is secreted in very large amount into the systemic circulation. It has been noted to be about 0.01–0.05% of the total systemically circulating proteins (45–49). Systemic concentration of adiponectin is ranged from 3 to 30 μg/mL (45). Adiponectin levels are sexually dimorphic as its concentration is higher in females than in males (45). In various metabolic disorders, such as obesity and diabetes, a marked reduction in plasma adiponectin levels has been reported (49, 50). Nevertheless, its levels are markedly elevated during fasting and are positively associated with severe weight reduction although in these situations the body has a greatly reduced adipose tissue mass (51, 52). This elevation in plasma adiponectin levels during food restriction condition is caused by adipose tissue in bone marrow. In contrast to other parts of the body, a prominent increase in the mass of adipose tissue in bone marrow has been noted in food restriction conditions (53).

Adiponectin exerts its biological action *via* two 7-transmembrane receptors, AdipoR1 and AdipoR2 (45, 51), which are structurally as well as functionally different from 7-transmembrane G protein-coupled receptors. These receptors constitute a subgroup of 7-transmembrane receptors together with 11 progestin AdipoQ receptors (45). Besides peripheral organs, studies have demonstrated expression of both AdipoR1 and AdipoR2 in various brain regions, including the hypothalamus, although evidence for the transport of adiponectin across the blood-brain barrier is still lacking (54–56).

Binding of adiponectin to its receptor leads to the activation of 5' AMP-activated protein kinase (AMPK). The activated AMPK acts to regulate energy homeostasis of the cell *via* fatty acid oxidation and stimulation of glucose uptake (45, 49, 51). Moreover, adiponectin has been shown to modulate the release of reproductive hormones. Adiponectin inhibits LH, GnRH-stimulated LH, and GnRH secretion while no impact on follicle-stimulating hormone (FSH) secretion was noted (54, 56). Recently, Wen et al. (57). analyzed the adiponectin effect on hypothalamic *Kiss1* mRNA expression in GT1-7 cells, which are immortalized mouse hypothalamic neuronal cells, and *in vivo* in rats. They showed that adiponectin, as well as a synthetic activator of AMPK, greatly reduced transcription of *Kiss1* mRNA while inhibition of AMPK caused an increase in expression of *Kiss1* mRNA in both *in vitro* and *in vivo* studies. Taken together, these findings suggest a negative impact of adiponectin on the activities of KP-containing neurons. The negative impact of adiponectin on Kiss1 expression suggests that it might be involved in short-term fasting induced suppression of the reproductive axis. In fasting condition, an increase in systemic levels of adiponectin has been reported.

Leptin

Leptin is another important adipokine of white adipose tissue. In contrast to systemic adiponectin levels, leptin levels in the bloodstream are directly related to the body mass of adipose tissues. Leptin plays a vital role in the maintenance of energy balance in the body (58–60). One of the key functions of leptin is to communicate information on the body's current metabolic status to brain centers for energy homeostasis (61, 62). Systemic concentrations of leptin are reduced in food restriction conditions while food intake augments leptin concentrations (63). Available experimental data show that leptin is an important regulator of the metabolic deficiency/sufficiency-induced alterations in the neuroendocrine axes. Thereby, it also affects reproductive functions (37, 60, 64).

Besides peripheral reproductive organs, expression of the leptin receptor (LepR) has also been noted in several central neuronal networks in the hypothalamus, including KP-secreting neurons (33, 37). In situations of energy imbalance, low levels of leptin cause a clear reduction in *Kiss1* transcripts levels in the hypothalamus (28, 30, 31, 33, 40) while the elevation of systemic leptin concentrations *via* exogenous administration greatly ameliorates expression of *Kiss1* transcripts levels (30, 33). Similarly, ablation of leptin in *ob/ob* mice and hypoleptinemia in experimental diabetic rats diminish *Kiss1* mRNA expression while leptin infusion in both, ob/ob mice and in the rat model augments *Kiss1* transcript levels (30, 31, 33). Leptin can also indirectly change activities of KP-secreting neurons because many studies have reported the expression of LepR in numerous discrete regions of the hypothalamus (58). Important neuronal populations that express LepR include the GABAergic, neuropeptide Y (NPY), proopiomelanocortin (POMC), and agouti-related peptide (AgRP) populations (44, 58, 65, 66). These neurons are known to communicate with KP neurons (43, 44, 67). The indirect impact of leptin on KP neurons is supported by the evidence that exogenous leptin injection was unable to induce signal transducer and activator of transcription-3 (STAT3), a leptin action mediating intracellular signaling pathway, expression in KP neurons (65).

However, Donato et al. (68) have recently shown that hypothalamic KP neuronal LepR deletion did not change LH secretion. Likewise, re-expression of LepR on KP cells in LepR null mice also did not improve hypogonadotropic hypogonadism phenotype in these mice (69). These observations, together with above mentioned findings (28, 30, 31, 33, 40) of a pivotal role of leptin in KP secretion, suggest a potential developmental compensation or an indirect effect of leptin in modulating KP secretion in mice. Nevertheless, more studies are required in other species to further clarify the link between leptin and KP.

Ghrelin

Ghrelin, an orexigenic peptide hormone of the upper gastrointestinal track, is a ligand of growth hormone secretagogue receptor (GHSR), which is also a member of the seven transmembrane receptor family (70–72). Ghrelin has been implicated in the short-term regulation of food intake. The systemic concentrations of ghrelin increase at the preprandial time, whereas they decrease postprandially (70, 72, 73). In food restriction conditions, increased ghrelin levels in the circulation are associated with a decrease in reproductive hormones (74). Exogenous ghrelin administration rapidly induces food intake and inhibits the reproductive axis (70, 72, 74, 75). Besides short-term actions on food intake, ghrelin is also involved in the regulation of long-term body weight. Chronic administration of ghrelin increases the body weight through a number of mechanisms, including continuous stimulation of food intake, alterations in energy expenditure, and induction of adiposity (75). In mice, congenital loss of ghrelin or of the *GHSR* gene causes resistance to high-fat-diet-induced adiposity and weight gain (76, 77). Likewise, ablation of both ligand and receptor resulted in reduced body weight of mice, high energy expenditure, and increased motor activity on a standard chow without exposure to a high-fat diet (78). All in all, the available data pinpoint an important role of ghrelin in monitoring and transferring metabolic information to the brain centers implicated in the regulation of reproduction and intake of food intake.

Ghrelin acts centrally in the brain *via* GHSR in the hypothalamus to stimulate food intake and to alter reproduction (72, 75). Expression of GHSRs has been observed on a subset of *Kiss1*-expressing neurons. In 2009, Forbes et al. (79) reported a reduction in the hypothalamic transcript levels of *Kiss1* in response to an increase in circulating ghrelin levels either due to food deprivation or exogenous injection of ghrelin. Besides this direct action of ghrelin on the hypothalamic *Kiss1* gene expression, an indirect action *via* interneurons like the AgRP/NPY neurons (75), which will be discussed below, is also possible.

An important role of estradiol has been reported in the modulation of KP neuronal response to ghrelin by Frazao et al. (80). These researchers found that elevated levels of estradiol augment transcript levels of GHSR in the hypothalamic ARC. Moreover, an increase in the number of KPergic neurons responding to ghrelin was noted (80). Very recently, it has been reported that an increase in ghrelin levels during the short-term fasting condition leads to a stimulatory effect of central KP on growth hormone secretion. This effect has not been observed in normal fed condition. Moreover, a ghrelin receptor antagonist or a block of increase in its systemic levels abolishes this effect of KP on growth hormone secretion. On the basis of these findings, it has been proposed that central KP neuronal networks might transfer reproductive and metabolic status related cues onto the somatotropic axis thus causing a change in the release of growth hormone (81).

Insulin

Insulin, a metabolic hormone secreted by the pancreatic β cells, is involved in metabolic regulation of reproduction through actions on both central and peripheral components of the reproductive axis (82, 83). Central injections of insulin cause a dose-dependent attenuation in feeding and body weight (84). Ablation of insulin receptor (IR) from neurons results in hypogonadotropic hypogonadism in mice *via* central hypothalamic mechanism (85). Moreover, central injection of insulin has been reported to reinstate normal LH secretion in an experimental rat model of diabetes (86).

Besides many other neurons, expression of IRs has been noted on the ARC KP cells (87). It has been found that the specific deletion of the IR gene from KP neurons delayed the onset of puberty in mice but reproductive capacity was normal in adulthood (87). Therefore, these observations indicate that insulin signaling in KP neurons is important for the normal pubertal awakening of the reproductive axis but not an absolutely critical signal for the achievement of ultimate pubertal hallmarks. Additionally, reproductive ability, feeding, glucose regulation, distribution of fat, and body weight were normal in adult mutants. Of note, administration of insulin in the late follicular ovarian phase significantly stimulated expression of the c-fos protein in sheep ARC KP neurons (88), although it is not clear whether this effect is direct or indirect.

Some indirect evidence supports a possible role of insulin in altering the activity of hypothalamic KP-secreting neurons. In rats, experimental chronic diabetes has been noted to cause a marked reduction of *Kiss1* transcript levels in the hypothalamus (31, 89). Likewise, during short-term fasting, which is characterized by reduced levels of insulin, a reduction in *Kiss1* expression was reported (28, 90). However, exogenous injections of insulin did not reverse the decreased *Kiss1* gene expression, which was induced by fasting- and diabetes-associated metabolic perturbations (30, 31). Additionally, *in vitro* data did not show any effect of insulin on KP expression in the mouse hypothalamic cell line N6 (30). Of note, leptin and NPY applications have stimulated *Kiss1* expression in this cell line.

Indirect Impact of Metabolic Cues on the Hypothalamic KPergic Neurons

Besides the *per se* impact of metabolic cues on the hypothalamic KPergic neurons, a number of other hypothalamic neuronal networks are also sensitive to metabolic status-related cues. The major hypothalamic neuronal systems, which express the LepR, the GHSR, and the IR, include gamma-aminobutyric acid (GABA), glutamate, NPY/AgRP, and POMC/CART neurons. Many of these neurons, in turn, can alter activities of the hypothalamic KPergic neurons either directly or indirectly.

Glutamate and GABA Expressing Neurons' Input to KP Secreting Neurons in the Hypothalamus

Glutamate and GABA neurons are playing important roles in the regulation of reproduction (91). These neurons have been documented to contain receptors for metabolic hormones, and their activities are modulated by metabolic cues (44, 67, 92–94). In a recent study, we checked changes in the hypothalamic glutamate and GABA systems in fed and 48 hours fasted monkeys *via* checking transcripts levels of *Kiss1*, *Kiss1r*, *NR1* (*N*-methyl-D-aspartate receptor subunit 1) and *GAD67* (glutamic acid decarboxylase67) in the mediobasal hypothalamus (MBH) and pre-optic area (POA) of the adult male rhesus macaque (*Macaca mulatta*) (95). The expression of *Kiss1*, *Kiss1r,* and *NR1* mRNA was greatly decreased in fasted macaques as compared to *ad libitum* fed monkeys. A noteworthy reduction was also noted in the expression of KP and the interactions of NR1 with KPergic neurons in the hypothalamus of fasted monkeys. Taken together, these observations indicate that a reduction in inputs of glutamate-containing neurons to KPergic neurons may be responsible for the reduction in the hypothalamic KP signaling in the fasted monkey. However, no obvious change in expression of *GAD67* mRNA between fed and fasted monkey was observed, suggesting that the fasting-induced reduction in the hypothalamic KP signaling is not mediated through GABAergic neurons (95).

RFamide-Related Peptide-3 Expressing Neurons Input to KP-Secreting Neurons in the Hypothalamus

The hypothalamic gonadotropin-inhibitory hormone and its mammalian ortholog RFamide-related peptide-3 (RFRP-3) neurons have been implicated as the potent inhibitors of reproduction in a number of vertebrate species (26, 96–98). RFRP-3 binds to a G protein-coupled receptor namely GPR147. GPR147 is expressed in different regions of the hypothalamus including a subset of the hypothalamic KPergic neurons in the ARC (99). Moreover, a direct contact between GnIH fibers and about 35% of ARC KPergic neurons was also noted.

Different studies in animal models and human subjects analyzed of RFRP-3 effect on KP stimulation of GnRH (99, 100). In human subjects, although RFRP-3 exerts an inhibitory effect on LH secretion in postmenopausal women, no noteworthy effect of RFRP-3 was observed on KP-stimulated LH secretion in men during concomitant KP and RFRP-3 administration (100). In mouse hypothalamic explant culture, research from Tsutsui's group showed that RFRP3 significantly reduced KP-induced GnRH release (99). Of note, no effect of RFRP-3 on KP-induced GnRH release was noted in the mouse hypothalamic GT1-7 cells (101).

Leon et al. performed an analysis of the GPR147 ablation on the hypothalamic *Kiss1* mRNA expression (102). They reported that GPR147 null mice showed normal pubertal awakening of the reproductive axis. Of note, an increase in expression of *Kiss1* mRNA was noted in the hypothalamic ARC of the adult GPR147 null male mice. Additionally, an increase in systemic levels of FSH and response of LH to GnRH stimulation was observed in GPR147 null mice. However, ablation of GPR147 did not rescue hypogonadotropic hypogonadism in *Kiss1r*-ablated mice. More importantly, in the GPR147 null mouse energy imbalance conditions induced a lesser degree of disruption in the secretion of LH (102). These findings indicate that a lack of RFRP3 signaling may partly prevent metabolic perturbation induced inhibition of the reproductive axis. However, expression of *Kiss1* mRNA was not checked in GPR147 ablated mice in these conditions of metabolic perturbations. Therefore, it will be important to check *Kiss1* expression in GPR147 null mice in situations of metabolic insufficiency in order to know whether RFRP-3 signaling mediates nutritional challenge induced suppression of the reproductive axis.

Orexigenic Neuronal Input to the KP Secreting Neurons in Hypothalamus

Hypothalamic orexigenic neurons include NPY and AgRP neurons among others (103–108). AgRP is utterly secreted by a specific neuronal population in the ARC, which also co-expresses NPY. These neurons are playing a crucial role in feeding. They stimulate feeding when they are activated by metabolic deficiency-associated signals (105, 109).

These neurons express receptors for several key metabolic hormones like leptin, insulin, and ghrelin (103, 107, 109). Hence, AgRP/NPY neurons are direct targets of leptin action (109, 110) Exogenous injection of leptin induces a mark activation of STAT3, a prominent leptin action mediating intracellular signaling pathway, in AgRP/NPY neurons (109–111). Insulin has been noted to inhibit the electrophysiological properties of NPY/AgRP neurons. Insulin causes inhibition of NPY/AgRP neurons through activation of ATP-sensitive K^+ channels (112). However, ablation of IR from AgRP/NPY neurons does not induce prominent alterations of the reproductive axis, while deletion of both, IR and LepR, adversely affected the reproductive axis (113, 114). Ghrelin stimulates the activity of ARC AgRP/NPY neurons (75, 109, 115) *via* activation of GHSR present on these neurons (109, 116). More importantly, it has been reported that ghrelin's orexigenic effects are lost in *Agrp* and *Npy* knockout mice, suggesting that intact NPY and AgRP neurons are essential for orexigenic effects of ghrelin (115).

Although a large body of data established the perception of metabolic cues by the AgRP/NPY neurons, there is only very limited information on possible routes *via* which the effects are transmitted to KP neurons in the ARC. In the ovine brain, Backholer et al. (43) observed the occurrence of reciprocal trans-synaptic neural connections between the hypothalamic NPY-containing cells and the perikarya of the KP-expressing neurons. This anatomical evidence indicates that NPY-containing neurons can affect the output of KP neurons. More importantly, a normal NPY neuronal circuitry is essential for proper functioning of hypothalamic KPergic neurons, as mice with NPY deficiency have defective hypothalamic KP expression (30).

Recently, Foradori et al. provided more comprehensive evidence for cross-talk between KP and NPY neurons (81), especially from KP to NPY neurons in presence of the fasting-induced alteration in metabolic cues. KP administration in fasted ewe has been noted to cause a significant increase in growth hormone level *via* stimulation of NPY neurons and growth hormone releasing-hormone in ARC and an inhibition of somatostatin neurons in the periventricular nucleus.

Anorexigenic Neuronal Input to KP-Containing Neurons in the ARC

The hypothalamic ARC POMC (POMC)/CART (cocaine- and amphetamine-regulated transcript) neurons have been implicated as a pivotal central controller of metabolic homeostasis (109, 117, 118). These neurons have been described to constitute a major part of the hypothalamic satiety center. The anorexigenic role of POMC neurons is pinpointed by the evidence that ablation of the *Pomc* gene results in a state of severe hyperphagia, which ultimately leads to an enormous amount of weight gain (119). Moreover, food deprivation reduces mRNA levels of *Pomc* in the hypothalamic ARC, whereas transcript levels of hypothalamic *Pomc* are augmented in overfed rats (120). Similarly, *CART* mRNA expression is also at the nadir in fasting, while food intake restores ARC *CART* mRNA expression (121).

The possible metabolic cues that may be sensed by POMC neurons include leptin and insulin. Presence of both LepR and IR has been noted on POMC neurons (109, 117, 122–124). Recently, researchers documented *via* the whole-cell recording that both leptin and insulin excite POMC neurons and nearby KP cells *via* stimulation of TRPC5 (short transient receptor potential channel 5) channels (112), which are abundantly present in these hypothalamic neurons. Moreover, central administration of exogenous insulin greatly suppressed feeding and enhanced expression of the c-fos protein in ARC POMC neurons (112).

Indeed, POMC neurons are strategically located in the hypothalamus. Thereby, they can integrate the information provided by many different metabolic cues and can link these to the KP neurons. A direct action of POMC neurons is supported by the presence of reciprocal connections between POMC and KP neurons in the hypothalamus (43, 125). POMC neuronal projections were observed in close apposition with a number of other neurons which cross-talk with KP neurons. This also suggests an indirect connection.

Very recently, Tena-Sempere's group uncovered a melanocortin-KP-GnRH regulatory pathway (126). This pathway was reported to be involved in transmitting leptin actions and plays an important role in regulating the onset of puberty. Of important note, KP neurons were noted to play a vital role in relaying the stimulatory effects of melanocortin signaling onto the reproductive centers (126). In this regard, they reported the existence of a close contact between α-MSH fibers and KP-containing neuronal cell bodies in the ARC of pubertal female rats while the chronic block of the melanocortin receptor, MC3/4R, results in a significant reduction of *Kiss1* transcript levels. Moreover, the LH responses to the MT-II melanocortin agonist, which stimulates LH release, greatly reduced in Gpr54-ablated mice and also in DREADD-induced inhibition of ARC Kiss1 neurons. Altogether, these findings suggest central role KP in mediating impact of POMC neurons on to GnRH neurons during development and metabolic cues related changes.

Very recently, True et al. have reported several differences in coexpression patterns of various hypothalamic neuropeptides in female nonhuman primates as compared to rodents (127). They did not observe coexpression of CART with POMC but instead with NPY. They also noted co-expression of the CART in a subpopulation of KP cells. These CARP + KP neurons were noted to show close appositions with GnRH neurons. In contrast, the single-labeled KP and CART fibers were in synaptic contacts with GnRH neurons.

Heppner et al. (128) reported that KP neurons in the hypothalamic ARC receive synaptic input from glucagon-like peptide 1 (GLP-1), which is an anorexigenic neuropeptide. Moreover, KP neurons also express Glp1r mRNA. More importantly, they noted an increase in KP neurons action potential firing after application of the GLP-1R agonist. GLP-1R agonist also results in a direct membrane depolarization of ARC KP cells. However, central infusions of the GLP-1R antagonist, exendin (9–39), did not exert any effect on expression of ARC Kiss1mRNA or plasma LH in the normal fed mice (128).

CONCLUSION AND FUTURE RECOMMENDATIONS

In summary, emerging and increasing evidence indicates that metabolic cues exert a profound impact on the hypothalamic *Kiss1*-expressing neurons, both directly and indirectly. The direct sensing of metabolic cues is indicated by the presence of metabolic hormone receptors on *Kiss1*-expressing neurons while indirect sensing of metabolic information is suggested by cross-talk of these neurons with other hypothalamic neuronal populations which also respond to metabolic cues.

Most of the current evidence for the metabolic regulation of the hypothalamic KP system is provided by non-primate studies. Therefore, in the future, further studies in nonhuman primates are required to get more insight into the mechanism by which various peripheral metabolic cues (leptin, adiponectin, testosterone, estrogen, cortisol/corticosterone, ghrelin, insulin, glucagon, thyroid hormones, etc.) exert effects on their central neuronal targets (KPergic, AgRP/NPY, POMC/CART, GABA, corticotropin-releasing hormone, etc.). Indeed, deeper understanding of the metabolic impact on the hypothalamic KP signaling in animal models phylogenetically closer to humans and therefore with high clinical significance will more likely put Kiss1-Kiss1r signaling in the focus as a potential drug target. This may include improvement and management of reproductive functions as well as treatment of disorders of energy balance. Notably, an important role of KP has been shown in the restoration of the reproductive axis after its quiescence in metabolic disorders such as diabetes and hypothalamic amenorrhea (129, 130).

In 2014, Tolson et al. (131) have shown that ablation of KP signaling leads to a reduction in the body's metabolic activities. They also noted that a lack of the KP system leads to glucose intolerance and obesity (131). However, it is not known whether KP exerts an impact on metabolic activities peripherally or centrally or both. Therefore, it will be interesting to check the impact of various organ-specific knockdowns of KP signaling on metabolism. Very recently, De Bond et al. (132) compared the expression of different metabolically important genes, such as *Npy*, *Pomc*, *lepr*, *Ghsr* (ghrelin receptor), *Mc3r* (melanocortin

receptors 3), and *Mc4r* (melanocortin receptors 4). Unexpectedly, they observed no clear alterations in gonadectomized kiss1r-ablated mice compared to intact controls. These findings indicate that the etiology of obesity in the lack of KP-Kiss1r signaling may show an impairment in metabolic cues peripherally instead of central metabolic impairments (132).

AUTHOR CONTRIBUTIONS

FW and BA have written first draft of this review article and have drawn figures. FU, MS, and RB have edited and revised the review article. All authors have read and approved the final version of the manuscript.

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REVIEW
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The Emerging Role(s) for Kisspeptin in Metabolism in Mammals

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Kisspeptin was initially identified as a metastasis suppressor. Shortly after the initial discovery, a key physiologic role for kisspeptin emerged in the regulation of fertility, with kisspeptin acting as a neurotransmitter *via* the kisspeptin receptor, its cognate receptor, to regulate hypothalamic GnRH neurons, thereby affecting pituitary–gonadal function. Recent work has demonstrated a more expansive role for kisspeptin signaling in a variety of organ systems. Kisspeptin has been revealed as a significant player in regulating glucose homeostasis, feeding behavior, body composition as well as cardiac function. The direct impact of kisspeptin on peripheral metabolic tissues has only recently been recognized. Here, we review the emerging endocrine role of kisspeptin in regulating metabolic function. Controversies and current limitations in the field as well as areas of future studies toward kisspeptin's diverse array of functions will be highlighted.

Keywords: kisspeptin, obesity, pancreas, liver, adipocytes, mouse models

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INTRODUCTION

Historical Summary of Kisspeptin

The kisspeptin (*Kiss1*) gene was first identified in a screen of human genes that reduced the metastatic potential of human melanoma cells (1). Since its discovery, kisspeptin has been a focus of study for a series of different fields including cancer biology, reproductive neuroendocrinology, reproductive biology, and, most recently, metabolism. While the kisspeptin gene and its activation of the kisspeptin receptor (KISS1R) were initially characterized by cancer biologists (1, 2), in 2003, the study of kisspeptin accelerated following the demonstration by two groups of its essential role in regulating reproductive function (3, 4). This can be dramatically illustrated by performing a literature search for articles on kisspeptin from the years between 1996 and 2002 (18 articles) and the subsequent 7 years, 2003–2009 (458 articles), after the seminal studies from Seminara and de Roux. The studies from this latter period define a critical role for kisspeptin signaling in the regulation of GnRH neurons, demonstrating kisspeptin involvement with puberty (3, 4), mediating gonadal steroid hormone negative (5, 6) and positive (7, 8) feedback and serving as an afferent pathway for metabolic control of the reproductive hormone axis (9–11). Interest in kisspeptin has further accelerated in the past 7 years (1,540 articles) as novel peripheral roles for kisspeptin have been identified in both reproductive, metabolic, and developmental processes (12–14). The aim of this review is to provide a summary of studies describing a role for kisspeptin in the peripheral regulation of metabolism.

Kisspeptin and the KISS1R

The kisspeptin gene, located on chromosome 1 in human, was originally reported to encode a 145 amino acid preprotein (15), though recent updates to the human genome sequence include a one-bp change resulting in an earlier stop codon and indicating that the human protein product is likely

138 amino acids. The preprotein can be further processed to the biologically active 54 amino acid C-terminally amidated peptide (Kp54, metastin) that was demonstrated to activate the KISS1R previously referred to as the orphan receptor GPR54 (2, 16). In mouse, the *Kiss1* gene, as in human, is located on chromosome 1, and in rat, the gene is located on chromosome 13. However, for the rat and mouse genes, regulation is complicated by the expression of multiple splice variants, although, in both, the protein precursor is also processed to Kp54. For example, the rodent *Kiss1* gene (mouse gene shown in **Figure 1**) consists of a number of splice variants that produce the same protein product (17, 18), suggesting that key differences in cell-specific regulation may be mediated by alternative promoter elements. This has been borne out in studies which have defined cyclic AMP response element binding protein (CREB) (19) and estrogen receptor (17) regulation of the mouse *Kiss1* gene (**Figure 1**).

The *Kiss1* gene is expressed in a variety of tissues besides the brain (hypothalamus, amygdala) (20–23), including the liver (13, 24), testis (24–26), ovary (27, 28), fetal adrenal (12), heart (29) fat (24, 30, 31), and placenta (32). This provides a number of possibilities for sources of circulating kisspeptin, with strong experimental evidence suggesting that the liver and placenta can contribute to biologically significant levels in the circulation (13, 32).

The KISS1R gene (*Kiss1r,* also called *Gpr54*, *Axor12,* and hOT7T175) is a member of the G-protein-coupled receptor family and is located on chromosome 19 in human and chromosome 10 in mouse. It consists of five exons and encodes a 398 amino acid protein in humans and a 395 amino acid protein in the mouse (33).

The Kiss1R has significant homology in the transmembrane regions with the galanin receptors, yet has little affinity for galanin (34). Kiss1R was demonstrated to signal through a $G_{q/11}$-mediated pathway to increase intracellular Ca^{2+} (35, 36) and activate the extracellular signal-regulated kinase (ERK)-signaling pathway, stimulating GnRH secretion. Recently, in the GnRH neuron, KISS1R was also demonstrated to signal *via* a $G_{q/11}$-independent but β-arrestin-dependent pathway leading to the activation of ERK (37, 38).

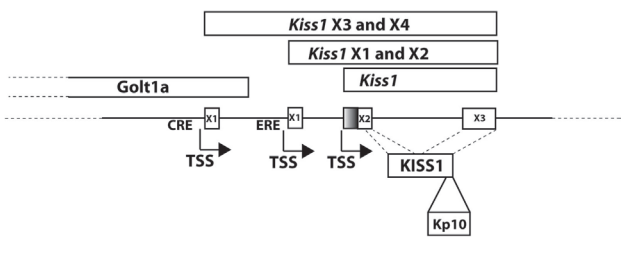

FIGURE 1 | Mouse *Kiss1* gene. Transcript variants of mouse *Kiss1* are expressed from two different first exons, X3 (XM_006529681) and X4 (XM_006529682), that are regulated by cyclic AMP response element binding protein at a CRE (19). The transcriptional start site for *Kiss1X*3 and X4 is located in an exon of the Golt1a gene. *Kiss1X*1(XM_006529679) and X2 (XM_006529680) are regulated by ESR1 at an ERE (17). *Kiss1* is a transcript including just the second and third exons (NM_178260.3). X1 and X3 include a larger second exon (including the shaded region) and X2 and X4 include a smaller second exon. All variants produce the KISS1 protein. KP-10 is the active region of all bioactive KISS1 peptides.

Besides the hypothalamus (39, 40), *Kiss1r* has been reported to be expressed in peripheral tissues including the pituitary, adipocyte, heart, spinal cord, gonads, and pancreas (13, 16, 29–31, 41–43). These findings suggest that a range of physiological systems may be impacted by kisspeptin.

Though not activated by galanin, the Kiss1R has been demonstrated to be activated by ligands other than the kisspeptins. RFamide-related peptide-3 (RFRP-3) and its receptor, neuropeptide FF receptor 1 (NPFFR1), are the mammalian orthologs of avian gonadotrope inhibitory hormone (GnIH) and its receptor GnIHR. RF9 is an antagonist to the GnIHR (44) that directly activates GnRH neuron firing (45) and LH secretion in a KISS1R-dependent manner (46). While kisspeptin independent activation of the KISS1R is documented, high levels of kisspeptin can also exert effects independent of the KISS1R (13) though the mechanism of action for these effects is not yet established. These data likely provide a biological rationale for the more severe reproductive phenotype observed in the *Kiss1R* KO mouse than in the *Kiss1* KO mouse (47).

Kisspeptin Neurons Mediate Central Regulation of Reproduction by Peripheral Metabolic Signals

Since the initial observations describing an essential role for kisspeptin signaling in puberty (3, 4), it has emerged that kisspeptin neurons are also relays of steroid feedback regulation to GnRH neurons (5, 6, 21, 22, 39, 48, 49) and are important components of the circuitry controlling GnRH pulse generation (50–54).

Kisspeptin neurons in the brain have also been proposed to integrate signals relaying peripheral metabolic status to the reproductive hormone axis, specifically to the GnRH neurons [reviewed in Ref. (9, 55, 56)]. There is evidence for changes in central kisspeptin expression both in response to food restriction (negative energy balance) or in genetic or diet-induced models of obesity (positive energy balance). However, to date, reports on the modulation of kisspeptin expression by caloric surfeit and obesity vary and are at times conflicting such that no firm consensus exists on the topic.

In studies on calorically restricted models, most, but not all, investigators report a reduction in *Kiss1* expression in both hypothalamic kisspeptin neuron populations. Long-term diet-restricted ewes were shown to express reduced *Kiss1* mRNA in both the ARC and POA when compared with normal weight ewes (57). And in fasted male mice, reduced hypothalamic *Kiss1* mRNA levels relative to fed controls were reported (58). In rats, one group reported 72 h of fasting caused a significant reduction in hypothalamic *Kiss1* expression in both males and females (59). However, another group observed no change in ARC *Kiss1* expression in response to a 48-h fast and a reduction in AVPV *Kiss1* mRNA only in ovariectomized/estrogen-replaced female rats (60). Clearly, more studies will be required to address the role of hypothalamic kisspeptin in mediating the suppression of the reproductive axis in states of negative energy balance.

In diet-induced obese female mice, *Kiss1* expression in both the AVPV and ARC decreases relative to normal chow-fed controls (61), perhaps contributing to a reduced reproductive function. And in a genetic model of obesity, the leptin-deficient

Ob/Ob mouse, ARC *Kiss1* mRNA levels are reduced (49, 61, 62) or unchanged (58) as compared to control mice. In the study by Smith et al., leptin treatment of Ob/Ob mice increased *Kiss1* expression, but this represented only a partial rescue of a reduced *Kiss1* expression reported for the Ob/Ob mouse (21, 22, 49). Leptin has long been known to play a permissive role in reproductive function (63) and signaling *via* the kisspeptin neurons, which may contribute to the functional regulation of fertility by leptin.

The gut-derived hormone ghrelin could also impact the reproductive axis *via* kisspeptin neuronal afferents, though here again, the evidence is not clear. Some investigators have suggested that only the AVPV KISS1 neurons are regulated by ghrelin (64), and others have also identified the regulation of ARC KISS1 neurons by ghrelin, interestingly with a strong interacting effect by E2 (65). Kisspeptin neurons have been shown to express the ghrelin receptor [GHSR (65)], though they are not thought to express the leptin receptor (62, 66), suggesting leptin-sensitive afferent neuronal regulation of kisspeptin and/or GnRH neurons (62, 66). ARC kisspeptin neurons send projections to the kisspeptin neurons in the AVPV (67, 68), suggesting that for some processes, a two kisspeptin neuron circuit could be required. Therefore, the relative role that ARC or AVPV kisspeptin neurons play in mediating metabolic signals is not clear (60, 61, 64, 69, 70).

The *Kiss1R* Knockout Mouse Exhibits Metabolic Dysfunction and Suggests a Key Role for Kisspeptin Signaling in Regulating Metabolism

Evidence for a broader role for kisspeptin signaling in the regulation of metabolism comes from detailed analysis of the KISS1R knockout mouse (*Kiss1r* KO) (71). These studies reported striking differences in body weight and glucose metabolism in female mice, but also differences in body composition and increased circulating leptin in both sexes. Because of the well-established obesity associated with a reduced estrogen signaling (72–74), Tolson et al. ovariectomized the female mice to assess those effects occurring independently of reduced estrogen. They found that a small, but significant, component of the obesity observed in female mice is due to loss of kisspeptin signaling and is not secondary to the reduction in estrogen levels, resulting from hypothalamic hypogonadism (71). However, *Kiss1r* KO males, because they exhibited no KISS1R-mediated weight difference, were not as carefully studied, despite having an increased adiposity and circulating leptin compared to controls. In addition, not reported in either sex was any evaluation of gluconeogenic capacity or whether there were differences in lipid metabolism despite an observed reduction in nocturnal respiratory exchange ratio (RER) in female KO mice compared to controls as assessed by indirect calorimetry. A reduced RER is suggestive of an increased use of lipids for energy metabolism (75). In follow-up studies in female mice, the Kauffman laboratory demonstrated that the changes in body composition, leptin levels, and RER were present in 6-week-old female KO mice, which preceded the increased body weight phenotype (76). The developmental progression of the phenotype observed in the male KO mice (increased adiposity, leptin levels, and reduced RER) remains still to be performed. The studies by the Kauffman laboratory have spurred a number of investigators to try to assess the tissue-specific mechanisms by which kisspeptin may regulate glucose and lipid metabolism, food intake, and body weight.

One possibility is that the body weight phenotype in Kiss1r KO mice is in part the result of an altered hypothalamic control of food intake or energy expenditure. Kisspeptin treatment alters both neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) neuronal activity in mice (77). In sheep, kisspeptin treatment increases *Npy* gene expression and decreases *Pomc* expression (57). Using optogenetic technology, ARC kisspeptin neurons were activated in mice and demonstrated glutamine, secreted from kisspeptin neurons regulation of both POMC and agouti-related peptide neurons. Interestingly, this resulted in the excitation of POMC neurons, *via* Gq/G11 coupled glutamate receptor and the inhibition of AgRP neurons, *via* a G_i/G_o coupled glutamate receptor (78).

To address whether the underlying cause of the obesity in female *Kiss1r* KO mice was at the level of the hypothalamus, De Bond et al. used quantitative PCR and *in situ* hybridization histochemistry to examine the expression of key genes in the hypothalamic appetite-regulating system, including *Pomc* and *Npy* as well as the genes expressing the receptors for leptin, ghrelin, and the melanocortins (79). They established that there were no differences in the expression of any of these genes between ovariectomized *Kiss1r* KO and ovariectomized control mice, suggesting peripheral sites of action of kisspeptin as potentially being a primary contributor to altered metabolism in the *Kiss1r* KO mouse. However, these sorts of assessments of mRNA levels do not preclude possible changes in neuronal activity and/or neurotransmitter release. They also do not fully address heterogeneity of the relevant neuronal populations and suggest that additional studies are needed to fully clarify a possible central role for kisspeptin signaling in energy metabolism. The following sections address the potential role of kisspeptin signaling in the major metabolic organs in the body, specifically the liver, muscle, fat, and pancreas.

Liver-Derived Kisspeptin Participates in Islet Hormone Cross-Talk

A long-standing question in pancreatic islet biology is how glucagon, produced in α-cells in response to low glucose levels, influences insulin secretion from β-cells that occurs in states of hyperglycemia. These considerations have clinical relevance since patients with type 1 diabetes mellitus (T1DM) exhibit hyperglucagonemia (80). In addition, many patients with type 2 diabetes mellitus (T2DM) exhibit elevated glucagon levels and insufficient insulin secretion to control glucose levels (81, 82). High glucagon levels are also apparent in prediabetic patients, who exhibit impaired glucose tolerance, suggesting that impaired glucagon suppression may contribute to the development of T2DM (83, 84).

The glucagon receptor is expressed on hepatocytes, where its activation rapidly stimulates cyclic AMP (cAMP) production (85), activating the PKA-signaling cascade. The PKA regulatory complex consists of two catalytic subunits (C) and two regulatory subunits (R). The C subunits are sequestered by the R subunits in

the absence of cAMP. Increases in cAMP result in the release of the C subunits which phosphorylate and activate CREB and increase the transcription of CREB-responsive genes. These include genes for the rate-limiting enzymes for gluconeogenesis, such as glucose-6-phosphatase (*G6pase*) or phosphoenolpyruvate carboxykinase (*Pepck*) (86–88), providing the adaptive response of an increased hepatic glucose production in response to hypoglycemia.

Constitutive activation of liver PKA-dependent signaling stimulates gluconeogenesis, leading to hyperglycemia, which would be expected to stimulate insulin secretion from β-cells. However, experimentally, the opposite has been observed. In 2005, the McKnight laboratory developed a mouse model with liver-specific expression of a mutant PKA C subunit (tryptophan 196 to arginine, called CαR) that exhibits impaired binding to the PKA R subunit and is thus less sequestered (inactive) in the absence of cAMP. The CαR mice did not have an increased expression of the gluconeogenic enzymes *G6pase* or *Pepck*, but did have reduced hepatic glycogen levels and were found to have modest hyperglycemia, but this was not associated with higher insulin levels, but rather with a reduced insulin secretion (89). Similar results were observed by our group using a more robust model of constitutive hepatic PKA activation, one in which the PKA R subunit gene is completely disrupted by Cre/LoxP technology (L-ΔPrkar1a mice). These mice have an increase in *G6pase* and *Pepck* gene expression in the liver causing fasting hyperglycemia and, notably, insufficient insulin secretion to correct glycemia during intraperitoneal glucose tolerance tests (13). These data suggest that an increased PKA signaling in the liver could be indirectly acting on pancreatic β-cells to suppress insulin secretion.

Evidence that a secreted factor was mediating this effect came from a bioassay in which plasma from L-Prkar1a mice suppressed insulin secretion from wild-type (WT) mouse islets *in vitro* (13).

To identify the factor, we compared hepatic gene expression in the L-ΔPrkar1a mouse with WT mice infused with glucose to achieve hyperglycemia equivalent to that in the L-ΔPrkar1a mouse. Of note, glucose-infused WT mice exhibited a robust and significantly elevated insulin secretion in contrast to the L-ΔPrkar1a counterparts. A liver gene expression array combined with bioinformatic analysis to identify genes for secreted proteins that were upregulated in the liver of L-ΔPrkar1a mice surprisingly yielded a single candidate gene, *Kiss1,* that was significantly upregulated in L-ΔPrkar1a liver (13). This result was confirmed by direct assessment of liver kisspeptin mRNA expression by quantitative PCR as well as kisspeptin protein levels by immunoblot.

Glucagon is secreted during fasting to participate in adaptive energy mobilization in the liver and fat. We demonstrated that *Kiss1* expression was increased in overnight fasted WT mice, but not in mice with a liver-specific deletion of the glucagon receptor gene. These results suggest that liver glucagon receptor activation can both stimulate insulin secretion by increasing blood glucose levels and inhibit insulin secretion by stimulating kisspeptin production.

To confirm the functional regulation of insulin secretion by the kisspeptin receptor, we used mice with selective ablation of the pancreatic *Kiss1r* gene (Panc-Kiss1R mouse) using the pancreas-specific PDX-1 CRE driver mouse and a *Kiss1r* floxed mouse that we developed (40). Acute treatment of control mice with kisspeptin preceding a glucose injection resulted in impaired glucose tolerance and attenuated insulin secretion, while Panc-Kiss1R mice injected with kisspeptin before glucose injection had glucose tolerance and insulin secretion similar to vehicle-injected mice (13).

Attempts to assess a role for kisspeptin on insulin secretion have yielded conflicting results with some noting that kisspeptin stimulates glucose-stimulated insulin secretion (GSIS) (90, 91) and others reporting the opposite (92, 93). We noted a wide range of concentrations for kisspeptin used in these various studies, with kisspeptin concentrations in the nM range usually suppressing GSIS (92, 93) and μM kisspeptin concentrations usually stimulating GSIS (90, 91, 94). To directly address this controversy, we tested different concentrations of kisspeptin on GSIS in islets from control and Panc-Kiss1R mice. We found that kisspeptin at nM concentrations suppressed GSIS from control islets but not from islets lacking the KISS1R. By contrast, kisspeptin at μM concentrations stimulated GSIS even in the absence of the KISS1R (Panc-Kiss1R islets). Based on these studies, it is clear that the suppression of GSIS by nanomolar concentrations of kisspeptin is mediated by the KISS1R. At supraphysiological levels, kisspeptin stimulates GSIS through a non-KISS1R-mediated pathway.

We assessed liver expression of kisspeptin in mouse models of obesity. Both high-fat diet (HFD) fed obese and genetic models of obesity (db/db and Ob/Ob mice) had an increased liver kisspeptin expression as well as increased circulating plasma kisspeptin concentrations (13). To assess whether these results translated to humans, liver biopsies taken from patients diagnosed with T2DM were analyzed and exhibited a higher kisspeptin expression than liver tissue from non-diabetic subjects. This was associated with higher circulating kisspeptin levels in diabetic subjects than in non-diabetic subjects (13). These findings suggest that in T2DM, kisspeptin production is elevated in the liver and that this increased kisspeptin production is secondary to increased glucagon levels, and, indeed, treatment with a glucagon receptor antagonist in db/db mice reduced liver kisspeptin production and improved glucose homeostasis (13).

Therefore, these data demonstrate the existence of a hepatopancreatic circuit in which glucagon, from the pancreas, stimulates hepatic expression of the genes regulating gluconeogenesis and kisspeptin. While the increased expression of *Pepck* and *G6pase* increases hepatic glucose output, increases blood glucose levels and stimulates insulin secretion, the increased secretion of kisspeptin serves to suppress insulin secretion (**Figure 2**). Kisspeptin could therefore be developed as a therapeutic in the treatment of some metabolic disease.

Placenta Is a Major Source of Circulating Kisspeptin in Humans

Our data suggest that the liver contributes to circulating levels of kisspeptin that in metabolically challenged states can increase 2- to 10-fold above basal (13); however, these levels are far lower than those secreted in women at the end of pregnancy by the placenta [elevated nearly 10,000-fold (32)]. This dramatic increase has been corroborated in a recent study in which urine kisspeptin levels were over 200-fold higher in third trimester pregnant women than in non-pregnant women (95). Kisspeptin

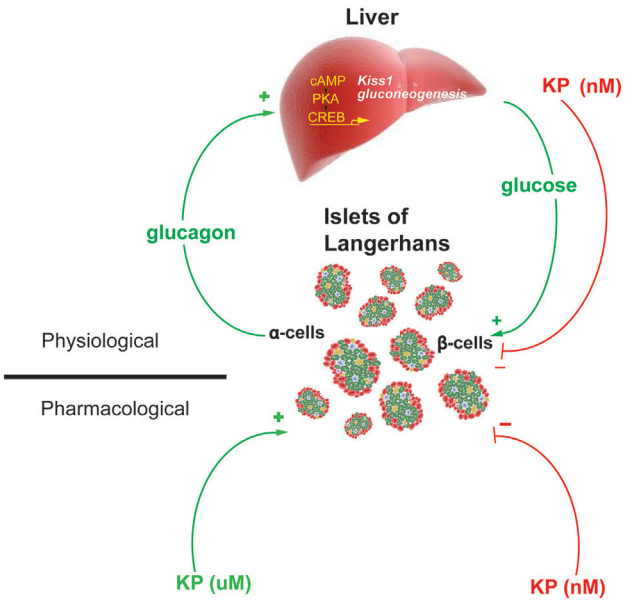

FIGURE 2 | Model of the proposed Hepato-Islet circuit. Glucagon secreted from the pancreatic α-cell activates its receptor on the liver increasing PKA activity and gene expression of gluconeogenic genes and the *Kiss1* gene. Increased glucose output from the liver increases blood glucose levels, increasing insulin secretion from the pancreatic β-cells. Increased kisspeptin secretion from the liver acts to suppress glucose-stimulated insulin secretion from the pancreatic β-cells. Pharmacologically delivered kisspeptin can stimulate (μM levels) or inhibit (nM levels) GSIS. Figure adapted from Song et al. (13).

levels decline rapidly after delivery, supporting the placenta as the source (32). Human term placenta was analyzed for *Kiss1* mRNA by *in situ* hybridization and for kisspeptin by immunohistochemistry, and kisspeptin expression in the outer syncytiotrophoblasts was reported, ideally located for secreting kisspeptin into the maternal circulation (32). The authors noted that there are similarities between invasive placental cells and invasive cancer cells (32), and during the establishment of the maternal–fetal interface, it is important to limit the interaction between the trophoblasts and uterine cells. Perhaps, kisspeptin plays a role in this process, mirroring its originally defined role as a metastasis inhibitor (1). Therefore, the increase in the production of kisspeptin in syncytiotrophoblasts in the first trimester may also play a role in negatively regulating trophoblast invasion, and kisspeptin signaling might be required for implantation and placentation (96).

A metabolic role for placentally derived kisspeptin can also be envisioned. During normal human pregnancy, insulin resistance develops and peaks during late pregnancy [34–36 weeks of gestation (97)]. This may be an adaptive response to preserve a slight excess of energy substrates in the blood for use by the developing fetus. A number of hormones have been proposed to contribute to the development of insulin resistance, including human placental lactogen, human placental growth hormone, progesterone, cortisol, tumor necrosis factor α, and leptin (97). One could envision two possible explanations for the high kisspeptin levels late in pregnancy. If kisspeptin is acting *via* the KISS1R, it would serve to tamp down increased insulin secretion to maintain a modest excess in the blood levels of energy substrates (glucose, free fatty acids). Intriguingly, insulin sensitivity recovers very quickly after delivery in parallel with decreasing kisspeptin levels (32, 97). One could also propose that the very high levels of kisspeptin circulating late in pregnancy could act *via* a kisspeptin receptor independent mechanism and serve to augment insulin secretion to compensate for the increased insulin resistance in late pregnancy.

Further investigation of placental kisspeptin will be difficult using many animal models. Very modest levels of placental kisspeptin expression have been reported in the rat, dog, and mouse (98–100). And while a gestational increase in kisspeptin expression was noted for mouse (99) and dog (98), circulating levels of kisspeptin are unlikely to reach the levels observed in humans and may not play the same role in these animal models as compared to humans.

Fat May Also Be a Source of Circulating Kisspeptin

The adipocyte could also be a source of circulating kisspeptin. *Kiss1* mRNA has been detected in rat adipose tissue (30, 31), and food restriction increased Kiss1 mRNA in the fat of both male and female rats (30). T1DM but not T2DM was associated with roughly 100-fold higher *Kiss1* mRNA levels in adipocytes compared to non-diabetic rats (24), suggesting that insulin plays a key role in the regulation of adipocyte *Kiss1* expression. Interestingly, the large increase in *Kiss1* mRNA was not associated with increased kisspeptin protein levels in the adipocytes of T1DM rats, suggesting either increased secretion or reduced protein translation. By contrast, Kiss1 mRNA was reduced in obese HFD fed and obese Zucker rats (30). The Wilkerson group also demonstrated a sex steroid regulation of adipocyte *Kiss1* expression. Estradiol stimulated expression in female adipocytes and testosterone stimulated expression in male adipocytes (30).

The adipocyte also expresses the KISS1R (101). Therefore, kisspeptins secreted by adipose tissue could either act as adipokines or as autocrine/paracrine regulators of adipocyte function. To explore kisspeptin's effects in fat, 3T3-L1 and primary rat hepatocytes were treated with Kp10 and lipid metabolism, glucose uptake and leptin and adiponectin secretion assessed (101). These studies demonstrated that Kp10 reduced adipogenesis in 3T3-L1 cells, likely as a result of a reduced expression of peroxisome proliferator-activated receptor gamma (PPAR-γ) and CCAAT/enhancer binding protein beta (CEBPβ), transcription factors involved in stimulating adipogenesis. Kp10 increased lipolysis in 3T3-L1 cells and rat adipocytes by enhancing the expression of periliphin and hormone-sensitive lipase and decreased glucose uptake and lipogenesis. Kp10 also stimulated the secretion of leptin and decreased the secretion of adiponectin from rat adipocytes. While these studies suggest a role for kisspeptin in regulating adipocyte development and function, the effects were largely seen at near μM levels of Kp10, calling into question the physiological relevance of the findings. It is possible that local levels of paracrine/autocrine secretion of kisspeptin could reach these levels, or that the very high levels of kisspeptin observed during human pregnancy could achieve levels that functionally

regulate fat, although this is unlikely to play a role in mouse or rat given the relatively modest levels of kisspeptin during gestation in these rodent models.

Human fat has also been demonstrated to express *Kiss1* (31). In women, a positive correlation between *Kiss1* mRNA in visceral adipose tissue and body mass index (BMI) was reported (31). Exclusions for this study included women under 19 years old and those that were post-menopausal. Not excluded were subjects with diabetes. These findings agree with our observation that circulating kisspeptin levels are increased 2- to 4-fold in HFD fed and db/db obese mice and nearly 10-fold in humans with T2DM when compared to lean mice and non-diabetic humans, respectively (13). These findings are at odds with the rodent data showing a reduced *Kiss1* expression in obese rats (30), and they also appear to differ from studies showing decreased circulating kisspeptin levels in obese patients with BMIs above 35 kg/m^2 when compared to non-obese controls with BMIs below 25 kg/m^2 (102). However, the high BMI subjects in the Kolodziejskii study specifically excluded those with diabetes and they would not exhibit hyperglucagonemia and the resulting increased hepatic *Kiss1* expression (102).

The contribution of fat to circulating levels of kisspeptin is unclear, making it difficult to discern whether kisspeptin from fat serves as an endocrine factor. A cell-specific KO of the kisspeptin gene from adipocytes would help address this question. These studies may ultimately demonstrate an exclusively paracrine/autocrine role of kisspeptin in fat regulation.

Other Potential Effects of Kisspeptin on Peripheral Metabolic Function

Evidence for the muscle as a target or a source of kisspeptin is limited. While skeletal muscle has not been demonstrated to synthesize kisspeptin or express significant levels of the KISS1R, there is evidence that smooth and cardiac muscles are regulated by kisspeptin.

Kisspeptin receptor has been localized in cardiomyocytes as well as the smooth muscle cells of the intramyocardial blood vessels (29, 103), and kisspeptin has been demonstrated to induce inotropic actions on cardiac function with the effects confirmed to be mediated by the KISS1R (29). The relevance during normal

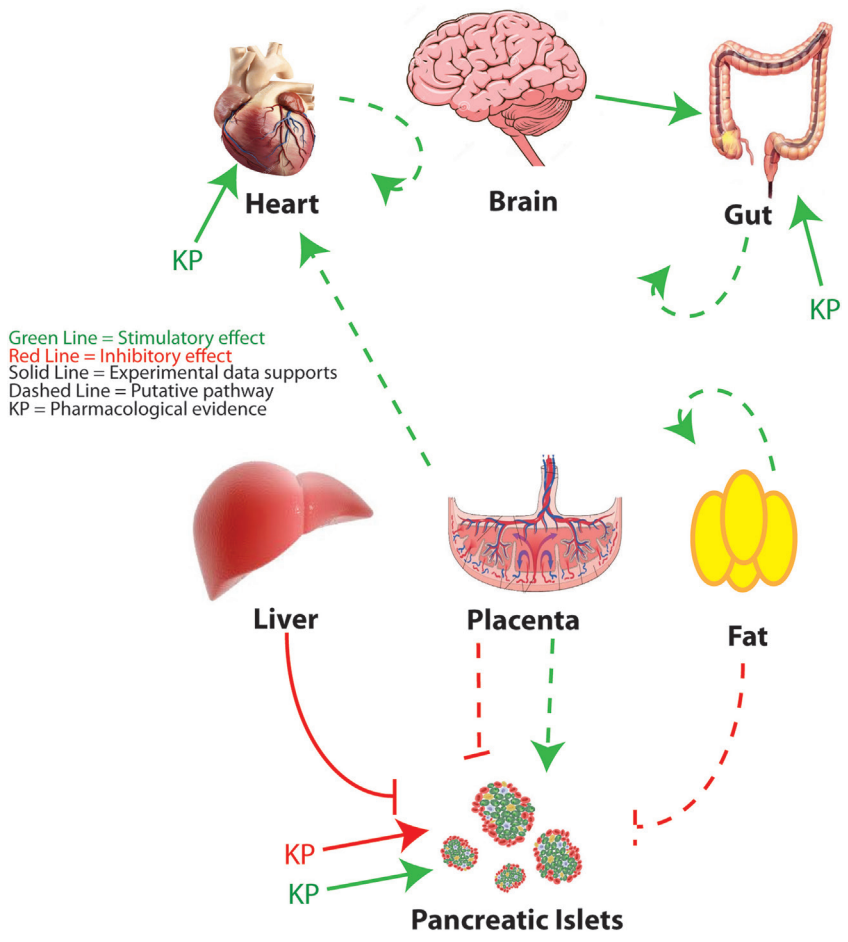

FIGURE 3 | Peripheral metabolic regulation by kisspeptin. An overview of kisspeptin regulatory pathways is discussed in this review. Red lines indicate putative suppressive effects. Green lines indicate putative stimulatory effects. Solid lines indicate that specific experimental evidence is provided to support the pathway. Dashed lines indicate a speculative pathway based on the available evidence. KP indicates targets for which pharmacological roles for kisspeptin have been proposed. Figure of brain adapted from Dreamstime.com.

physiology is unclear, however, since no cardiac dysfunction is reported in either humans or mice lacking the KISS1R (29). It was proposed that the high levels of kisspeptin secreted from the placenta could play a role in the adaptive increase in cardiac output during pregnancy (29, 104). However, the local expression of kisspeptin-like immunoreactivity was noted in human, mouse, and rat vascular and endocardial endothelial cells and in human cardiomyocytes (29) and could also be a source of high kisspeptin levels. Local secretion of kisspeptin could be a mechanism for intracardial regulation of cardiac output. Interestingly, the level of kisspeptin immunoreactivity was significantly lower in the right atria of patients transplanted for ischemic heart disease when compared to controls. While these changes could be secondary to low oxygen levels, it could suggest a possible role for kisspeptin in maintaining proper blood flow to the heart during atherosclerotic arterial narrowing.

Regulation of gut motility also contributes to metabolic status. A recent report suggests that kisspeptin can stimulate gastrointestinal motility by both central and peripheral mechanisms (105). While ICV infusion of kisspeptin stimulated gastrointestinal motility and fecal output at low nM concentrations, kisspeptin also exerted direct effects on the contractility of the circular smooth muscle of the colon. However, the peripheral effects of kisspeptin in the colon were only apparent at μM concentrations and could indicate a non-KISS1R-mediated mechanism of action such as observed for the effects of high kisspeptin concentrations on the beta cell (13). Local secretion of kisspeptin could achieve μM levels and represent an endogenous regulatory mechanism in the gastrointestinal system. Alternatively, these studies may help define a therapeutic role for pharmacological kisspeptin.

Kisspeptin does not appear to directly impact energy metabolism of skeletal muscle, but the literature does indicate a potentially important role on cardiac function and gut motility. Leveraging conditional knockout mouse models of both kisspeptin and the KISS1R will be required to fully understand kisspeptin's role in regulating heart contractility and gut motility.

Summary and Conclusion

As the study of kisspeptin enters its third decade, and new functions are attributed to the peptide, more animal and human studies are needed to understand its complex pleiotropic effects. The widespread expression of kisspeptin and its receptor indicates an ever-expanding array of roles in normal physiology, but also during the extreme physiological, developmental, and metabolic challenges of pregnancy or in pathophysiological states such as diabetes (**Figure 3**). In reviewing the literature, several challenges emerge. The first is that a spectrum of kisspeptin doses is being used, both *in vivo* and *in vitro*, and more attention needs to be paid to whether the effects of kisspeptin are physiological or pharmacological. This is not meant to disparage the latter since there is evidence that kisspeptins show a therapeutic potential in a variety of systems. A second challenge is trying to understand whether kisspeptins' effects are being mediated by the kisspeptin receptor and, if not, to determine those mechanisms of action not mediated by the cognate receptor. When possible, it is invaluable to validate the mechanism of action using the Kiss1R KO mouse (13, 29). The development of novel mouse models, including mice with floxed alleles of both the kisspeptin (in development) and KISS1R (40, 106) genes and optogenetic tools to assess neurobiological circuitries (50, 51, 107), will help define the sources of kisspeptin and the relevant sites of action. However, the development of additional animal and human models will be imperative to adequately study phenomenon not recapitulated in rodent models.

AUTHOR CONTRIBUTIONS

AW and MH jointly conceived of and wrote the manuscript.

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Kisspeptin and Metabolism: The Brain and Beyond

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Apart from the well-established role of kisspeptin (Kp) in the regulation of reproductive functions, recent data described its action in the control of metabolism. Of particular interest for the review is the population of Kp neurons localized in the arcuate nucleus (ARC) of the hypothalamus, the site of the brain where reproductive and metabolic cross talk occurs. However, within the hypothalamus Kp does not work alone, but rather interacts with other neuropeptides, e.g., neurokinin B, dynorphin A, proopiomelanocortin, the cocaine- and amphetamine-regulated transcript, agouti-related peptide, and neuropeptide Y. Beyond the brain, Kp is expressed in peripheral tissues involved in metabolic functions. In this review, we will mainly focus on the local action of this peptide in peripheral organs such as the pancreas, liver, and the adipose tissue. We will concentrate on dysregulation of the Kp system in cases of metabolic imbalance, e.g., obesity and diabetes. Importantly, these patients besides metabolic health problems often suffer from disruptions of the reproductive system, manifested by abnormalities in menstrual cycles, premature child birth, miscarriages in women, decreased testosterone levels and spermatogenesis in men, hypogonadism, and infertility. We will review the evidence from animal models and clinical data indicating that Kp could serve as a promising agent with clinical applications in regulation of reproductive problems in individuals with obesity and diabetes. Finally, emerging data indicate a role of Kp in regulation of insulin secretion, potentially leading to development of further therapeutic uses of this peptide to treat metabolic problems in patients with these lifestyle diseases.

Keywords: kisspeptin, obesity, diabetes, undernutrition, metabolism, reproduction

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DOUBLE BURDEN OF MALNUTRITION AS A SERIOUS WORLDWIDE CONCERN

A serious public health challenge facing many countries worldwide, recognized by the World Health Organization (WHO), is the double burden of malnutrition. It is characterized by the coexistence of contrasting forms of malnourishment: undernutrition along with overweight (1). Actions proposed by the WHO include interventions, programs and policies to reduce the risk or this burden. Understanding the neuroendocrine background for such conditions could lead to improvement of therapeutic strategies.

KISSPEPTIN (Kp) AS A POSSIBLE LINK BETWEEN METABOLISM AND REPRODUCTION

All organisms need food to meet their functional requirements for immediate metabolic needs and conserve energy excess in the form of fat for times of food scarcity (2). Another crucial and energy demanding process, i.e., reproduction, is needed for the perpetuation of species (3). Kp,

encoded by *KISS1* in the human and non-human primates and *Kiss1* in non-humans, its receptor *KISS1R* and *Kiss1r* or *GPR54*, respectively, are involved in the regulation of the hypothalamic–pituitary–gonadal (HPG) axis (4, 5). Importantly, energy metabolism is tuned to distinct sex-specific functions. Whereas in males metabolism may represent a default state, in females it is linked to specific requirements during gestation, parturition, and lactation (6). The arcuate nucleus (ARC) of the hypothalamus, expressing numerous neuropeptides including Kp, has been an area of focus for both circuits regulating metabolism and reproduction (7, 8).

ROLE OF Kp AND OTHER NEUROPEPTIDES IN METABOLISM CONTROL: FOCUS ON THE BRAIN

Extensive data support a major role of the ARC Kp neurons population in conveying information on metabolism to the gonadotropin-releasing hormone (GnRH) neurons, localized at the apex of the HPG axis (9). Disturbances in positive and negative energy balance often result in impairments of fertility, such as hypothalamic hypogonadism, frequently found in case of obesity and diabetes (10–12). However, Kp does not work alone, as Kp immunoreactive (-ir) neurons in the ARC also express neurokinin B (NKB) and dynorphin A (DYN A) and are termed KNDy neurons, and their degree of colocalization varies among species (13–18). NKB belongs to tachykinins, is encoded by the *Tac2* or *TAC3* gene (in rodents and humans, respectively), and works *via* the NK3R receptor [Tacr3 encoded by *Tacr3* and *TACR3*, animals and human genes, respectively (19)]. DYN A is an endogenous opioid (encoded by *pDyn* and *PDYN*, animal and human genes, respectively) working alongside the kappa-opioid receptor, encoded by the *Oprk1* in animals and *OPRK1* in humans genes (13, 20). KNDy neurons regulate the secretion of GnRH, while NKB stimulates and DYN A inhibits Kp (21–24).

Neuropeptide expression in KNDy neurons depends on the metabolic status. In chronically obese female mice a decrease was observed in *Kiss1* mRNA in the ARC (25), while fasting reduced *Kiss1* mRNA expression in the hypothalamus in rats and mice (10, 26, 27). In diabetic male rats, a decrease in *Kiss1* mRNA levels was found in the hypothalamus and a lack of an increase in *Kiss1* mRNA after gonadectomy [GDX (28)]. Finally, an increased number of Kp-ir neurons and an altered response after GDX in the ARC were reported in streptozotocin (STZ)-induced diabetic rats (11). Current studies in our laboratory are exploring the effects of obesity, diabetes and GDX in female rats (29).

Neurokinin B expression is also dependent on the metabolic status, as in pubertal female rats fed high-fat diet (HFD) higher expression of *Tac2* mRNA in the ARC was found (30). In db/db diabetic mice, *Tac2* mRNA levels increased in the hypothalamus (31), while in diabetic type 2 (DM2) male rats the number of NKB-ir neurons in the ARC increased (11). By contrast, under fasting conditions in female rats the expression of *Tacr3* and *Tac2* mRNA in the ARC decreased (19). The metabolic status was found to influence the DYN system, with an increase in DYN concentrations in the dorsomedial hypothalamic nucleus reported in obese ob/ob mice (32). In HFD-fed adult mice and obese rats, no changes were observed in prodynorphin (*pro-Dyn*) expression in the ARC (33, 34). In STZ-induced diabetic rats, an increase in *proDyn* mRNA and protein level in the hypothalamus was shown (35, 36), while DM2 male rats had higher numbers of DYN A-ir in the ARC (11). Food restriction increased *proDyn* mRNA levels in the lateral hypothalamus (**Figure 1**) (37).

These results indicate effects of the metabolic status on the expression of KNDy peptides; however, they may depend on species, strains, sex of animals, as well as the experimental design, e.g., diet composition and duration or diabetes induction methods.

Besides KNDy neurons, in the ARC two other neuron populations may be identified as a link between metabolism and reproduction: cells expressing proopiomelanocortin (POMC) and the cocaine and amphetamine-regulated transcript (CART) and a population expressing agouti-related peptide (AgRP) and neuropeptide Y [NPY (38, 39)]. First, while POMC neurons are suggested to decrease food intake, and catabolic and anabolic effects of CART in the ARC and lateral hypothalamic area, respectively, were reported, NPY/AgRP cells play an important orexigenic role in energy homeostasis (38, 40–42). Second, POMC and NPY/AgRP neurons respond to insulin. Electrophysiological studies have shown that this hormone excites POMC and inhibits NPY/AgRP neurons (43, 44). Third, NPY/AgRP- and POMC-derived peptides, e.g., alpha-melanocyte stimulating hormone, directly influence GnRH neuron excitability (45). Fourth, anatomical data indicate a direct contact of GnRH cells with POMC/CART and NPY/AgRP neurons (8, 46, 47). Fifth, there is evidence that KNDy cells in the ARC are locally connected with AgRP and POMC neurons (48, 49). However, there are differences in the ARC neuropeptides coexpression in the monkey compared to rodents (8). Finally, Kp shows stimulatory effects on POMC and inhibitory effects on NPY neurons in the ARC (50). Thus, both POMC/CART and AgRP/NPY cell populations appear to be good candidates as GnRH regulators (51).

EVIDENCE FOR THE ROLE OF Kp IN METABOLISM CONTROL: BEYOND THE BRAIN

The Kp gene and its receptor are expressed not only in the central nervous system (CNS) but also in the peripheral tissues (52, 53). In this review, we discuss its role in the pancreas, liver, and adipose tissue. In view of two extensive reviews on the subject (52, 53), we will focus mainly on the latest findings in the field.

Several lines of evidence indicate a role of Kp in metabolism regulation in the peripheral tissues (53, 54, 55). First, Kp and its receptor are expressed in peripheral tissues controlling metabolism (26, 54, 56). Second, at times of metabolic imbalance (e.g., undernutrition, obesity, and diabetes), alterations of the Kp system in these tissues were observed (57). Third, *in vitro* experiments indicate an action of Kp on lipid metabolism (55). Fourth, evidence for a function of Kp in metabolism control came from a study of *Kiss1r* KO animals. *Kiss1r* KO females

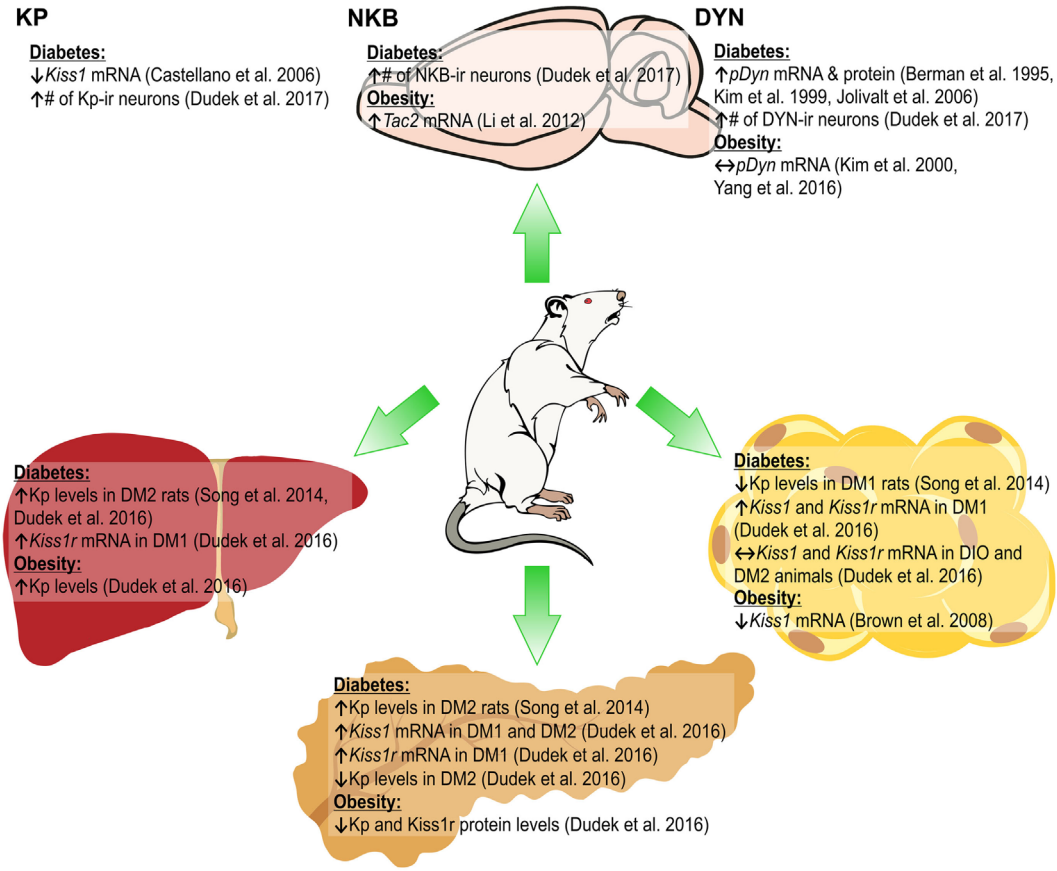

FIGURE 1 | Changes in the expression of KNDy peptides [kisspeptin (Kp), neurokinin B (NKB), and dynorphin (DYN)] in the arcuate nucleus of the hypothalamus (ARC; upper part) and Kiss1/Kiss1r in peripheral organs (lower part) of rat due to diabetes obesity. ↑, increase; ↓, decrease; ↔, no effect (11, 26, 28, 30, 33–36, 54, 56).

had a greater body weight (BW), hyperinsulinemia, increased adiposity, elevated fasting basal glucose levels as well as impaired glucose tolerance (58). However, the observed obesity cases were not due to hyperphagia, but rather reduced metabolism. *Kiss1r* KO females had dramatically decreased energy expenditure. By contrast, *Kiss1r* KO males were characterized by normal BW and glucose tolerance. It would be of interest to study possible insulin resistance in this experimental model. Of particular relevance for this review, in the DM2 state insulin resistance is observed. Moreover, DM2 is often associated with obesity and accounts for roughly 90% of all diabetes cases (59). Adiposity, hyperinsulinemia and decreased metabolism were already seen at a younger age in *Kiss1r* KO females, with impaired glucose tolerance and feeding developing later in adulthood, after BW was significantly increased. Thus, an early life decrease in metabolism and energy expenditure may underline the later emergency of the obese phenotype of adult *Kiss1r* KO females (60). This phenotype could arise from defective signaling in the brain, and/or peripherally. Data on the expression of NPY, POMC, leptin receptor, ghrelin receptor, and melanocortin receptor 3 and 4 genes involved in the appetite regulating system of the hypothalamus generally showed no changes in the above-mentioned genes in *Kiss1r* KO mice with GDX. This suggests that obesity revealed in *Kiss1r* KO mice may reflect peripheral rather than central metabolic impairments (61). Below, we will update information on Kp in the peripheral tissues involved in control of metabolism.

Kp, PANCREAS, AND GLUCOSE METABOLISM

Glucose homeostasis is regulated by insulin secreted from beta cells in the pancreatic islets (62). Moreover, insulin release is modulated by numerous G protein-coupled receptors including *Kiss1r* (63). Expression of the *Kiss1* gene in the pancreatic tissue was shown by Lee et al. (64), while later Hauge-Evans et al. (65) detected the expression of the Kp gene and its receptor in alpha and beta cells in human and murine islets, respectively. Both Kp and its receptor immunoreactivities colocalized with alpha and beta pancreas cells (65). Kisspeptin-54 (Kp-54) increased glucose-induced insulin secretion from human and murine islets, without any effect on basal secretion (65). Studies on rhesus monkeys (66) and rats (67) using kisspeptin-10 (Kp-10) confirmed *in vitro* observations. In addition, intracerebroventricular (icv.) administration of the peptide in rats had no effect on insulin levels, suggesting the peripheral site of action of Kp (67). Indeed, Kp-10 and kisspeptin-13 act directly at beta

cells to potentiate insulin secretion stimulated by glucose in murine, porcine and human islets (63, 67, 68). Kp enhanced insulin secretion from murine islets in a dose-dependent manner through a $G_{\beta\gamma}$-dependent pathway (63, 67). However, there are contradictory results indicating that Kp could inhibit (69, 70) or have no effect on insulin secretion (67), which may be related to the used doses and differences in protocols. Data from our laboratory confirmed expression of *Kiss1* and *Kiss1r* in the pancreas [mRNA and peptide (56)]. We have shown that diabetic type 1 (DM1) and DM2 male rats had increased pancreatic *Kiss1* mRNA levels. However, in the case of *Kiss1r*, an increase was recorded only in the DM1 group. By contrast, protein levels decreased in diet-induced obese (DIO) and DM2 animals. In addition, Kp and its receptor levels were undetected in the DM1 group (as a result of damage to pancreatic cells caused by STZ). Levels of Kiss1r protein were reduced only in the DIO group (56). Thus, in DM2/DM1 animals, the Kiss1/Kiss1r system may not function properly, thus being unable to control insulin levels (**Figures 1** and **2**).

Kp IN THE LIVER

The liver is an important organ, which acts as the body's glucose reservoir (71). It is also responsible for maintaining steady circulating blood sugar levels by storing and producing glucose depending upon the body's need (71). Only few studies focused on Kp in the liver. Kp-10 administered peripherally in male rats had antioxidant effects and increased the levels of free radical scavengers (superoxide dismutase and adenosine deaminase), suggesting protective effects on liver metabolism (72). Kp-10 in rats exposed to heat-induced oxidative stress decreased plasma corticosterone levels (73). Stressors enhance the activity of the hypothalamus–pituitary–adrenal axis, increasing corticosteroid levels. Since corticosterone may affect glucagon sensitivity and promote glycogenolysis, the authors hypothesized that glycogenolysis may be suppressed by the Kp-dependent reduction in corticosterone release (73). An extensive study conducted by Song et al. (54) revealed that in hyperglucagonemic diabetic animals (in HFD-induced diabetes and genetic leptin receptor-deficient db/db mice) liver Kp levels were increased. *In vitro* stimulation of mouse hepatocytes also increased *Kiss1* mRNA and protein levels. Moreover, DM2 patients exhibit increased liver and plasma Kp levels. In addition, we showed an increase in liver Kp expression in both DIO and DM2 male rats (56). Kp-10 and Kp-54 also impaired glucose tolerance, while selective liver Kp knock-down depressed glucose-sensitive insulin secretion (GSIS) and improved glucose tolerance and mice lacking liver Kiss1r exhibited improved glucose tolerance when fed HFD (54).

Based on the above findings, a tri-hormonal regulatory circuit between pancreatic alpha cells, hepatocytes, and beta cells was proposed, with Kp playing an important role in the liver to islet endocrine signaling. It was suggested that activation of the liver glucagon receptor stimulates insulin secretion by increased hepatic glucose production and hyperglycemia. However, liver glucagon action may also inhibit insulin secretion by stimulating Kp production. According to this model, in pancreatic beta cells in DM2 patients, the GSIS can be stimulated by hyperglycemia but inhibited by Kp. These findings extend a potential for Kp antagonism as a therapeutic tool to improve beta cell function in diabetic patients (**Figure 2**) (54).

Kp IN THE ADIPOSE TISSUE

The major function of adipocytes is to store fat for periods of greater energy requirements (74). Control of these lipogenic and lipolytic processes is modulated by hormonal signals, with the adipose tissue emerging as an active participant in regulating physiologic and pathologic processes (75).

Kiss1 mRNA was detected in rat (26, 56) and *KISS1* mRNA in human (76) adipose tissue and was shown to regulate the metabolic status. While fasting of rats increased adipose *Kiss1* mRNA expression in both sexes, a decrease was reported in *Kiss1* mRNA expression in adipose tissue of HFD and obese Zucker rats. These alterations were seen in visceral (epididymal) and subcutaneous (in the case of Zucker rats) adipose tissue (26). In women, a

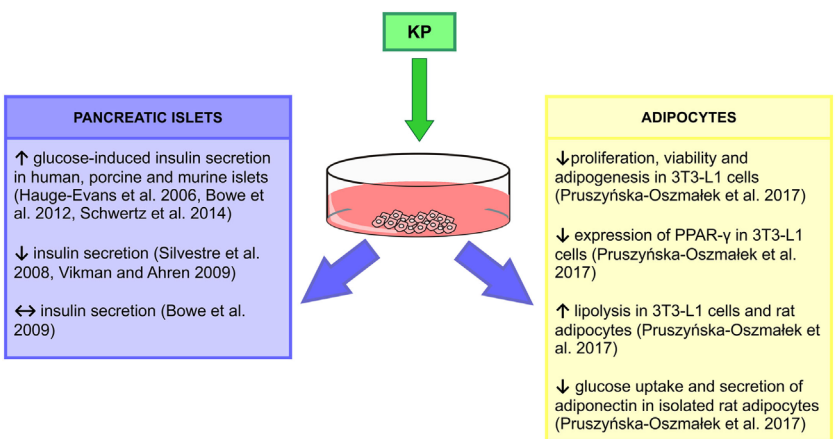

FIGURE 2 | *In vitro* actions of kisspeptin (Kp). ↑, increase; ↓, decrease; ↔, no effect (55, 63, 65, 67–70).

positive correlation between body mass index (BMI) and *KISS1* mRNA levels was found in the omental adipose tissue, but not in subcutaneous fat (76). *Kiss1* mRNA expression in adipose tissue is sensitive to sex steroids; while GDX in both sexes had no effect on *Kiss1* mRNA, supplementation of castrated animals with testosterone (males) and estradiol (females) increased *Kiss1* mRNA in rats (26).

We have revealed expression of mRNA and peptide for Kp in gonadal fat of male rats (56). While in DIO and DM2 animals expression of *Kiss1* and *Kiss1r* mRNA did not change, we were not able to detect peptides in these animals. By contrast, in DM1 rats there was a marked decrease in Kp and no change in *Kiss1r* levels. Our *in vitro* study showed that isolated rat adipocytes and mouse 3T3-L1 cells express mRNA and peptide for *Kiss1* and *Kiss1r* genes, and we found multiple actions of Kp-10 in these cells (55). First, Kp inhibited proliferation, viability, and adipogenesis in 3T3-L1 cells and decreased expression of PPAR-γ and CEBPβ—genes, involved in differentiation processes and adipogenesis. Second, Kp-10 increased lipolysis in rat adipocytes and 3T3-L1 cells by enhancing the expression of perilipin and hormone-sensitive lipase. Third, Kp-10 modulated lipogenesis. Finally, Kp-10 decreased glucose uptake and adiponectin secretion, while it stimulated leptin secretion from rat adipocytes (55). Thus, Kp could be a major factor in the regulation of adipocyte metabolism, at least *in vitro.* In particular, Kp-10 may slow the process of lipid accumulation *via* decreasing lipogenesis and increasing lipolysis. Further experiments are required to reveal the effects of Kp on adipose tissue *in vivo.* The stimulatory effect of Kp-10 on adiponectin, but not leptin levels was already shown in male rhesus monkeys, both fed and fasting (**Figures 1** and **2**) (66).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

We reviewed data supporting the hypothesis that Kp is a link between metabolic cues and reproductive functions. On one hand, there have to be a proper amount of fat in the body to enter puberty, but on the other hand obesity can cause disturbances in puberty, followed by irregularities in testosterone production in men and ovulation in women (77, 78). Moreover, both puberty onset and obesity seems to be related to changes in Kp expression in the hypothalamus (7). However, peripheral Kp signaling may also play an important role in obesity or increasing body mass during adolescence. Within the hypothalamus, Kp does not act alone, but with other neuropeptides, e.g., NKB and DYN A, also being sensitive to the metabolic status. Moreover, relationships between the expression of Kp, NPY/AgRP, and POMC/CART were found. These neuropeptides have an impact on the hypothalamic GnRH functions, and complex interactions between these neurons in integration of metabolic and reproductive functions need to be revealed.

Although Kp is successfully used as a therapeutic target in human reproduction, including treatment of delayed and precocious puberty, subfertility, and contraception, much less is known on its role in the metabolic syndrome (79, 80). Kp-10 administered to men with DM2, and central hypogonadism enhanced endogenous testosterone secretion (81). Studies in animals indicate that Kp may be involved in the regulation of insulin secretion (54, 55, 63, 65, 69, 70). Recently, it was also revealed that Kp plasma concentration in the human is negatively correlated with BMI and waist circumference. Moreover, in non-diabetic individuals, Kp was shown to be associated with reduced glucose-stimulated insulin secretion (82). Further studies are needed to explore interactions between Kp and insulin in diabetic patients.

As most experiments are conducted on males and differences between sexes exist, there is a need to untangle alterations between females and males in the regulation of metabolic and reproductive functions. More research is required to understand interactions between Kp and metabolism in the peripheral tissues, where it can act in the paracrine manner. In addition, as tachykinins (neurokinins B and A, substance P) are extensively expressed within the CNS and at the periphery (83) and studies suggest a role of substance P in the regulation of metabolic functions (84, 85), it would be interesting to assess potential NKB/NK3R expression in peripheral tissues related to metabolism. More *in vivo* studies are needed on Kp action in the pancreas, liver, and fat tissue, while genetically modified animal models should be developed to elucidate mechanism(s) of its action. Basic scientists should participate in the WHO actions that have the potential to reduce the risk or burden of undernutrition and overweight by revealing the underlying mechanism(s). This may lead to the development of individually oriented therapeutic strategies against these lifestyle diseases.

AUTHOR CONTRIBUTIONS

Both MD and KZ participated in drafting, writing, and editing the manuscript. JHS participated in writing and editing the manuscript.

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Biological Significance of Kisspeptin–Kiss 1 Receptor Signaling in the Habenula of Teleost Species

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Kisspeptin is a neuropeptide, encoded by kisspeptin 1 (KISS1)/Kiss1 gene, which primarily acts as the regulator of reproductive functions *via* its receptor, kisspeptin receptor (KissR) in vertebrates. In the brain, Kiss1 gene is mainly expressed in the hypothalamic region, but *KissR* gene is widely distributed throughout the brain, suggesting that kisspeptin–KissR system may be involved in not only reproductive, but also non-reproductive functions. In non-mammalian vertebrates, there are two or more kisspeptin and KissR types. The zebrafish (*Danio rerio*) possess two kisspeptin (Kiss1 and Kiss2) and their respective receptors [Kiss1 receptor (KissR1) and KissR2]. In the brain of zebrafish, while Kiss2 is expressed in the preoptic-hypothalamic area, Kiss1 is predominantly expressed in the habenula, an evolutionarily conserved epithalamic structure. Similarly, KissR1 is expressed only in the habenula, while KissR2 is widely distributed in the brain, suggesting that the two kisspeptin systems play specific roles in the brain. The habenular Kiss1 is involved in the modulation of the raphe nuclei and serotonin-related behaviors such as fear response in the zebrafish. This review summarizes the roles of multiple kisspeptin–KissR systems in reproductive and non-reproductive functions and neuronal mechanism, and debates the biological and evolutional significance of habenular kisspeptin–KissR systems in teleost species.

Keywords: kisspeptin 1, teleosts fish, zebrafish, non-hypothalamic, Gpr54/Kiss1r

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INTRODUCTION

During the past decade, the field of reproductive neuroendocrinology has shifted from its major focus on the hypothalamus–pituitary–gonadal (HPG) axis comprising gonadotropin-releasing hormone (GnRH), gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)], and gonadal steroids to the next level with the discovery of RFamides, which include kisspeptin and gonadotropin-inhibitory hormone (also known as LPXRFamide). Kisspeptin is a neuropeptide, encoded by kisspeptin 1 (KISS1)/Kiss1 gene which was originally identified as a metastasis suppressor gene (1). Kisspeptin binds to its receptor, kisspeptin receptor (KissR), which was originally identified as an orphan G-protein-coupled receptor-54 (GPR54, also known as hOT7T175) (2). Kisspeptin consists of core peptides, including 54-, 14-, and 13-amino acids peptides and its processed mature, biologically active 10-amino acid peptide (Kiss1-10). In 2003, two studies reported consecutively the role of kisspeptin–KissR signaling in reproduction, particularly the control of GnRH-LH secretion during the onset of puberty in mammals (3, 4). Since then, numerous studies have demonstrated the role of kisspeptin–KissR signaling, neuroanatomy, and neuro-molecular mechanisms underlying

the control of reproductive physiology in mammalian species (5). We have been interested to understand the importance of kisspeptin–KissR signaling in the vertebrate reproduction from an evolutional perspective using non-mammalian vertebrates. In 2004, we were the first to identify the non-mammalian KissR-like sequence from a cichlid fish, Nile tilapia (*Oreochromis niloticus*) and also demonstrated their gene expression in GnRH neurons using a laser capture microdissection technique (6). As for kisspeptin in teleosts, fish Kiss1 gene was first reported in zebrafish (*Danio rerio*) (7, 8). Interestingly, we found another gene encoding kisspeptin-like structure, which is slightly different from Kiss1 in the zebrafish and medaka (*Oryzias latipes*), we, therefore, named it Kiss2 (9). Similar to kisspeptins in teleosts, there are two or more KissR types, which are distributed in different patterns in the brain (10), suggesting specific role for two kisspeptin types in fish brain. Although in the mammalian species, kisspeptin–KissR system primarily targets GnRH neurons, in fact, kisspeptin neurons actually send their projections to a large number of brain areas and KissR are widely distributed in the brain (11–13). In addition, Kiss1 gene is also expressed in some extra-hypothalamic regions, such as the hippocampal dentate gyrus (14) and the medial amygdala (15). Surprisingly, in the zebrafish and medaka, knockout of two kisspeptins (*kiss1* and *kiss2*) and KissRs [Kiss1 receptor (*kissr1)* and *kissr2*] genes had no obvious effect on their reproductive capability (16, 17). These observations indicate that kisspeptin–KissR system may play roles in processing several non-reproductive functions. In fact, a functional MRI study in humans has recently revealed that kisspeptin modulates limbic brain activity in response to sexual and emotional stimuli, and influences mood in healthy men (18). Given that the zebrafish model has clear distinct neuroanatomical patterns of two kisspeptin–KissR systems, the zebrafish is believed to be an ideal model to understand differential role and regulatory mechanism of the two kisspeptin–KissR systems. We have been particularly interested to understand the role of Kiss1 in the habenula. The habenula is an evolutionary conserved epithalamic structure, which is involved in certain forms of emotive decision making in primates. Recent discoveries in primates by Dr. Hikosaka's group indicate that the habenula plays a prominent role in emotive behavioral choice through neuromodulation of the dopamine and the serotonin systems (19). In addition, the habenula is involved in behavioral responses to pain, stress, anxiety, sleep, and reward. The dysfunction of the habenula is associated with neurological problems, such as depression, schizophrenia, and drug-induced psychosis (20). Therefore, the habenula has been a recent focus as a potential therapeutic target for neuropsychiatric disorders.

The anatomy, molecular biology, functions, and regulatory mechanism of hypothalamic kisspeptin–KissR system have been extensively studied, and summarized in a number of review articles for mammalian species (5, 21–23) and for non-mammalian species (10, 24–29). However, the knowledge on non-hypothalamic kisspeptin–KissR system is still limited (14, 30–33). The role of non-hypothalamic kisspeptin signaling is scarcely examined in non-mammalian vertebrates. In this review, we provide an overview and recent updates of non-hypothalamic kisspeptin–KissR systems in non-mammalian vertebrates, with specific emphasis on the habenular Kiss1-KissR1 system in the zebrafish model.

TWO KISSPEPTINS AND KissR TYPES IN FISH

Since their first identification in zebrafish and medaka (9), two kisspeptin types have been identified in several teleost species (24, 25), but some fish species, such as tilapia (34), *Astatotilapia burtoni* (27), red seabream (35), Atlantic halibut (36), flatfish Senegalese sole (37), black rockfish (38), Japanese flounder (39), and puffer fish (40) are likely to possess only one (Kiss2) type. Similar to multiple kisspeptin forms, multiple KissR (KissR1, KissR2, KissR3, and KissR4) types have also been identified in various fish species (25, 28, 41). In zebrafish, there are two KissR types, KissR2 and KissR3 (also designated as KissR1a and KissR1b, respectively) (41, 42). Pharmacological assays verified the ligand-receptor affinities for two kisspeptins and their receptors (28). In zebrafish, while zebrafish Kiss1 peptide (zfKiss1-10) activates KissR3 more efficiently than zebrafish Kiss2 peptide (zfKiss2-10), KissR2 is activated by both zfKiss1-10 and zfKiss2-10 in zebrafish (42). Distribution of two KissR types in the brain further verified the classification of their relationship with two kisspeptin types. In zebrafish, KissR3 gene is widely expressed in the brain, whereas KissR2 gene and its protein product are mainly expressed in the habenula (43–45), where Kiss1 gene is expressed. Therefore, based on these characteristics, we designate zebrafish KissR2 and KissR3 as KissR1 (*kissr1*) and KissR2 (*kissr2*), respectively in our articles (10). However, in some teleost species, relationship between multiple kisspeptins and their receptors has not been clearly characterized because multiple kisspeptins and receptors can cross talk with each other and have different neuroanatomical distributions. Nevertheless, it is very clear that two kisspeptin–KissR types are highly conserved in teleosts species, which are, however, involved in wide range of functions in the brain.

Similar to mammalian species, several functional assays have revealed the major role of Kiss-KissR systems in the control of reproduction in fish. In some fish, including zebrafish, *in vivo* assays show that Kiss2 (Kiss2–10 or Kiss2–12) rather than Kiss1 (Kiss1–10 or Kiss1–15) mainly exhibits its stimulatory effect on gonadotropin synthesis and release (9, 46–48). In chub mackerel, Kiss2 dodecapeptide (Kiss2–12) but not Kiss1 pentadecapeptide (Kiss1–15) administration alters GnRH1, LHβ, and FSHβ genes expression (47), which is further supported by co-expression or proximity of KissR2 in preoptic-hypothalamic GnRH neurons reported in several fish species (6, 49, 50). On the other hand, in some species, such as medaka, Kiss1 seems to be more potent than Kiss2 in the regulation of gonadotropin stimulation. In chub mackerel, Kiss1–15 is more potent than Kiss2–12 on stimulation of gonadal maturation when it was administered chronically (51, 52). In male yellowtail kingfish, Kiss1–10 and Kiss2–10 administration resulted in different effects depends on duration of treatment and reproductive stages of fish (53). These results indicate that regardless of kisspeptin types, fish kisspeptin can stimulate reproductive functions, which, however, may vary dependent on reproductive stages, gender, fish species, and treatment methods.

EXPRESSION OF Kiss1–KissR1 IN THE TELEOST HABENULA

Expression of Kiss1 gene in the ventral part of the habenula has been shown in the zebrafish (9, 44, 45) as well as in the medaka (9, 54), goldfish (55), European sea bass (56), and the orange spotted grouper (57). However, unlike zebrafish Kiss1 gene expression, in other fish species, Kiss1 gene is also expressed in some brain regions, such as the preoptic-hypothalamic area, suggesting that Kiss1 can act on multiple action sites and have different roles in these species. In contrast to habenular kisspeptin, the expression of habenular KissR1 in the zebrafish, is seen in only a limited fish species. In medaka, KissR1 gene is expressed in the habenula and preoptic nuclei (58). In the European sea bass, not only KissR1 but also KissR2 expression has been reported in the habenula (56). Interestingly, in some species such as the chum mackerel and striped bass, KissR1 gene is expressed in the ventral habenula and preoptic area, in spite of the lack of Kiss1 gene expression in the habenula (50, 59). These results suggest that expression of KissR1 in the habenula is conserved at least among teleosts species that possess two kisspeptin types. In zebrafish, immunohistochemical localization using antibodies specific to zebrafish-Kiss1 and KissR1 reveal that habenular Kiss1/KissR1 cells project to the ventro-anterior corner of the median raphe (vaMR), a subregion of the MR [a division of the superior raphe (SR)] through the fasciculus retroflexus (FR) (44, 45, 60), which has also been confirmed in a *kiss1*:mCherry transgenic zebrafish (61). However, in the zebrafish brain, the KissR1 antibody also labeled cells in other brain area, such as the telencephalon, diencephalon, and spinal cord regions (60). This is because of the cross-reactivity of the KissR1 antibody against kissr1b-derived protein 2, an alternative splice variant of the KissR1 gene, which shares the epitope of the KissR1 antibody (62). Zebrafish KissR1 gene possess four additional alternative splice variants encoding different protein lengths (KRBDP 1–4), which, however, are functionally incapable of mediating kisspeptin-derived cellular responses (62). In the zebrafish, Kiss1 and its receptor are co-expressed in the same neurons within the habenula (63). Furthermore, central administration of Kiss1 peptides significantly suppresses Kiss1 gene expression, suggesting an autocrine regulation of the Kiss1 gene (63).

MODULATION OF SEROTONIN AND ITS RELATED BEHAVIORS BY HABENULAR Kiss1/KissR1

Habenular Kiss1/KissR1 cells send projections in the vicinity of serotonin [5-hydroxytryptamine (5-HT)]-containing neurons located the median raphe (60). Mammalian habenula consists of two major subnuclei, the medial and lateral habenula. The medial habenula projects to the interpeduncular nucleus (IPN), while the lateral habenula directly projects to the ventral tegmental area and raphe, which are dopaminergic and serotonergic structures, respectively (64). Similar to mammalian habenula, the fish habenula can also be neuroanatomically subdivided into two major nuclei, the dorsal and ventral habenula based on difference in their cytoarchitectual structures (65). In zebrafish, the dorsal habenula project to the IPN *via* FR, while the ventral habenula project to the vaMR (60, 66). In addition, the dorsal habenula express the POU-domain transcription factor *brn3a*, a marker for the mice medial habenula (67, 68), while the ventral habenula express *protocadherin 10a*, a specific marker of the rat lateral habenula (66, 69). Therefore, the fish dorsal and ventral habenula have been elucidated as the homolog of the mammalian medial and lateral habenula, respectively. In mammals, the lateral habenula has been implicated as a pivotal regulator of dopaminergic and serotonergic neurons (19, 70). Furthermore, the lateral habenula is involved in sleep, locomotion, motivation, reward, and behavioral stress responses (19). In the brain of zebrafish treated with zfKiss1–15 peptides, expression of genes associated with serotonin, *pet1* and *sert* (*slc6a4a*), and *c-fos* genes are significantly upregulated within the raphe nucleus (63). Central administration of zfKiss1–15 has no effect on anxiety, but shows a trend in anxiolytic effect (increase in number of transition) in zebrafish (45) when observed using a novel-tank diving test (71). Fish administered with zfKiss1–15 peptides failed to exhibit fear (45), characterized by erratic and freezing behaviors in response to an aversive stimulus from skin extract (alarm substance) (72, 73). Such effects were not observed when zfKiss2–10 was administered, suggesting these effects could be mainly modulated by Kiss1–KissR1 pathway. In addition, injections of zfKiss1–15 peptides conjugated with saporin; a ribosome-inactivating cytotoxic protein (74) induced cell death of Kiss1 neurons, the immunoreactivity of KissR1 was significantly reduced in the habenula and median raphe, and in these fish, alarm substance-induced fear response was significantly reduced (45). A recent study using *kiss1*-mutant fish revealed the potential involvement of habenular Kiss1 neurons to avoid punishment (75). These Kiss1 gene-mutants have a stop codon upstream of the active Kiss1 peptide, which causes deficiency in learning to avoid a shock that is predicted by light. These studies suggest that Kiss1–KissR1 signaling modulates behavioral response to uncontrollable aversive stimuli in the habenula. However, possible involvement of KissR2 or other receptor signaling pathways in this behavioral response should not be ignored, because KissR2 or other GPCRs are also activated by both Kiss1–10 and Kiss2–10 (42, 76).

MECHANISM OF SEROTONERGIC MODULATION BY Kiss1–KissR1 SIGNALING

zfKiss1–15 administration effects serotonin-related genes, although KissR1 is not expressed in serotonergic neurons (60, 63), indicating that Kiss1 neurons act indirectly through interneurons on serotonergic system in zebrafish. Habenular Kiss1 neurons are glutamatergic in nature and their axons form close association with either glutamatergic or GABAergic interneurons in the median raphe region (60) (**Figure 1**). This suggests that Kiss1 might regulate serotonergic neural activities *via* the modulation of glutamatergic presynaptic neurotransmission. We speculate KissR1 might function as a presynaptic autoreceptor on habenula

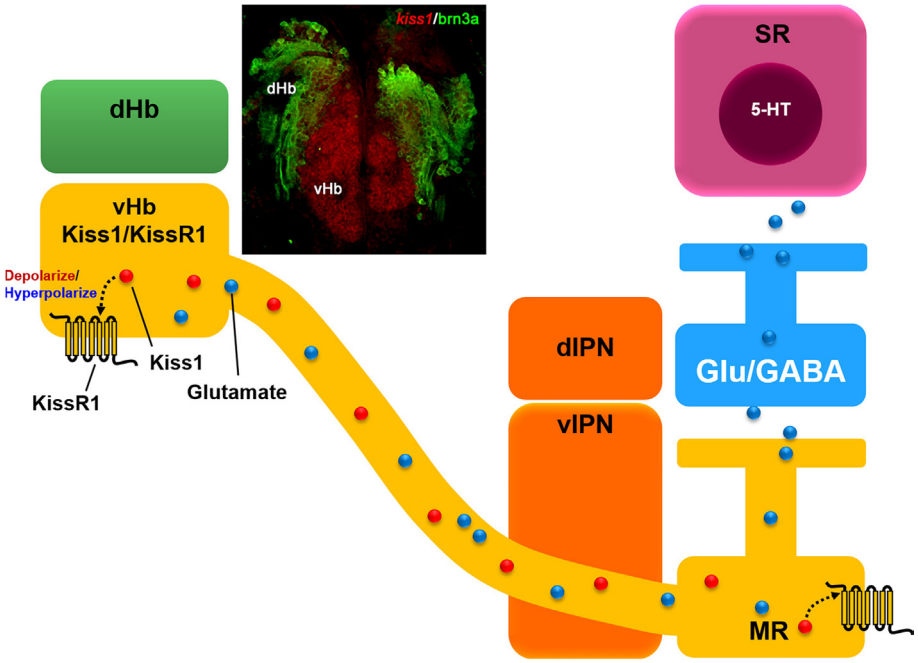

FIGURE 1 | Schematic drawing of hypothetical neural circuit of habenular kisspeptin 1 (Kiss1) neurons. Kiss1 (red dot) modulate ventral habenular (vHb) neuronal activities *via* concentration-dependent mechanism through co-expressing Kiss1 receptor (KissR1). Photomicrograph shows a coronal brain section image of *kiss1* mRNA expression in the vHb (red) but not in the dorsal habenula (dHb, green) expressing *brn3a*, a marker gene for the dHb in transgenic (*brn3a-hsp70:GFP*) zebrafish. Habenular Kiss1 neurons send their projections to the median raphe (MR), a division of the superior raphe (SR). Kiss1 cells are glutamatergic in nature and it is hypothesized that the presynaptic action of the Kiss1/KissR1 system causes the release of glutamate (blue dot) in Kiss1 cells from the vHb that potentially regulates the serotonin (5-HT) system in the SR directly or *via* glutamatergic and GABAergic neurotransmission. Abbreviations: dIPN, dorsal interpeduncular nucleus; vIPN, ventral interpeduncular nucleus modified from Nathan et al. (60).

Kiss1 nerve terminals to facilitate glutamatergic transmission on serotonergic neurons, which remains to be confirmed.

In the zebrafish, although Kiss1 inhibits alarm substance-induced fear response, but its involvement through the serotonergic system remained unclear. The effect of Kiss1 on alarm substance-induced fear responses was blocked in the presence of serotonin receptor antagonists (77). Interestingly, treatment with different antagonist against two serotonin receptor types (5-HT_{1A} and 5-HT_{2}) results in different behavioral responses. Anxiolytic effect of Kiss1 is modulated *via* 5-HT_{1A} receptor, while inhibitory effect of Kiss1 on freezing behavior is modulated *via* 5-HT_{2} receptor (77). Further, calcium imaging study has shown that Kiss1-gene mutant zebrafish larvae have reduced activation of raphe neurons by aversive stimulus (75). These studies indicate that Kiss1–KissR1 signaling is involved in the modulation of serotonergic neural activity under uncontrollable aversive conditions. Administration of exogenous Kiss1 or ablations of Kiss1 neurons suppresses alarm substance-induced fear response (45). Surprisingly, administration of Kiss1 also elevates *c-fos* gene expression by cellular excitation in the ventral habenula neurons (63). Optogenetic stimulation of ventral habenula neurons evokes place avoidance behavior (78), which is contradictory to the results of *c-fos* expression. This issue has been recently resolved by an electrophysiological approach (75), where Kiss1 has been shown to have a concentration-dependent effect on ventral habenula neurons: depolarizing at low concentrations and hyperpolarizing at high concentrations. Furthermore, *c-fos* expression was induced by a concentration of 10^{-11} mol/fish of Kiss1 peptides, but not with a higher concentration of 10^{-9} mol/fish (63). Therefore, suppression of alarm response by exogenous Kiss1 peptides could be due to hyperpolarization of ventral habenula neurons, which, however, remains to be further examined by functional assays.

Although Kiss1–KissR1 signaling modulates serotonin in response to uncontrolled aversive stimulus, but it remains unclear what regulates Kiss1 neurons, including Kiss1 peptide synthesis and secretion and Kiss1 neuronal activities. More importantly, the role of Kiss1–KissR1 signaling within the habenula neurons is still unknown. It is also important to identify the upstream control of the ventral habenula neurons, which could be from several afferent projections from brain regions, such as the entopeduncular nucleus, preoptic area, and hypothalamus (79, 80). Recent studies have revealed a functional connection between a thalamic nucleus and the habenula in zebrafish, and this pathway mediates light-evoked locomotor activity, including circadian behavior (81, 82). In mammals, the habenula (lateral habenula) neurons have been suggested to act as circadian oscillators (83–85). In addition, in the goldfish, Kiss1 and KissR gene expression are influenced by different light spectra (86). Therefore, Kiss1–KissR1 signaling in the habenula could be involved in mediating circadian controlled innate behaviors, such as sexual behavior and sleep–wake cycle, which remain to be studied.

BIOLOGICAL SIGNIFICANCE OF HABENULAR KISSPEPTIN

In a series of our studies, we have provided some evidences for the involvement of kisspeptin–KissR signaling in the zebrafish vertebrate habenula (45, 60, 63, 77). However, the presence of Kiss1 in the fish habenula nuclei has been shown in a limited number of fish species. Some fish species possess only one type of kisspeptin gene (Kiss2), which is expressed in the hypothalamic area (10). Some fish species that possess two kisspeptin types such as the chum mackerel show no expression of Kiss1 in the habenula (59). On the other hand, expression of KissR in the habenula has been identified in several fish species and also in mammals (11, 87) (**Table 1**) and kisspeptin neurons in the anteroventral periventricular nucleus have been shown to innervate the habenula (88), suggesting that the action of kisspeptin–KissR signaling and its role within the habenula might be evolutionarily conserved regardless of the source of kisspeptin. Interestingly, in the fetal mouse brain, Kiss1R containing cells are seen in a highly restricted population of cells in the habenula as early as embryonic day 17 (89), which is a period when the formation of habenula-IPN pathway is completing (90). Similarly, in embryonic zebrafish, Kiss1-positive cells are first appearing in the ventral habenula by 5-days post fertilization, when the innervation of the ventral habenula axon has reached their target, the medial raphe (91). Furthermore, in a mutant fish that lacks functional Tcf7l2, a downstream modulator of the Wnt signaling cascade, Kiss1 gene expression is lost and the median raphe are not innervated by ventral habenula axons (91). Therefore, the habenular Kiss1–KissR1 signaling may play a role in the habenula axonal formation during brain development. Previous studies have demonstrated the role of the lateral habenula in the hormonal onset of maternal behavior in female rats (92, 93). In both rodent and fish, the habenula is known to be sensitive to steroid hormones and express estrogen receptors (94, 95). In addition, the habenula in the zebrafish produces neurosteroids locally (96, 97). Furthermore, goldfish Kiss1 gene promoter contains putative binding sites for estrogen receptors (55), and in orange-spotted grouper, Kiss1 neurons express estrogen receptors in the habenula (57). Estrogen has effect on mood, mental state, and memory, which are closely related to serotonergic functions (98). Interestingly, in larval zebrafish, treatment with an estrogen receptor β agonist increased *c-fos* expression in the habenula with anxiolytic effect (increase in exploration behavior) (95). Therefore, kisspeptin–KissR signaling pathway within the habenula could be involved in the neuromodulatory processes

TABLE 1 | Kisspeptin and kisspeptin receptor (KissR) types and their expression in the habenula.

Species	Kisspeptin types	Expression (cell body) in the habenula	KissR types	Expression in the habenula	Reference
Rat	Kisspeptin 1 (Kiss1)	–	Kiss1R	+	(11)
Mouse	Kiss1	–	Kiss1R	+	(13, 87, 89)
Syrian hamster	Kiss1	–	Kiss1R	+	(99)
Xenopus laevis	Kiss1 Kiss2	– –	GPR54-1a GPR54-1b GPR54-2	ND ND ND	(42)
Zebrafish (*Danio rerio*)	Kiss1 Kiss2	+ –	Kiss1 receptor (KissR1) KissR2	+ –	(9, 44, 63)
Medaka (*Oryzias latipes*)	Kiss1 Kiss2	+ –	KissR1 KissR2	+ –	(9, 54, 58)
Goldfish (*Carassius auratus*)	Kiss1 Kiss2	+ –	KissR1 KissR2	ND ND	(55)
Striped bass (*Morone saxatilis*)	Kiss1 Kiss2	– –	KissR1 KissR2	+ –	(50)
European sea bass (*Dicentrarchus labrax*)	Kiss1 Kiss2	+ –	KissR1 KissR2	+ +	(56)
Orange-spotted grouper (*Epinephelus coioides*)	Kiss1 Kiss2	+ –	KissR1 KissR2	ND ND	(57)
Chum mackerel (*Scomber japonicas*)	Kiss1 Kiss2	– –	KissR1 KissR2	+ –	(59)
Sapphire devil (*Chrysiptera cyanea*)	Kiss1 Kiss2	+ –	KissR1 KissR2	ND ND	(100)
Nile tilapia (*Oreochromis niloticus*)	Kiss2	–	KissR2	ND	(34)
Astatotilapia burtoni	Kiss2	ND	KissR2	+	(49)
Red seabream (*Pagrus major*)	Kiss2	–	NA	ND	(35)
Grass puffer (*Takifugu niphobles*)	Kiss2	–	KissR2	ND	(101)

+, confirmed expression of kisspeptin and KissR types in the habenula; –, confirmed lack of expression of kisspeptin and KissR types in the habenula; ND, expression of kisspeptin and KissR types in the habenula has not been determined.

of emotional and goal-directed behaviors, which could also be influenced by reproductive conditions.

AUTHOR CONTRIBUTIONS

Both authors researched and wrote/edited the article and designed the figures.

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Regulation and Possible Functions of Kisspeptin in the Medial Amygdala

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Kisspeptin, encoded by the *Kiss1* gene, is required for reproduction. Humans and mice lacking kisspeptin or its receptor, Kiss1r, have impairments in reproductive physiology and fertility. In addition to being located in the hypothalamus in the anteroventral periventricular and arcuate nuclei, kisspeptin neurons are also present in several extra-hypothalamic regions, such as the medial amygdala (MeA). However, while there has been a significant focus on the reproductive roles of hypothalamic kisspeptin neurons, the regulation and function(s) of MeA and other extra-hypothalamic kisspeptin neurons have received far less attention. This review summarizes what is currently known about the regulation, development, neural projections, and potential functions of MeA kisspeptin neurons, as well as kisspeptin signaling directly within the MeA, with emphasis on data gathered from rodent models. Recent data are summarized and compared between rodent species and also between males and females. In addition, critical gaps in knowledge and important future directions are discussed.

Keywords: kisspeptin, *Kiss1*, Kiss1r, amygdala, GnRH, LH, reproduction, puberty

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INTRODUCTION

Kisspeptin, encoded by the *Kiss1* gene, is essential for reproduction. Humans and mice lacking *Kiss1* or its receptor, *Kiss1r*, have deficits in puberty onset, reproductive hormone release, and fertility (1–4). In humans and rodents, kisspeptin treatment directly stimulates GnRH neurons to increase downstream LH and FSH secretion (5–12). Kisspeptin-synthesizing neurons are primarily located in two regions of the hypothalamus, the anteroventral periventricular (AVPV)/rostral periventricular (PeN) continuum and the arcuate nucleus (ARC) (9, 13–17). The AVPV/PeN and ARC *Kiss1* populations are differentially regulated by testosterone (T) and estradiol (E_2). In the AVPV/PeN, E_2 increases *Kiss1* levels and gonadectomy (GDX) decreases *Kiss1* levels, supporting the proposed role of these neurons in mediating E_2-positive feedback induction of the pre-ovulatory LH surge in females (13–15). Supporting this, *Kiss1* neurons are sexually differentiated, being more numerous, and expressing higher *Kiss1* mRNA levels in females (15, 18). By contrast, *Kiss1* expression in the ARC increases following GDX and decreases with T or E_2 treatment. Thus, ARC *Kiss1* neurons are thought to participate in gonadal steroid negative feedback and the pulsatile release of GnRH secretion (13–15).

Smaller populations of *Kiss1* neurons have also recently been identified in several extra-hypothalamic areas, including the medial amygdala (MeA), bed nucleus of the stria terminalis (BnST), and lateral septum (9, 19–23). However, these extra-hypothalamic neurons have not been studied extensively, and their regulation and functions are only now beginning to be determined. In particular, the MeA region, part of the limbic system, is known to have numerous behavioral and physiological functions, including (but not limited to) roles in sexual behavior and reproductive physiology (24–31). This review summarizes what is currently known about the regulation, development, and function(s) of MeA kisspeptin neurons as well as kisspeptin signaling directly within the MeA.

IDENTIFICATION AND REGULATION OF KISSPEPTIN NEURONS IN THE MeA

Kiss1 neurons in the MeA were first observed in male mice in 2004 (9) but were not directly studied until 2011 when Kim et al. first tested whether rodent MeA *Kiss1* neurons are regulated by sex steroids (19), as occurs for hypothalamic kisspeptin neurons. The authors found that, as in the AVPV/PeN, *Kiss1* levels in the MeA are strongly upregulated by sex steroids in both mice and rats. Specifically, in adult, gonadectomized (GDX) mice and rats, there are few, if any, detectable MeA *Kiss1* cells, whereas exogenous treatment with T or E_2 significantly increases MeA *Kiss1* cell number (19) (**Figure 1A**). Unlike E_2 treatment, DHT treatment did not increase MeA *Kiss1* expression, indicating the sex steroid upregulation of MeA *Kiss1* occurs through estrogen receptors (ERs) rather than androgen receptors (19). Xu and colleagues (21) similarly determined that exogenous E_2 treatment in male and female rats upregulates both MeA *Kiss1* mRNA and MeA kisspeptin protein expression (21).

The MeA region is known to express high levels of sex steroid receptors, including both ERα and ERβ (the latter is not highly expressed in the ARC or AVPV/PeN), but it was not initially known which ER mediates E_2 stimulation of MeA *Kiss1* neurons. Recent data from ERαKO mice indicate that both the hypothalamic (13, 14, 32, 33) and MeA (33) *Kiss1* cells are primarily regulated *via* ERα. In the ARC and AVPV/PeN, E_2 treatment alters *Kiss1* expression in wild-type (WT) mice but not in ERαKO mice (13, 14, 32, 33). Similarly, in the MeA, E_2 robustly increases *Kiss1* expression in WT mice, whereas ERαKO mice given E_2 failed to show comparable large increases in MeA *Kiss1* levels (33). Thus, substantial E_2 stimulation of MeA *Kiss1* requires ERα signaling. However, E_2-treated ERαKO mice did show a minor increase in MeA *Kiss1* levels compared with non-E_2-treated ERαKOs, indicating that another ER may partially compensate for the loss of ERα. This partial increase in *Kiss1* expression in E_2-treated ERαKO mice is unique to the MeA, as hypothalamic *Kiss1* levels in E_2-treated ERαKOs were comparable to that of non-E_2-treated ERαKOs (33). By contrast, *Kiss1* expression in the MeA (and hypothalamus) of ERβKO mice of both sexes mirrored that of WT mice under all hormonal conditions (13, 33). Thus, unlike ERα, ERβ is not required for E_2's regulation of either hypothalamic or MeA *Kiss1*.

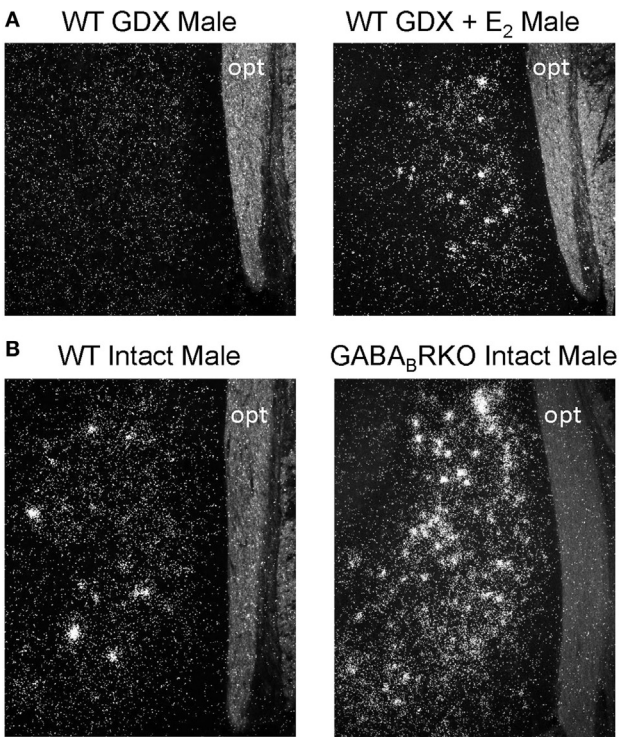

FIGURE 1 | Medial amygdala (MeA) *Kiss1* expression (silver grains in *in situ* hybridization photomicrographs) is regulated by E_2 and GABA signaling. **(A)** Gonadectomized (GDX) mice have few, if any, *Kiss1* cells in the MeA, whereas E_2 treatment significantly increases MeA *Kiss1* expression. **(B)** Gonad-intact $GABA_BR$ KO mice have substantially more MeA *Kiss1* than gonad-intact wild-type (WT) males, despite comparable circulating sex steroid levels. opt, optic tract.

In the MeA of gonad-intact rats and mice, *Kiss1* expression is higher in males than in diestrus females (19). However, gonad-intact female rats have increased MeA *Kiss1* expression during proestrus (when circulating E_2 levels are highest), and relatively low levels during estrus and diestrus (19). Thus, the sex difference in MeA *Kiss1* expression between gonad-intact males and females is likely due to differences in circulating sex steroid levels. Indeed, when E_2 levels are equalized between males and females, the previously observed sex difference in MeA *Kiss1* levels disappears, with males and females now showing comparable elevated MeA *Kiss1* (19, 21) and kisspeptin (21) expression.

In addition to E_2, GABA signaling *via* $GABA_BR$ also strongly regulates MeA *Kiss1* expression. In gonad-intact $GABA_BR$ KO mice, MeA *Kiss1* levels are drastically elevated in comparison to WT mice (22) (**Figure 1B**). This large increase in MeA *Kiss1* expression is not due to altered sex steroid levels, as circulating T (males) or E_2 (females) were similar between WT and $GABA_BR$ KOs (22). Interestingly, the elevated *Kiss1* levels in the MeA of $GABA_BR$ KOs are typically much greater than observed for E_2 stimulation of *Kiss1* in this region in WT mice. This suggests that endogenous $GABA_BR$ signaling is a very potent regulator of MeA *Kiss1*. Interestingly, this large upregulation of *Kiss1* levels in $GABA_BR$ KO mice was exclusive to extra-hypothalamic *Kiss1* expression, as AVPV and ARC *Kiss1* expression were normal in $GABA_BR$ KO mice (22). Thus, GABA signaling *via* $GABA_BR$ may normally serve to inhibit MeA *Kiss1* expression, whereas $GABA_BR$ signaling seems to have no major effect on hypothalamic *Kiss1* levels. Whether this observed effect is due to $GABA_BR$ signaling directly in MeA *Kiss1* neurons remains to be determined, though MeA *Kiss1* neurons do express $GABA_BR$ (22).

DEVELOPMENTAL EXPRESSION OF KISSPEPTIN NEURONS IN THE MeA

Although MeA *Kiss1* expression is detectable in adult rodents, especially when sex steroids are elevated, MeA *Kiss1* expression is not detected in prepubertal rodents, at postnatal day (PND) 14 in mice (22) or PND 19 and earlier in rats (34). Similarly, MeA

Kiss1 expression was absent at PND 14 in $GABA_BR$ KO mice despite being dramatically elevated in these mice in adulthood (22). These findings indicate that in juvenile and prepubertal rodents either (1) MeA *Kiss1* neurons are not yet present or (2) MeA *Kiss1* neurons are present but not able to express *Kiss1*. The latter possibility might reflect the absence of elevated circulating sex steroids before puberty, therefore precluding notable MeA *Kiss1* expression at young ages.

The developmental pattern of MeA *Kiss1* expression was recently examined every 5 days, from PND 15 until PND 40, in gonad-intact C57BL6 male mice, with puberty occurring around PND 35 (33). MeA *Kiss1* was first detected at very low levels around PND 20–25, but did not significantly increase until PND 35, with highest expression at PND 40 (the oldest age examined). Circulating T levels in the same mice mirrored the developmental pattern of MeA *Kiss1* expression, similarly increasing at PND 35 (as expected with puberty) (33). However, whether the pubertal increase in T caused or resulted from the increase in MeA *Kiss1* expression is unclear. Therefore, the second study treated juvenile (PND 14) male mice with high-dose E_2 for 4 days to determine if elevated E_2 exposure at this prepubertal age could prematurely increase MeA *Kiss1* expression (33). Indeed, juvenile E_2 treatment increased MeA *Kiss1* at PND 18 versus untreated controls, demonstrating that by PND 18, MeA *Kiss1* neurons are present and capable of expressing notable *Kiss1* if sex steroids are sufficiently elevated (33). Thus, increases in MeA *Kiss1* expression around puberty are likely a response to rising circulating sex steroid levels at this time. Whether this emergence of notable MeA *Kiss1* levels at puberty is functionally relevant to the pubertal process or to other physiological/behavioral processes is currently unknown.

MeA KISSPEPTIN NEURON PROJECTIONS AND POSSIBLE FUNCTIONS

Understanding where MeA kisspeptin neurons project to can inform upon their potential functions. Research examining the afferent and efferent projections of MeA kisspeptin neurons only recently began, and thus, little is currently known. Using double-label immunohistochemistry, Pineda and colleagues demonstrated in male rats that some MeA kisspeptin neurons receive neural appositions from "upstream" neurons containing tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, and vasopressin (35). 25 and 11% of MeA kisspeptin neurons receive inputs from TH and vasopressin neurons, respectively (35). However, it is currently unknown if MeA kisspeptin neurons express either dopamine receptors or vasopressin receptors or from where in the brain the dopamine or vasopressin signaling originates. It is also unknown whether dopamine or vasopressin alters MeA *Kiss1* or kisspeptin expression or neuronal activity, and if so, whether such regulation is positive or negative. Regardless, these data suggest that MeA kisspeptin neurons may have a role in participating in dopamine- or vasopressin-dependent physiology/behaviors, such as motivation and reward-seeking behaviors or social behaviors.

In rodents, the MeA has been implicated in regulating reproductive physiology because MeA lesions in rats disrupt ovarian cycles and alter pubertal timing (24–26, 31, 36–38). However, the specific cell types in the MeA that influence the reproductive axis are unknown. Given kisspeptin's potent actions on GnRH neurons, *Kiss1* neurons in the MeA are good candidates to serve as reproductive signalers from this brain region. Supporting this possibility, in female mice, MeA kisspeptin neurons send axonal projections to the preoptic area (POA), where many GnRH neurons reside (23). Moreover, injections of an AAV-DIO-YFP virus into the MeA of male KissCre-GFP mice revealed that ~15% of GnRH neurons in the POA receive close fiber appositions from MeA kisspeptin neurons (35). These anatomical data suggest that MeA kisspeptin neurons may have the potential to modulate a subset of GnRH neurons, though it should be noted that *most* GnRH neurons did not receive MeA kisspeptin contacts (which may be a result of technical/methodological limitations). Additional studies are therefore needed to determine if—and to what degree—MeA kisspeptin cells project to GnRH neurons.

Anterograde tracing studies in male rats recently demonstrated that the accessory olfactory bulb (AOB), but not the main olfactory bulb, projects to MeA kisspeptin neurons (35). This suggests that MeA kisspeptin neurons may also have a role in processing or responding to olfactory/pheromone cues, potentially social signals. Supporting this, selective chemogenetic activation of MeA kisspeptin neurons in male mice increased the amount of time males spent investigating estrous females (39). In that study, male KissCre-GFP mice received bilateral injections of a stimulatory viral DREADD receptor construct into the posterodorsal MeA, followed 4 weeks later with peripheral injection of clozapine-N-oxide (CNO) to selectively activate MeA kisspeptin neurons (39). Although selective DREADD activation of MeA kisspeptin neurons increased males' investigation of females, it also increased the amount of time males spent with juvenile conspecifics (39), indicating that MeA kisspeptin neurons may modulate behavioral responses to any social odors, not just opposite-sex odors. Importantly, it remains unknown whether these induced behavioral changes are due to kisspeptin or another neuropeptide/neurotransmitter co-released from MeA kisspeptin neurons when activated. A prior study in *Kiss1r* KO mice showed that kisspeptin signaling is required for proper opposite-sex odor preference (40). Thus, it may be specifically kisspeptin (rather than another signaling factor) from these MeA neurons that are modulating opposite-sex and juvenile odor preference, but this still needs to be determined. Interestingly, in addition to receiving afferent projections from the AOB, MeA kisspeptin neurons also send reciprocal projections back to the AOB region, specifically the mitral and granule layers in mice and the granule layer in rats (23, 35). Mitral and granule cells are part of a reciprocal feedback circuit, with mitral cells exciting granule cells *via* glutamate and granule cells inhibiting mitral cells *via* GABA signaling (41–46). The functional relevance of such MeA kisspeptin signaling to the AOB cells remains to be determined.

Finally, Adekunbi and colleagues recently found that selective DREADD activation of MeA kisspeptin neurons reduces anxiety, with CNO-treated mice spending more time exploring the open arms in an elevated-plus maze than control mice. This

suggests that MeA kisspeptin neurons may lower anxiety-related behaviors. However, these results differ from previous data showing intracerebroventricular (icv) injection of kisspeptin-13 in male rats decreased time spent in the open arms of the elevated-plus maze, suggesting that kisspeptin increased anxiety (47). These conflicting findings may reflect species differences or different methodologies (i.e., selective DREADD activation of just MeA kisspeptin neurons versus increased kisspeptin signaling throughout the brain). It is also possible that another neuropeptide/neurotransmitter released from MeA kisspeptin neurons caused the anxiolytic behavior in the DREADD study. Additional research is needed to clarify the role of MeA kisspeptin neurons in modulating anxiety.

KISSPEPTIN SIGNALING WITHIN THE MeA

Thus far, this review has focused on the regulation, projections, and functions of kisspeptin neurons residing in the MeA, but several studies have also examined the role of kisspeptin signaling within the MeA. Intra-MeA injections of kisspeptin-10 in GDX + E_2 (diestrus E_2 levels) female rats dose dependently increased LH within an hour (48). Thus, kisspeptin acting directly within the MeA may also modulate GnRH release. Supporting this hypothesis, intra-MeA injection of a kisspeptin antagonist, peptide-234, in GDX + E_2-treated females decreased LH secretion 2–4 h later (48). This suggests that *endogenous* kisspeptin signaling acting within the MeA is important for normal GnRH/LH secretion. Although it is possible that the kisspeptin or antagonist treatment spread outside of the MeA to other brain areas, other studies showed that icv injections of this same dose, 100 pmol, had no effect on LH levels (49, 50). Therefore, in female rats, kisspeptin may act directly within the MeA to modulate GnRH/LH release. In male rats, a similar dose of 100 pmol of kisspeptin-10 injected directly into the MeA also increased LH levels (51), with a greater LH increase after a higher dose, 1 nmol (51). Peptide-234 injected directly into the MeA of male rats did not alter LH (51), unlike in females, which may reflect a sex difference in endogenous MeA kisspeptin signaling targeting the MeA. In addition to increasing LH, intra-MeA injections of kisspeptin-10 also increased ex-copula erections in rats, which was prevented by concurrent kisspeptin antagonist treatment (51). Thus, kisspeptin signaling in the MeA of male rodents may regulate reproductive physiology and behavior.

The functional consequences of kisspeptin signaling directly within the MeA discussed above have only been examined in rats, for which there is some evidence of kisspeptin receptor, *Kiss1r*, in the MeA. Radiolabeled *in situ* hybridization found abundant expression of *Kiss1r* in the rat amygdala (52). However, non-radioactive ISH found no *Kiss1r* expression in the MeA (53), perhaps because non-radioactive ISH is less sensitive and, therefore, unable to detect low *Kiss1r* expression (53). *Kiss1r* expression has not been detected in the MeA of mice (54), indicating the functions of kisspeptin signaling acting in the MeA may be species dependent, though further studies on this issue are needed.

If kisspeptin can in fact act directly in the MeA, then where is such kisspeptin signaling coming from? It is currently unknown if any kisspeptin neuronal population, hypothalamic or extra-hypothalamic, projects to the MeA to be the potential source of kisspeptin acting in this area. One possibility could be that MeA kisspeptin neurons project locally, within the MeA, to regulate other neurons in this region, but this has not been studied. Additional research is therefore needed to determine if MeA-derived kisspeptin acts locally within the MeA or if kisspeptin action within the MeA is due to kisspeptin release from other areas.

GAPS IN KNOWLEDGE AND FUTURE DIRECTIONS

The MeA region is implicated in many diverse functions and behavioral processes, and deciphering the specific function(s) of kisspeptin neurons in this region is therefore not simple. **Figure 2** summarizes our current understanding of the regulation and function(s) of MeA kisspeptin neurons. Although MeA kisspeptin neurons are clearly regulated by E_2 and GABA signaling, *via* ERα and $GABA_BR$, respectively, whether this regulation occurs directly or indirectly on MeA kisspeptin neurons is unknown. Supporting a possible direct regulation, MeA kisspeptin neurons express $GABA_BR$, and ERα is heavily expressed in the MeA region (though ERα specifically in MeA kisspeptin neurons has not been determined). TH and

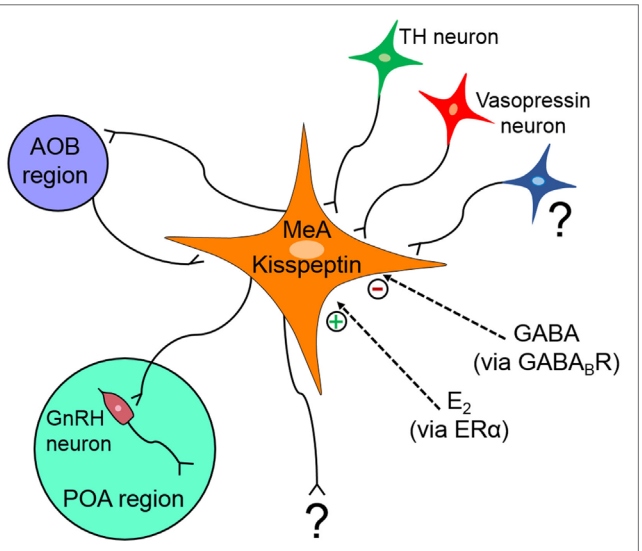

FIGURE 2 | Schematic diagram summarizing what is known about the regulation and projections of medial amygdala (MeA) kisspeptin neurons. MeA kisspeptin neurons receive projections from tyrosine hydroxylase (TH) and vasopressin neurons, as well as from neurons originating in the accessory olfactory bulb (AOB). MeA kisspeptin neurons have efferent projections back to the AOB and also to some GnRH neurons in the preoptic area (POA). MeA *Kiss1* neurons are upregulated by E_2 *via* ERα and downregulated by GABA signaling *via* $GABA_BR$, but it is currently unknown if this E_2 and GABA regulation occurs directly on MeA *Kiss1* cells or indirectly via intermediary neurons. ? = unknown afferent or efferent projections of MeA kisspeptin neurons.

vasopressin neurons project to MeA kisspeptin neurons; however, whether MeA kisspeptin neurons express dopamine or vasopressin receptors is unknown, as is any effect of dopamine or vasopressin on MeA kisspeptin neurons. Additional research is also needed to determine what other factors may regulate MeA kisspeptin neurons. Other than kisspeptin, it is also currently unknown what other signaling factors are produced by MeA *Kiss1* neurons. This is important for knowing whether the LH or behavioral responses following DREADD activation of these neurons are due to kisspeptin or some other co-released neuropeptide/neurotransmitter.

MeA *Kiss1* expression is first detected around puberty, when gonadal steroids are also rising. Data suggest that the increase in MeA *Kiss1* at this time is likely caused by the pubertal increases in gonadal sex steroids, but this requires further examination. Regardless, the presence of notable *Kiss1* in the MeA only at puberty and beyond suggests that the functional relevance of kisspeptin released from the MeA is restricted to processes during sexual maturation and/or adulthood.

MeA kisspeptin neurons send axon projections to some GnRH neurons, which may indicate MeA kisspeptin neurons can modulate the reproductive axis. This is supported by LH increases after MeA *Kiss1* neuron DREADD activation. Lesions of the entire MeA disrupt ovarian cycles and alter puberty, perhaps because of ablated MeA kisspeptin neurons, but this has not been studied. MeA kisspeptin neurons also form a reciprocal circuit with the AOB, and activation of MeA kisspeptin neurons increases social interactions in mice, indicating MeA kisspeptin neurons may influence social and/or sexual olfactory processing. Activation of MeA kisspeptin neurons also decreases anxiety behavior, suggesting kisspeptin or another neuropeptide/neurotransmitter released from these neurons influences anxiety. Other possible neural targets of MeA kisspeptin neurons remain to be determined and are needed to understand the functions, reproductive or otherwise, of these particular MeA neurons.

AUTHOR CONTRIBUTIONS

Both authors researched and wrote/edited the article and designed the figures.

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