MOLECULAR EVOLUTION OF GPCRS

# Kisspeptin/kisspeptin receptors

**Jérémy Pasquier, Nédia Kamech, Anne-Gaëlle Lafont, Hubert Vaudry[1], Karine Rousseau and Sylvie Dufour**

Laboratory of Biology of Aquatic Organisms and Ecosystems (BOREA), UMR CNRS 7208, IRD207, Université Pierre and Marie Curie – Paris 6, Muséum National d'Histoire Naturelle, 7 rue Cuvier, CP32, 75231 Paris Cedex 05, France
[1]Laboratory of Neuronal and Neuroendocrine Differentiation and Communication, INSERM U982, Institute for Research and Innovation in Biomedicine (IRIB), University of Rouen, 76821 Mont-Saint-Aignan, France

Correspondence should be addressed to S Dufour
**Email**
dufour@mnhn.fr

## Abstract

Following the discovery of kisspeptin (Kiss) and its receptor (GPR54 or KissR) in mammals, phylogenetic studies revealed up to three Kiss and four KissR paralogous genes in other vertebrates. The multiplicity of Kiss and KissR types in vertebrates probably originated from the two rounds of whole-genome duplication (1R and 2R) that occurred in early vertebrates. This review examines compelling recent advances on molecular diversity and phylogenetic evolution of vertebrate Kiss and KissR. It also addresses, from an evolutionary point of view, the issues of the structure–activity relationships and interaction of Kiss with KissR and of their signaling pathways. Independent gene losses, during vertebrate evolution, have shaped the repertoire of *Kiss* and *KissR* in the extant vertebrate species. In particular, there is no conserved combination of a given *Kiss* type with a *KissR* type, across vertebrate evolution. The striking conservation of the biologically active ten-amino-acid C-terminal sequence of all vertebrate kisspeptins, probably allowed this evolutionary flexibility of Kiss/KissR pairs. KissR mutations, responsible for hypogonadotropic hypogonadism in humans, mostly occurred at highly conserved amino acid positions among vertebrate KissR. This further highlights the key role of these amino acids in KissR function. In contrast, less conserved KissR regions, notably in the intracellular C-terminal domain, may account for differential intracellular signaling pathways between vertebrate KissR. Cross talk between evolutionary and biomedical studies should contribute to further understanding of the Kiss/KissR structure–activity relationships and biological functions.

**Key Words**

- kisspeptin
- receptor
- evolution
- vertebrates

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## Introduction

The kisspeptin system, composed of the ligand, kisspeptin (Kiss), and its receptor, kisspeptin receptor (KissR), has attracted the attention of most reproductive physiologists during the past 10 years. Even though the involvement of this neuroendocrine system in reproductive physiology was unveiled only in 2003, the first characterizations of its components date back to the 1990s. In 1996, Welch's group in Hershey (Pensylvania) isolated a novel cDNA from malignant melanoma cells that had lost the potential to metastasize after the introduction of human chromosome 6 (Lee *et al*. 1996). They designated this cDNA, Kiss1, in reference to Hershey's candy specialty, the

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'Hershey Chocolate Kiss' and demonstrated that its transfection into C8161 melanoma cells suppressed metastasis. In 1999, a novel G protein-coupled receptor (GPCR), designated GPR54, was isolated in rat (Lee *et al.* 1999). The ortholog of GPR54 in human was cloned 2 years later and termed either AXOR12 (Muir *et al.* 2001) or hOT7T175 (Ohtaki *et al.* 2001). The same year, in order to identify the endogenous ligands of this orphan receptor, three research groups used heterologous cell models, i.e., CHO-K1 (Kotani *et al.* 2001), HEK293 (Muir *et al.* 2001) and B16-BL6 (Ohtaki *et al.* 2001), which they transfected with GPR54 (Kotani *et al.* 2001), AXOR12 (Muir *et al.* 2001), or hOT7T175 (Ohtaki *et al.* 2001). Three surrogate agonist peptides were thus isolated from the placenta extracts and named either kisspeptins (54, 14, and 13 amino acid peptides: (Kotani *et al.* 2001)) or metastin (54 amino acid peptide: (Ohtaki *et al.* 2001)).

In 2003, three groups revealed the link between hypogonadotropic hypogonadism (HH) and mutation in GPR54, via reports of clinical cases (de Roux *et al.* 2003, Seminara *et al.* 2003) and knockout mouse models (Funes *et al.* 2003, Seminara *et al.* 2003). These studies showed that humans or mice in which GPR54 was mutated or lacking could not go through normal puberty, had small gonads, exhibited low plasma concentrations of sex steroids and gonadotropins, and were sterile. This major breakthrough in reproductive physiology was thereafter fully documented and the role of the kisspeptin system in the onset of puberty and reproduction was investigated, revealing a major stimulatory action on gonadotropin-releasing hormone (GNRH) neurons and possible direct effects on the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (for reviews see Roa *et al.* (2008), Pinilla *et al.* (2012) and Gopurappilly *et al.* (2013)). Recent studies involving knockdown experiments in fish have revealed the importance of the kisspeptin system in early development, especially in neurulation (Hodne *et al.* 2013).

Characterization of the kisspeptin system has been carried out in various vertebrate species, leading to the discovery of multiple genes encoding kisspeptins (from *Kiss1* to *Kiss3*) (Biran *et al.* 2008, Felip *et al.* 2009, Kitahashi *et al.* 2009, Lee *et al.* 2009*b*, Pasquier *et al.* 2012*b*) as well as multiple genes encoding their receptors (from *KissR1* to *KissR4*) (Biran *et al.* 2008, Felip *et al.* 2009, Lee *et al.* 2009*b*, Pasquier *et al.* 2012*a*). This diversity among kisspeptin systems led to studies on its evolutionary history (Akazome *et al.* 2010, Tsutsui *et al.* 2010, Pasquier *et al.* 2012*a*,*b*, Tena-Sempere *et al.* 2012). We review here recent advances on molecular diversity and phylogenetic evolution, structure–activity relationships, and signaling mechanisms of vertebrate kisspeptins and their receptors.

## Molecular diversity and phylogenetic evolution of kisspeptins in vertebrates

Kisspeptins are members of the RFamide peptide family, named after the C-terminal motif, arginine (R) and phenylalanine (F) coupled to an amide function, shared by most peptides of this superfamily. The first characterized peptide of the RFamide family was isolated from the ganglia of the mollusc, *Macrocallista nimbosa*, and shown to act as a cardiac stimulator in this species (Price & Greenberg 1977*a*,*b*). Since this discovery, an increasing number of Rfamide-related peptides has been identified in other groups of metazoa including vertebrates. Besides kisspeptins, the RFamide superfamily also includes neuropeptides FF (NPFF), gonadotropin-inhibitory hormone (GNIH), prolactin-releasing peptides (PrRP), and 26RFamide peptides (Fukusumi *et al.* 2006, Tsutsui *et al.* 2010). Molecular phylogeny of the genes coding for these peptides allowed definition of the so-called 'RFamide' superfamily, even though some peptides do not end in 'RF', but for instance in 'RY' as exemplified by various vertebrate kisspeptins.

### *Kiss* gene, precursor structure and multiple mature peptide forms

In mammals, the *Kiss1* gene is composed of three exons and two introns as shown in human (Cartwright & Williams 2012), pig (*Sus scrofa*) (Tomikawa *et al.* 2010), and mouse (*Mus musculus*) (Tomikawa *et al.* 2012). The first exon only codes for the UTR, while the coding sequence (CDS) is split between exons 2 and 3 (Fig. 1). A similar gene structure of three exons and two introns has also been suggested for the zebrafish *Kiss1* gene (Kitahashi *et al.* 2009). In other vertebrates, only the sequences corresponding to exons 2 and 3 have been described so far, similarly containing the *Kiss* CDS (for review see Tena-Sempere *et al.* (2012)).

After the discovery of the single *Kiss1* gene in eutherian mammals, investigations of various vertebrates revealed the existence of up to three paralogous *Kiss* genes (*Kiss1*, *Kiss2*, and *Kiss3* (Pasquier *et al.* 2012*b*)).

While no alternative splicing of the *Kiss1* gene has been identified, to our knowledge, in mammals, a recent study in a teleost species, the Senegalese sole (*Solea senegalensis*), has revealed the existence of two *kiss2* transcript isoforms. These isoforms are generated by the retention of an intron between the two exons (Mechaly *et al.* 2011). The shortest isoform (*Kiss2_v1*) encodes a



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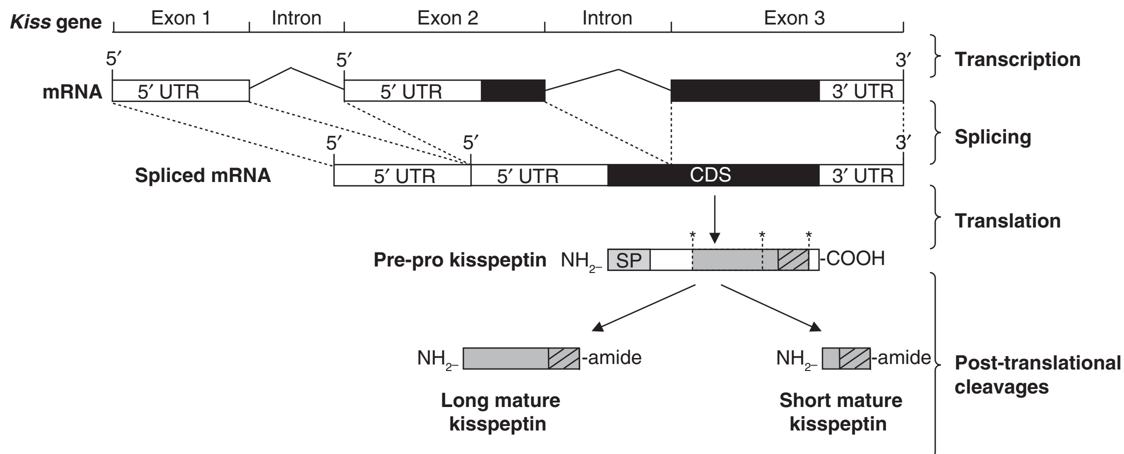


**Figure 1**
*Kiss* gene transcription, translation, and post-translational processing. CDS, coding sequence; SP, signal peptide. The minimal bioactive sequence, composed by ten amino acids, Kp(10), is represented by a hatched square.



classic Kiss2 precursor, while the longest one (*Kiss2_v2*) encodes a truncated and non-functional protein. To date, the Senegalese sole is the only species presenting such an alternative splicing process (Mechaly *et al*. 2011).

Each *Kiss* gene encodes a polypeptide precursor, which is secondarily processed to give size-variants of kisspeptins (Fig. 1). In humans, three mature peptides have been purified from placenta, i.e., kisspeptin1–54 (hKp1(54)), kisspeptin1–14 (hKp1(14)), and kisspeptin1–13 (hKp1(13)) (Kotani *et al*. 2001, Muir *et al*. 2001). To date, in other vertebrates, only a few mature forms of kisspeptin have been purified, i.e., Kp2(12) from Xenopus brain (Lee *et al*. 2009*b*), Kp2(12) from red-eared slider turtle brain, and Kp2(13) from Masu salmon brain (Osugi *et al*. 2013).

All these mature peptides encompass a C-terminal 10-amino acid sequence, (Kp(10)), which represents the minimal sequence for bioactivity (Fig. 2A; Kotani *et al*. 2001). This Kp(10) sequence is highly conserved among vertebrates (Fig. 2A). Positions 1 and 10 correspond to aromatic amino acids, Tyr or Phe, in all sequences. Positions 2, 4, 6, 7, 8, and 9 are fully conserved among vertebrates, except for mutations encountered in degenerating pseudogenes, such as the recently described mallard duck and crocodile Kp2-like genes (Fig. 2A). In all vertebrate species, the Kp(10) sequences are flanked at their C-terminal end by the sequence, ‘G-Basic-Basic’, or ‘G-Basic-Stop’, characteristic of the conserved proteolytic cleavage and α-amidation sites of neuropeptides (Eipper *et al*. 1992). In contrast, the remaining part of the precursor sequence is highly variable among vertebrates and between paralogous kisspeptin precursors within a given species. This latter feature has been exploited to generate specific antibodies against distinct kisspeptin precursors (Servili *et al*. 2011).

## Diversity of *Kiss* genes in vertebrates

Since the discovery of the first *Kiss1* gene in humans, several *Kiss* genes have been identified in most vertebrate lineages including cyclostomes, chondrichthyes, teleosts, amphibians, and sauropsids (Lee *et al*. 2009*b*, Pasquier *et al*. 2012*b*, Tena-Sempere *et al*. 2012; Fig. 3). Phylogenetic and syntenic analyses (Lee *et al*. 2009*b*, Kim *et al*. 2012, Pasquier *et al*. 2012*a*,*b*) revealed that the *Kiss* genes can be classified into three different clades (i.e., *Kiss1*, *Kiss2*, and *Kiss3* clades, according to the recent nomenclature devised by Pasquier *et al*. (2012*b*)). In addition, syntenic analysis supports the potential existence of a fourth *Kiss* gene (*Kiss4*) in early vertebrates, although it has not yet been identified in any extant vertebrate species (Pasquier *et al*. 2012*b*).

Among extant sarcopterygians, various situations concerning the number of paralogous *Kiss* genes have been observed. In mammals, prototherians such as platypus (*Ornithorhynchus anatinus*) possess the *Kiss1* and *Kiss2* genes (Lee *et al*. 2009*b*). Eutherian species were thought to possess only the *Kiss1* gene, but a recent study has revealed the presence of a pseudo-*Kiss2* gene, with a highly mutated Kp2(10) sequence (Osugi *et al*. 2013) in primates, including humans. In sauropsids, various situations have been reported. In squamates, the green anole (*Anolis carolinensis*) only possesses the *Kiss2* gene, while both the *Kiss1* and *Kiss2* genes have been predicted


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**A**

| | 1 2 3 4 5 6 7 8 9 10 | |
|---|---|---|
| Human Kp1 | YNWNSFGLRF | (Lee *et al.* 1996) |
| Mouse Kp1 | YNWNSFGLRY | (Tomikawa *et al.* 2012) |
| Opossum Kp1 | YNWNSFGLRY | (Lee *et al.* 2009*b*) |
| Platypus Kp1 | YNWNSFGLRY | (Lee *et al.* 2009*b*) |
| Crocodile Kp1 | YNWNSFGLRY | (Pasquier *et al.* 2014) |
| Chinese turtle Kp1 | YNWNSFGLRY | (Pasquier *et al.* 2014) |
| Python Kp1 | YNLNSFGLRY | (Pasquier *et al.* 2014) |
| Xenopus Kp1 | YNWNSFGLRY | (Lee *et al.* 2009*b*) |
| Coelacanth Kp1 | YNWNTFGLRY | (Pasquier *et al.* 2012*a*) |
| Sea bass Kp1 | YNLNSFGLRY | (Felip *et al.* 2009) |
| Zebrafish Kp1 | YNLNSFGLRY | (van Aerle 2008) |
| European eel Kp1 | YNWNSFGLRY | (Pasquier *et al.* 2012*a*) |
| Spotted gar Kp1 | YNWNSFGLRY | (Pasquier *et al.* 2012*a*) |
| Elephant shark Kp1 | YNWNSFGLRY | (Lee *et al.* 2009*b*) |
| Little skate Kp1 | FNWNSFGLRY | (Pasquier *et al.* 2014) |
| Platypus Kp2 | FNFNPFGLRF | (Lee *et al.* 2009*b*) |
| Mallard duck Kp2-like | FIFNPFGLGF | (Pasquier *et al.* 2014) |
| Crocodile Kp2-like | FNFNPFRFSF | (Osugi *et al.* 2013) |
| Chinese turtle Kp2 | FNFNPFGLRF | (Osugi *et al.* 2013, Pasquier *et al.* 2014) |
| Python Kp2 | FNFNSFGLRF | (Pasquier *et al.* 2014) |
| Green anole Kp2 | FNFNPFGLRF | (Pasquier *et al.* 2012*a*) |
| Xenopus Kp2 | FNFNPFGLRF | (Lee *et al.* 2009*b*) |
| Coelacanth Kp2 | FNFNPFGLRF | (Pasquier *et al.* 2012*a*) |
| Tetraodon Kp2 | FNLNPFGLRF | (Pasquier *et al.* 2012*a*) |
| Stickleback Kp2 | FNFNPFGLRF | (Lee *et al.* 2009*b*) |
| Sea bass Kp2 | FNFNPFGLRF | (Felip *et al.* 2009) |
| Zebrafish Kp2 | FNYNPFGLRF | (Biran *et al.* 2008) |
| European eel Kp2 | FNRNPFGLRF | (Pasquier *et al.* 2012*a*) |
| Spotted gar Kp2 | FNFNPFGLRF | (Pasquier *et al.* 2012*a*) |
| Elephant shark Kp2 | FNFNPFGLRF | (Lee *et al.* 2009) |
| Little skate Kp2 | FNFNPFGLRF | (Pasquier *et al.* 2014) |
| Xenopus Kp3 | YNVNSFGLRF | (Lee *et al.* 2009*b*) |
| Coelacanth Kp3-like | FNWNSFGLRF | (Pasquier *et al.* 2012*a*) |
| Elephant shark Kp3-like | YNLNSFGLKF | (Pasquier *et al.* 2012*a*) |
| Sea lamprey Kp-a | YNWNSFGLRF | (Lee *et al.* 2009*b*) |
| Sea lamprey Kp-b | FNYNPFGLRF | (Lee *et al.* 2009*b*) |

**B**

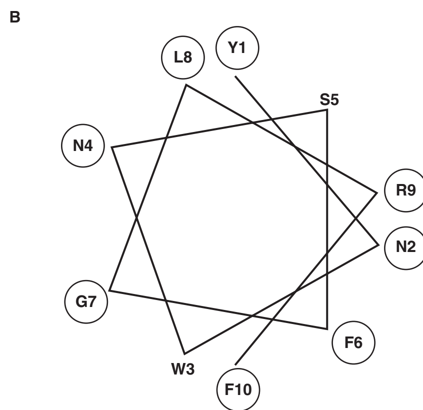


**Figure 2**
(A) Amino acid sequences of various vertebrate kisspeptin-10 (Kp10). Amino acids are colored according to their physicochemical properties. (B) Schematic helical top view of the human biological active kisspeptin Kp1(10). Conserved residues in Kp(10) vertebrate species are circled. Diagram reworked from http://bioweb2.pasteur.fr/docs/EMBOSS/pepwheel.html.

in the Indian python genome (Pasquier *et al.* 2014). Chelonians also possess the *Kiss1* and *Kiss2* genes, as both genes have been detected in multiple turtle genomes (Osugi *et al.* 2013, Pasquier *et al.* 2014). In crocodilians, only the *Kiss1* gene has been predicted to be potentially functional (Pasquier *et al.* 2014), while the *Kiss2* gene would represent a pseudogene with a highly mutated Kp2(10) (Osugi *et al.* 2013; Fig. 2A). Until recently, the *Kiss* genes were considered to be absent from the genomes of birds. However, our recent phylogenomic investigations revealed the existence of a *Kiss2* pseudogene in the genomes of three bird species, i.e., the mallard duck, the zebra finch, and the rock pigeon (Pasquier *et al.* 2014). The bird lineage represents, so far, the only example of the absence of functional *Kiss* genes among vertebrates. In amphibians, *Xenopus tropicalis* possesses three *Kiss* genes (*Kiss1*, *Kiss2*, and *Kiss3*) (Lee *et al.* 2009*b*). Only *Kiss1* and *Kiss2* transcripts have been described in *Xenopus laevis* and only *Kiss2* in the bullfrog (*Rana catesbeiana*) (Lee *et al.* 2009*b*). Concerning the early sarcopterygians, the *Kiss1*, *Kiss2*, and *Kiss3* genes have been predicted from the coelacanth (*Latimeria chalumnae*) genome (Pasquier *et al.* 2012*b*). However, considering the short open reading frame (ORF), *Kiss3* should be considered to be a pseudo-*Kiss3* gene in the absence of further functional evidence (Pasquier *et al.* 2012*b*).

In extant actinopterygians, a maximum of two *kiss* genes (*Kiss1* and *Kiss2*) have been identified. In a holostean species, the spotted gar (*Lepisosteus oculatus*), *Kiss1* and *Kiss2* have been predicted from the genomic data (Pasquier *et al.* 2012*b*). To date, in teleosts, two situations have been reported. Only the *Kiss2* gene is present in some species such as fugu (*Takifugu niphobles*), tetraodon (*Tetraodon nigroviridis*), and stickleback (*Gasterosteus aculeatus*) (Pasquier *et al.* 2012*b*). In contrast, the *Kiss1* and *Kiss2* genes have been characterized in some other species including zebrafish (*Danio rerio*) (Biran *et al.* 2008), goldfish (*Carassius auratus*) (Li *et al.* 2009), medaka (*Oryzias latipes*) (Lee *et al.* 2009*b*), chub mackerel (*Scomber japonicus*) (Selvaraj *et al.* 2010), striped bass (*Morone saxatilis*) (Zmora *et al.* 2012), and European eel (Pasquier *et al.* 2012*b*).

In chondrichthyes, only fragmental genomic databases are currently available. However, the occurrence of the *Kiss1*, *Kiss2*, and *Kiss3* genes could be predicted from the elephant shark genome (Lee *et al.* 2009*b*, Pasquier *et al.* 2012*b*) and the *Kiss1* and *Kiss2* genes from the little skate genome (*Leucoraja erinacea*) (Pasquier *et al.* 2014). In cyclostomes, two *kiss* genes have been predicted from sea lamprey genomic data (Lee *et al.* 2009*b*).

## Evolutionary history of the Kiss family

Syntenic analysis allowed us to investigate the origin of the *Kiss* gene diversity during vertebrate evolution. We found that the three conserved genomic regions containing *Kiss* genes comprise paralogs from at least eleven gene families (Pasquier *et al.* 2012*b*). The members of these families are



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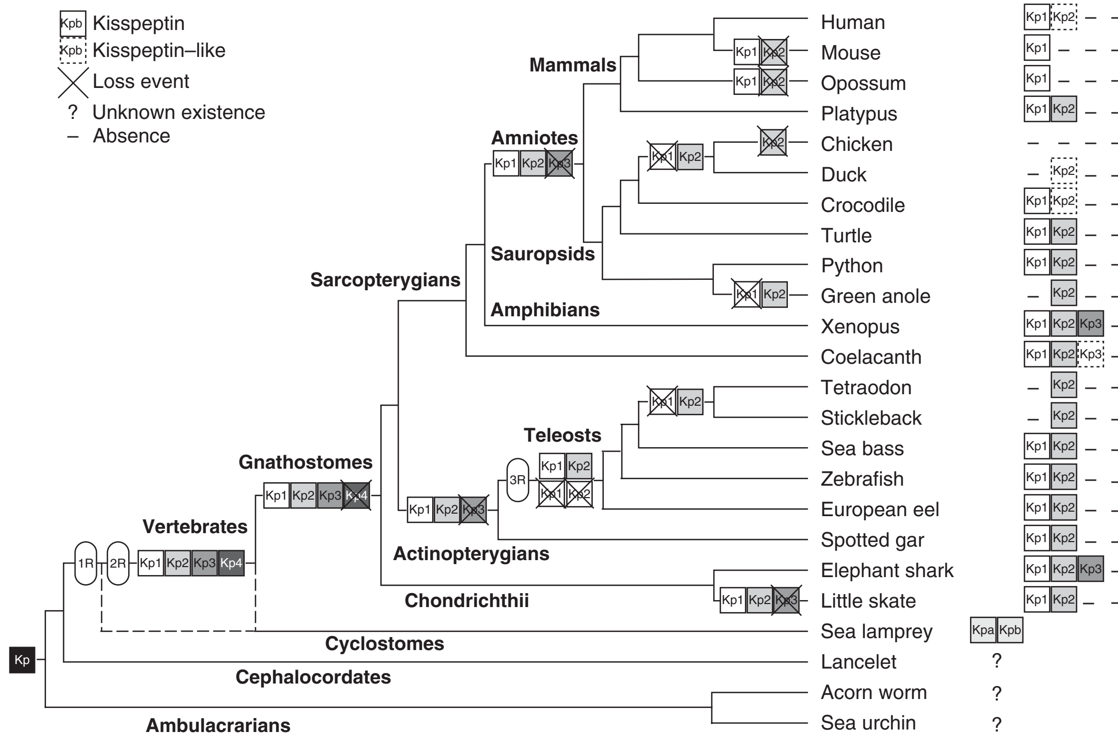


**Figure 3**

Current status and proposed evolutionary history of *Kiss* genes among deuterostomes. The common names of representative species of each lineage are given at the extremity of the final branches, together with the symbols for the *Kiss* genes they possess. These hypotheses assume the presence of a single *Kiss* gene ancestor in early chordates leading to four *Kiss* paralogous genes after the 1R and 2R in early vertebrates. Multiple pseudogenization processes and gene loss events shaped the current vertebrate *Kiss* gene diversity.

present among the three *Kiss* syntenic regions and they also delineate a fourth conserved region, which does not show any *Kiss* gene in the representative osteichthyan species investigated so far (Pasquier *et al*. 2012*b*). These gene families delineate a tetra-paralogon (Fig. 4). On the basis of these observations and in agreement with the vertebrate evolutionary model proposed by Nakatani *et al*. (2007) (model N), we hypothesize that this tetra-paralogon resulted from the duplications of one single genomic region localized on the proto-chromosome-D of the vertebrate ancestor. Therefore, we inferred that the current three *Kiss* genes may derive from a single ancestral gene duplicated through the two successive whole-genome duplications (1R and 2R) that occurred in the early stages of vertebrate evolution (Figs 3 and 4). After the 1R and 2R events, the history of the *Kiss* family was driven, all along vertebrate evolution, by multiple and independent pseudogenization processes and gene loss events, leading to the current diversity of *Kiss* described in the section Diversity of *Kiss* genes in vertebrates. and illustrated in Figs 3 and 4. The loss of the putative *Kiss4* gene would have occurred early in the history of vertebrates, as it is absent in extant species, as indicated by syntenic analysis. The *Kiss3* gene would have been lost independently in early actinopterygians and early amniotes (Fig. 3). The *Kiss1* and *Kiss2* genes are the most represented *Kiss* genes in extant vertebrates. *Kiss1* would have been lost independently in some teleosts and sauropsids, while *Kiss2* would have been lost in some sauropsids and mammals (Fig. 3). Notably, due to massive gene losses, no effect of the teleost-specific third round of whole-genome duplication (3R) can be recorded on the current number of *Kiss* genes in teleosts (Pasquier *et al*. 2012*a*,*b*; Figs 3 and 4).

Although the current hypothesis on the origin of *Kiss* diversity assumes the existence of an ancestral *Kiss* gene in early chordates, no *Kiss* genes have been identified yet in the genomes of representative species of cephalochordates, hemichordates, or echinoderms (Fig. 3).

## Molecular diversity and phylogenetic evolution of KissRs in vertebrates

KissRs are GPCRs belonging to the rhodopsin gamma family (Mirabeau & Joly 2013). In vertebrates, their closest



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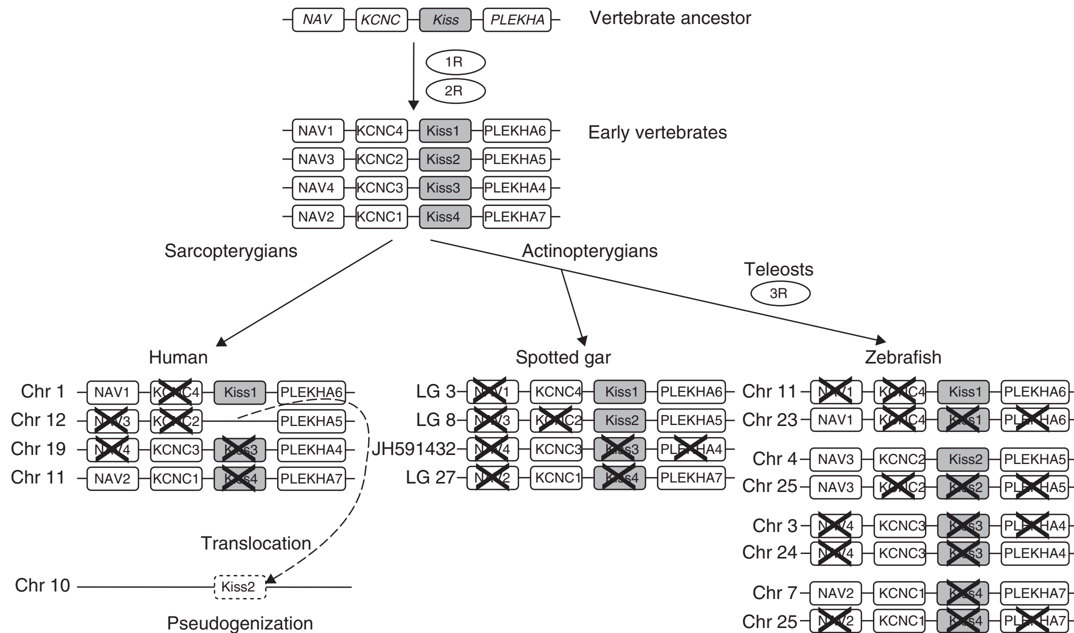


**Figure 4**

Proposed origin and evolution of vertebrate *Kiss* tetra-paralogons. The paralogous genes of several identified families, illustrated here by *NAV*, *KCNC*, and *PLEKHA*, delineate a tetra-paralogon in vertebrate (such as spotted gar and human) genomes that originated from 1R and 2R. In humans, a pseudo-*Kiss2* gene has been predicted on chromosome 10 (Osugi *et al*. 2013), indicating a translocation and pseudogenization. A duplicated tetra-paralogon is present in teleost (such as zebrafish) genomes, as a result of the 3R. Due to multiple *Kiss* gene loss, there is no effect of the 3R on the number of *Kiss* genes in the extant teleost species.

relatives are the galanin receptors (Lee *et al*. 1999, Mirabeau & Joly 2013). Following the characterization of the single *KissR1* gene in eutherian mammals, investigations in various vertebrates revealed the existence of up to four paralogous *KissR* genes (*KissR1*, *KissR2*, *KissR3*, and *KissR4*) (Pasquier *et al*. 2012*a*,*b*).

## *KissR* gene structure

The mammalian *KissR1* gene encompasses five exons and four introns, and its structure is well conserved among vertebrates (for review see Tena-Sempere *et al*. (2012)). However, it has been reported that the medaka *Kissr3* gene is made of six exons and five introns (Tena-Sempere *et al*. 2012).

The existence of *kissr* mRNA splicing isoforms has been reported in several teleost species including the senegalese sole (two *KissR2* isoforms) (Mechaly *et al*. 2009), the yellowtail kingfish (five *KissR2* isoforms) and Southern bluefin tuna (two *kissr2* isoforms) (Nocillado *et al*. 2012), the zebrafish (five *kissr3* isoforms) (Onuma & Duan 2012), and the European eel (three *kissr3* isoforms) (Pasquier *et al*. 2012*a*). The splicing variants can result from the retention of an intron as in the senegalese sole (Mechaly *et al*. 2009), yellowtail kingfish and bluefin tuna (Nocillado *et al*. 2012), or they can result from the deletion of exonic sequence as in the zebrafish (Onuma & Duan 2012), yellowtail kingfish (Nocillado *et al*. 2012), and European eel (Pasquier *et al*. 2012*a*). So far, these splicing variants are considered to give rise to non-functional proteins except for one of the zebrafish truncated isoforms (KRBDP3), which has been located in the nucleus and could exert a transactivation effect upon kisspeptin binding (Onuma & Duan 2012). The existence of these splicing isoforms for KissR2 and KissR3 in several teleost species indicates the existence of an ancestral post-transcriptional regulatory process for *KissR*.

## Diversity of *Kissr* genes in vertebrates

Since the discovery of the first *KissR* in rat, described at that time as a galanin-like receptor and named *Gpr54* (Lee *et al*. 1999), this gene has been identified in most of vertebrate lineages including cyclostomes, teleosts, amphibians, and sauropsids (Lee *et al*. 2009*b*, Pasquier *et al*. 2012*b*, Tena-Sempere *et al*. 2012). Phylogenetic and syntenic analyses (Lee *et al*. 2009*b*, Kim *et al*. 2012, Pasquier *et al*. 2012*a*,*b*) revealed that vertebrate species possess up to four paralogous *KissR*, i.e., *KissR1*, *KissR2*,



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*KissR3*, and *KissR4* genes according to the recent classification (Pasquier *et al*. 2012*a*).

Among vertebrates, various situations have been observed concerning the number of paralogous *KissR* genes. The maximal number of KissR has been recently detected in representative species of early sarcopterygians (coelacanth) and actinopterygians (spotted gar), which both possess the four *KissR* (Pasquier *et al*. 2012*a*,*b*).

In mammals, eutherians possess only the *KissR1* gene, while prototherians, such as platypus, have both the *KissR1* and *KissR4* genes (Fig. 5). In sauropsids, various situations have been reported (Pasquier *et al*. 2014). In squamates, the green anole (*A. carolinensis*) only possesses the *KissR4* gene, while both the *KissR1* and *KissR4* genes are predicted in the Indian python genome (Pasquier *et al*. 2014). Chelonians also possess the *KissR1* and *KissR4* genes, as both genes are predicted in the soft-shell Chinese turtle and the painted turtle genomes (Pasquier *et al*. 2014). In crocodilians, only the *KissR1* gene has been predicted in the salt-water crocodile, the American alligator, and the Indian gharial (Pasquier *et al*. 2014). Phylogenetic and syntenic analyses support the absence of *KissR1*, *KissR2*, and *KissR3* genes in birds; however, the possible existence of *Kissr4* in birds cannot be ruled out due to the incomplete sequencing of bird genomes in the putative region of *KissR4* (Pasquier *et al*. 2014). Among tetrapods, amphibians present the largest number of *KissR*, with three *KissR* in *X. tropicalis* (*KissR1*, *KissR2*, and *KissR3*) (Lee *et al*. 2009*b*), while only the *KissR2* transcript has been identified in the bullfrog (Moon *et al*. 2009) and the edible frog (Chianese *et al*. 2013).

In teleosts, different situations have been observed. All teleost species investigated so far express the *KissR2* gene (Tena-Sempere *et al*. 2012). However, some species also possess the *KissR3* gene, including zebrafish (*D. rerio*) (Biran *et al*. 2008), goldfish (*C. auratus*) (Li *et al*. 2009), medaka (*O. latipes*) (Lee *et al*. 2009*b*), striped bass (*M. saxatilis*) (Zmora *et al*. 2012), chub mackerel (*S. japonicus*) (Ohga *et al*. 2013), and European eel (*Anguilla anguilla*) (Pasquier *et al*. 2012*a*). In addition, the eel presents the peculiarity of being the only teleost species possessing a *kissR1* gene orthologous to the mammalian *KissR1*. With three different *kissr*, the eel is also the teleost species possessing the largest diversity of *kissr* genes.

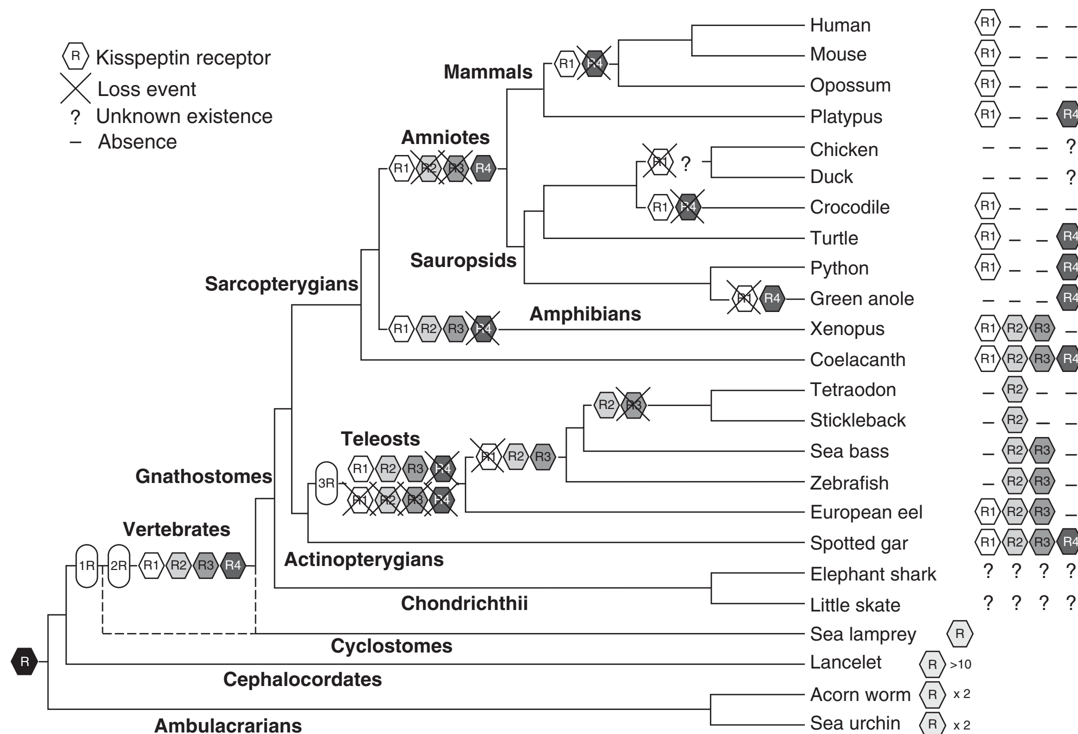


**Figure 5**

Current status and proposed evolutionary history of Kisspeptin receptor (*KissR*) genes among deuterostomes. The common names of representative species of each lineage are given at the extremity of the final branches, together with the symbols for the *KissR* genes they possess. These hypotheses assume the presence of a single *KissR* gene ancestor in early chordates leading to four *KissR* paralogous genes after the 1R and 2R in early vertebrates. Multiple pseudogenization processes and gene loss events shaped the current vertebrate *KissR* gene diversity.


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To date, no *KissR* gene has been identified in cartilaginous fish. In cyclostomes, one *KissR* gene has been predicted from the sea lamprey genome (Pasquier *et al*. 2012*a*). *KissR* homologous genes have been found in the genomes of representative species of non-vertebrate deuterostomes, such as cephalochordates (lancelet) (Holland *et al*. 2008), hemichordates (acorn worm), and echinoderms (sea urchin) (Pasquier *et al*. 2012*a*) (Fig. 5).

### Evolutionary history of KissR family

Syntenic analysis has revealed that the four genomic regions neighboring *KissR* genes are highly conserved, each presenting paralogs from at least eight gene families (Pasquier *et al*. 2012*a*). On the basis of these observations and in agreement with the chordate evolutionary models proposed by Putnam *et al*. (2008) (model P) and by Nakatani *et al*. (2007) (model N), we hypothesize that this tetra-paralogon originates from the duplications of one single genomic region localized on the chordate proto-chromosome-1 (according to model P) and the vertebrate proto-chromosome-A (according to model N). Therefore, we propose that the current four *KissR* genes may have resulted from a single ancestral gene duplicated through the whole-genome duplication events, 1R and 2R, that occurred in early steps of vertebrate evolution (Figs 5 and 6). Actually, four paralogous *KissR* genes are still present in some extant vertebrate species, including the coelacanth and the spotted gar (Figs 5 and 6). The hypothesis of the *KissR* tetra-paralogon suggests that a *KissR* ancestral gene was present before the emergence of vertebrates (Figs 5 and 6). In agreement with this hypothesis, *KissR* homologous genes have been identified in non-vertebrate deuterostomes (Fig. 5).

To date, phylogenetic analyses of the *KissR* family do not allow the homology relationships between the four *KissR* resulting from the 2R (Pasquier *et al*. 2012*a*,*b*) to be fully solved. However, one of the most recent phylogenetic reconstructions has indicated that *KissR1* and *KissR3* could be sister groups after the 2R (Pasquier *et al*. 2014). In addition, a recent study has proposed the phylogenetic reconstruction of a gene family associated with the *KissR* paralogon, the *PALM* family (Hultqvist *et al*. 2012). This study, which provides indirect insights into the relationships between the four *KissR*, strengthens the hypothesis of sister *KissR1* and *KissR3*, on the one hand, and sister *KissR2* and *KissR4*, on the other hand, resulting from the 2R.

After the 1R and 2R events, the history of the *KissR* family was driven, throughout vertebrate evolution, by

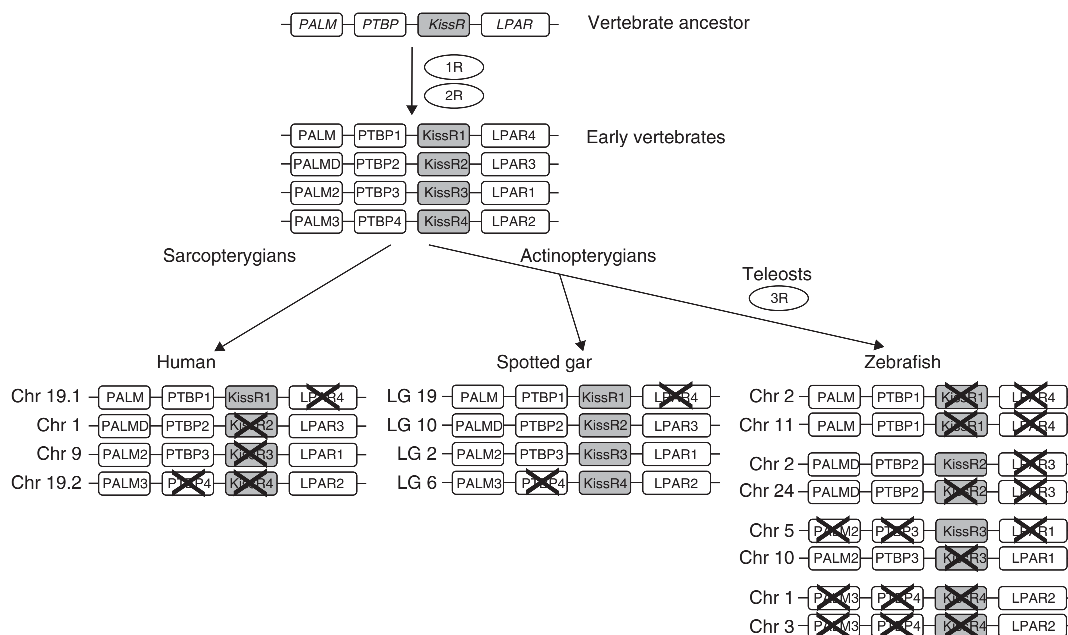


**Figure 6**

Proposed origin and evolution of vertebrate *KissR* tetra-paralogons. The paralogous genes of several identified families, illustrated here by *PALM*, *PTBP*, and *LPAR*, delineate a tetra-paralogon in vertebrate (such as spotted gar and human) genomes that originated from 1R and 2R. A duplicated tetra-paralogon is present in teleost (such as zebrafish) genomes, as a result of the 3R. Due to multiple *KissR* gene loss, there is no effect of the 3R on the number of *kissr* genes in the extant teleost species.


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multiple gene loss events leading to the current diversity of *KissR* described in the section Diversity of *KissR* genes in vertebrates and illustrated in Figs 5 and 6. *KissR4* would have been lost independently in the actinopterygian and sarcopterygian lineages. Among actinopterygians, this event would have occurred after the emergence of holosteans (such as the spotted gar) in a teleost ancestor. Among sarcopterygians, *KissR4* would have been lost independently in the amphibians, eutherian mammals, and some sauropsids. *KissR3* would have been lost independently in some teleosts and in early amniotes. *KissR2* would have been lost only in the amniote lineage. As mentioned for *Kiss* genes (see section Evolutionary history of the Kiss family), due to massive gene losses, no effect of the teleost-specific third round of whole-genome duplication (3R) can be recorded on the current number of *KissR* in teleosts (Pasquier *et al*. 2012*a*,*b*; Figs 5 and 6).

## Evolution of kisspeptin and KissR pairs

As a consequence of the independent losses of *Kiss* genes as well as of *KissR* genes, the extant vertebrate species present various combinations of putative *Kiss* and *KissR* couples. For example, the same *Kiss* genes, *Kiss1* and *Kiss2*, are present in various species from two distant lineages, amniotes and teleosts, while these species possess different *KissR* genes. In amniotes, only *Kissr1* and/or *Kissr4* are conserved. In contrast, in teleosts, all species kept *KissR2*, a few species also kept *KissR3*, and only the eel conserved the *KissR1* ortholog. This situation, which results from multiple independent loss events, challenges the former hypothesis of a conservation of *Kiss/KissR* pairs across vertebrate evolution. Another example of non-conservation of specific *Kiss/KissR* pairs is provided by some vertebrate species, such as the spotted gar, the European eel, and the coelacanth, presenting a larger number of *KissR* than *Kiss* genes.

The strong conservation of the biologically active Kp(10) sequences among *Kiss* types and vertebrate species (see section Molecular diversity and phylogenetic evolution of kisspeptins in vertebrates and Fig. 2A) may have favored the lack of conservation of specific *Kiss/KissR* pairs across vertebrate evolution. As a matter of fact, *in vitro* studies have shown that the different *KissR* can be independently activated by any kisspeptin (Lee *et al*. 2009*b*, Li *et al*. 2009, Onuma & Duan 2012).

Other evolutionary factors than co-evolution between specific *Kiss* and *KissR* may have therefore driven the conservation of multiple *Kiss* and *KissR* in various vertebrate species. They may concern differential properties and physiological roles, such as differential tissue expression, differential regulation, and mechanisms of action.

## Structure–activity relationships and evolution of kisspeptins

Human kisspeptin-10 (hKp1(10)) (YNWNSFGLRF-NH2) corresponds to the carboxy-terminal decapeptide sequence of kisspeptin1-54, (hKp1(54)), kisspeptin1–14 (hKp1(14)), and kisspeptin1–13 (hKp1(13)), three molecular forms purified from human placenta (Kotani *et al*. 2001). Synthetic hKp1(10) binds to KissR1, acts on intracellular messengers, and inhibits cell culture proliferation with the same efficacy as the longer kisspeptins (Kotani *et al*. 2001, Ohtaki *et al*. 2001). This decapeptide thus bears the biological activity of kisspeptins.

Many studies have been conducted to identify the amino acids involved in kisspeptin activity. Design and synthesis of downsized hKp1(54) analogs have demonstrated that the five hKp1(10) C-terminal residues play a significant role in receptor binding and activation (Niida *et al*. 2006). On rat Kp1(10) (that differs from human Kp1(10) by a C-terminal amidated tyrosine instead of an amidated phenylalanine), substituted analogs in which each residue was systematically replaced by alanine (Ala-scan) confirmed the importance of the aromatic residues Phe6 and Tyr10 in agonistic activity (Gutiérrez-Pascual *et al*. 2009).

NMR studies of hKp1(13) in a solution of SDS micelles showed that the peptide is structured in a helix on its C-terminal half with Phe9, Arg12, and Phe13 residues (respectively 6, 9 and 10 in hKp1(10) numbering) positioned on the same face of the helix (Orsini *et al*. 2007). In a schematic top view representation of the helix (Fig. 2B), the Asn2 residue appears to be also positioned on the same side, between Phe6 and Arg9. Ala-scan experiments on human Kp1(10) indeed demonstrated that this Asn2 residue is essential for receptor binding (Curtis *et al*. 2010). It should be noticed that the conformation of hKp1(10) depends on the membrane mimetic environment in which it is located. In dodecylphosphocholine (DPC) micelles, NMR analysis showed that the region encompassing the residues Trp3 to Phe10 contained several tight turns, but no helical conformation, with Leu8 in the same hydrophobic cluster as Phe6 and Phe10 (Lee *et al*. 2009*a*). Rat Kp1(10) mainly adopts an helical conformation in diphenylcarbodiimide (DPCD) micelles, from Asn4 to Tyr10, with a combination of α and 3(10) helix, and a disordered N-terminal region (Gutiérrez-Pascual *et al*. 2009).



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Residues that play a crucial role in kisspeptin activity, namely Asn2, Phe6, Arg9 and the C-terminal aromatic residue Phe10 or Tyr10, are conserved in all vertebrate species from coelacanth to human (Fig. 2A and B). The presence of Asn4 in the helix is also fundamental and this residue is strictly conserved in all species (Fig. 2A and B). Consistent with this notion, a Kiss1 variant has been recently described in a patient with idiopathic HH, where the substitution of Asn115 (position 4 in hKp1(10)) with a lysine altered the peptide activity, possibly by modifying its 3D structure (Topaloglu *et al.* 2012).

The chirality and the aromatic properties of the two C-terminal residues (Arg and Phe or Tyr) are also essential to hKp1(10) activity. Replacement of these residues by their D-enantiomers (Niida *et al.* 2006) or substitution by saturated-side-chain amino acids (Orsini *et al.* 2007) suppresses their biological activity. In contrast, substitution of some amino acids of the N-terminal end with their D-enantiomers can improve the peptide activity and/or its resistance to proteases. For instance, replacement of Trp3 by D-Trp yields to a compound with high metabolic stability in serum and good agonistic activity (Asami *et al.* 2012). Similarly, substitution of Tyr1 by D-Tyr increases the biological activity of the peptide *in vivo* (Curtis *et al.* 2010). A LC–MS/MS assay has been recently developed to quantify Kp(10) levels in rat plasma. Using this method, stability studies demonstrated that the peptide is rapidly degraded with a half-life of 1.7 min at 37 °C, the main degradation product being the N-terminal tyrosine-deleted peptide (Liu *et al.* 2013). It is also worth noting that Arg9 methylation confers greater resistance of the peptide to trypsin while improving its agonistic potency (Asami *et al.* 2012).

Several natural variants of the *Kiss1* gene in HH patients have now been reported (Semple *et al.* 2005), but only one occurred on the C-terminal end of the molecule at Asn115 (Asn4 in hKp1(10)), as described above (Topaloglu *et al.* 2012). In contrast, residues at positions 3 and 5 (Trp3 and Ser5 in hKp1(10)), that are the most variable in vertebrates (Fig. 2A and B), are relatively tolerant of Ala substitution (Gutiérrez-Pascual *et al.* 2009, Curtis *et al.* 2010).

## Structure–activity relationships and evolution of KissRs

Human *KissR1* (Q969F8 UniProtKB), a 398-amino acid protein, belongs to the superfamily of class A rhodopsin-like GPCRs. It comprises an extracellular N-terminal domain of ∼50 residues, followed by seven transmembrane helices (TM1 to TM7) and ends with a C-terminal cytoplasmic domain of about 70 residues (Supplementary Figures 1 and 2, see section on supplementary data given at the end of this article). The seven helices are connected by three intracellular loops, i1, i2, and i3, and three extracellular loops, e2, e3, and e4 (Supplementary Figure 1; Baldwin 1993, Bockaert & Pin 1999). Human *KissR1* has three N-glycosylated asparagine residues (N10, N18 and N28) located in the N-terminal domain (Clements *et al.* 2001) and a disulfide bridge involving cysteines 115 and 191, joining e2 and e3 (Supplementary Figure 1). These latter cysteines are conserved in vertebrate KissR sequences, as exemplified by the multiple KissR of an early teleost, the eel, and an early sarcopterygian, the coelacanth (Supplementary Figure 1). Globally, strong identity is observed in TM1 to TM4 and TM7 regions, whereas great variability occurs in the N- and C-terminal domains (Supplementary Figure 1). The three-dimensional conformations of eel and coelacanth Kiss receptors show the same typical overall structure of GPCRs as human KissR1, but with some variations at the N and C ends (Supplementary Figure 2).

Site-directed mutagenesis and molecular simulation showed the importance of Asp139 and Arg140 in TM3 for mediating receptor activation (Scheer *et al.* 1996), and these residues are conserved in other vertebrates, including eel and coelacanth KissR (Supplementary Figure 1). An increasing number of mutations involved in HH in humans and mice have been described in all regions of the KissR molecule (Wahab *et al.* 2011, Dungan Lemko & Elias 2012), for instance the substitution of Leu148 by serine in i2 (Seminara *et al.* 2003). Biochemical and pharmacological analyses have demonstrated that this Leu residue, which is highly conserved in class A GPCRs, is implicated in receptor coupling through hydrophobic interactions with the G α subunit (Wacker *et al.* 2008; Supplementary Figure 1).

The cytoplasmic C-terminal domain of the human receptor contains a proline (P) and arginine (R)-rich region, including three PRR repeats, that may be organized spatially in overlapping segments and constitute a binding domain for Src homology 3 (SH3) proteins (Saksela & Permi 2012). SH3 domains, which are directly involved in protein–protein associations, are found, for example, within proteins implicated in signal transduction, such as tyrosine kinases. This C-terminal intracytoplasmic region directly binds to the catalytic and regulatory subunits of phosphatase 2A and can form complexes with protein partners involved in receptor signaling (Evans *et al.* 2008). We also know that arginine–proline-rich motifs are implicated in folding and transport of


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multimeric membrane proteins (Michelsen *et al.* 2005). A human *KissR1* mutation in this region has been recently reported (Chevrier *et al.* 2013). It consists of the heterozygous insertion of an additional PRR motif in frame with the three PRR repeats, resulting in the synthesis of a receptor with four PRR motifs. This insertion has a dominant negative effect on the WT receptor and causes isolated HH. Molecular dynamics analysis showed that the addition of this PRR stiffened the C-terminal end giving it a polyproline type II helix (PPII), whereas in the WT *KissR1* this region had a very flexible conformation. The altered flexibility of the C-terminal tail modifies its interaction with partner proteins (Chevrier *et al.* 2013). In this regard, it is interesting to note that, in a case of precocious puberty, an Arg386Pro mutation located in the C-terminal region induces a sustained response to Kiss activation (Teles *et al.* 2008, Bianco *et al.* 2011). This PRR repeat pattern is not found in non-mammalian vertebrate KissR proteins (Supplementary Figure 1), which indicates an evolutionary variability in signal transduction and/or intracellular trafficking mechanisms.

Molecular modeling of the human KissR1 3D structure by the on-line platform I-Tasser (Roy *et al.* 2010; Supplementary Figure 2) predicted that the ligand-binding sites were in a cavity formed by residues of TM3 (Gln122, Gln123, Val126, Gln127), e3 (Tyr190, Cys191, Glu193), TM5 (Asn208, Tyr213), TM6 (Leu271, Trp276, Ile279, Gln280), and TM7 (His309, Tyr313). Remarkably, almost all these residues are conserved in vertebrates, as shown in Supplementary Figure 1 for the eel and coelacanth KissR, indicating that their putative function in ligand binding has been conserved through evolution.

## Interaction and evolution of Kiss/KissR pairs

The key C-terminal residues of Kp1(10), namely Phe6, Arg9, and Phe10 and to a lesser extent Leu8, are thought to play a major role in the access to a hydrophobic pocket of the receptor (Orsini *et al.* 2007). The nature and orientation of the C-terminal side chain residues of Kp1(10) are important for binding and receptor activation (Niida *et al.* 2006, Tomita *et al.* 2006), while the C-terminal aromatic residue (Tyr or Phe) is essential for binding (Gutiérrez-Pascual *et al.* 2009). Amidation of the peptide is also crucial, since Kp1(10) with a free C-terminal carboxyl group is inactive (Ohtaki *et al.* 2001). The carboxyamide group may interact with the receptor by hydrogen bonding (Tomita *et al.* 2006). The observation that human Kp1(13) and rat Kp1(10) have a common helical domain in their C-terminal part and that they are both full KissR1 agonists strongly indicates that the helix plays a role in the binding and the activation of the receptor. In support of this notion, substitution of the C-terminal Tyr of rat Kp1(10) by Ala, which disrupts the helical structure over the last two amino acids, suppresses binding of the analog to KissR1 (Orsini *et al.* 2007, Gutiérrez-Pascual *et al.* 2009).

To activate KissR, Kp1(10) may first need to be incorporated into the lipid membrane and transported to the receptor (Lee *et al.* 2009*a*). Indeed, structural studies based on Kp1(10) analogs in 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) micelles indicated that the peptide does not directly interact with KissR1, but has to be inserted in the lipid membrane first before diffusing laterally toward the receptor. NMR studies using DPC micelles have shown that the C-terminal end of Kp1(10) forms a hydrophobic cluster consisting of Phe6 and Phe10 and the aliphatic chain of Leu8, which probably interacts with the carbon chains of the inner membrane phospholipids (Lee *et al.* 2009*a*). The N-terminal region may also interact with the receptor as kisspeptin photoaffinity probe experiments with Kp1(54) and Kp1(14) show that the three N-terminal residues (Tyr, Asn, and Trp) can constitute a secondary binding site with KissR1 (Misu *et al.* 2013).

Thus, it is assumed that Kp1(10) adopts a helical conformation near the cell surface that enables the peptide to insert into the phospholipidic bilayer by hydrophobic and electrostatic interactions. This mechanism of insertion into eukaryotic cells is commonly described for anti-microbial peptides which are about the same size as Kp1(10), are carboxyamidated, adopt a helical conformation, and establish electrostatic and hydrophobic interactions with membrane phospholipids (Li *et al.* 2012).

Regarding the KissR, we do not know yet how it accommodates the ligand. For rhodopsin, whose 3D structure is known (Unger *et al.* 1997, Palczewski *et al.* 2000) and which belongs to the same class A GPCRs, the ligand binds in a pocket formed by TM2 to TM6. In this case, light causes a conformational change of retinal covalently bound in this cavity, leading to receptor activation. This activation is associated with an 'opening' of the receptor due to the rotation of TM6, which results in the uncovering of the G-protein-binding sites. The i2 and i3 intracellular loops are mainly involved in the interaction with the G protein (Farrens *et al.* 1996, Bourne 1997, Bockaert & Pin 1999) and are essential for the activation of Gs (Arora *et al.* 1998).

Finally, binding of Kiss to KissR1 may generate mono-, di- or oligomeric structures, as different quaternary structures are observed for Class A GPCRs, following agonist binding (Gurevich & Gurevich 2008,



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Pellissier *et al.* 2011). In the case of non-mammalian vertebrate species, which express several KissR types, formation of heterodimers may be hypothesized to occur as already shown for other GPCRs (Vilardaga *et al.* 2010).

## Signaling pathways activated by Kiss/KissR pairs

### Signaling pathways in mammals

The three initial studies reporting the characterization of KissR1 (GPR54, AXOR12, or hOT7T175) as the receptor for kisspeptins also investigated the intracellular signaling pathways activated by this receptor (Fig. 7; Kotani *et al.* 2001, Muir *et al.* 2001, Ohtaki *et al.* 2001, Asami *et al.* 2012). These studies, which used heterologous cell models, transfected with the rat or human KissR1 (CHO-K1 (Kotani *et al.* 2001), HEK293 (Muir *et al.* 2001), and B16-BL6 (Ohtaki *et al.* 2001)), showed that in mammals, the phospholipase C (PLC)/protein kinase C (PKC)/MAPK pathway was activated (for reviews Castaño *et al.* (2009) and Pinilla *et al.* (2012)). Using transient transfections of HEK293 (Muir *et al.* 2001) and CHO-K1 (Kotani *et al.* 2001) cell lines, the authors reported that calcium mobilization was observed after the activation of KissR and not altered by treatment with pertussis toxin. These results indicated that KissR was coupled to G proteins of the Gq/11 subfamily. This hypothesis was further strengthened by the fact that

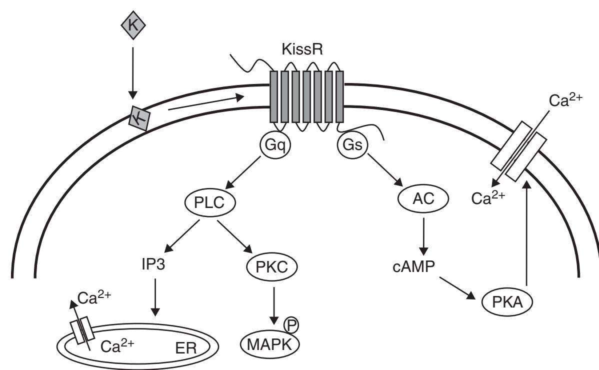


**Figure 7**
Schematic representation of the Kiss/KissR signaling pathways. Kisspeptin (K) is translocated along the phospholipid bilayer toward its receptor (KissR), a seven-transmembrane-domain G-protein-coupled receptor. The main signaling pathway involves Gq protein and activation of phospholipase C (PLC). PLC is a crossroads of two activation pathways: one leading to MAP kinase phosphorylation (MAPKP) via protein kinase C (PKC) and the other leading to endoplasmic reticulum (ER) calcium mobilization via phosphatidylinositol-3-kinase (IP3) activation. In teleosts, which possess more than one KissR, the adenylate cylcase (AC)/ protein kinase A (PKA) signaling pathway can also be activated, leading to a rise in extracellular $Ca^{2+}$ influx.

kisspeptin did not modify basal or forskolin-induced cAMP levels, showing that KissR does not couple strongly to Gs and/or Gi/o subfamilies. Kotani *et al.* (2001) demonstrated that kisspeptins stimulated arachidonic acid release and phosphorylation of MAP kinases (extracellular signal-regulated kinases, ERK1 and ERK2). They also reported a weak stimulation of p38 MAP kinase phosphorylation but not of stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK). Activation of the MAP kinase-related pathways by kisspeptins was also reported using B16-BL6 mouse melanoma cells stably expressing *KissR1* (Ohtaki *et al.* 2001). In COS-7 cells transiently transfected with cDNA-encoding mouse *KissR1*, kisspeptin increases intracellular IP3 production (Stafford *et al.* 2002).

Linked to the description of kisspeptin (metastin) as an inhibitor of metastasis, some *in vitro* studies have been carried out on cancer cell lines. In anaplastic thyroid cancer cells (ARO) expressing endogenous KissR1, Ringel *et al.* (2002) showed that treatment with kisspeptin (metastin) causes activation (phosphorylation) of ERK, but neither Akt nor p38. Three years later, Stathatos *et al.* (2005) created a stable KissR1 overexpression model using KissR1-null papillary thyroid cancer cells (NPA cell line), instead of using ARO cells which express endogenous KissR1. Similarly to ARO cells but in contrast to previous results (Ringel *et al.* 2002), exposure of transfected NPA cells to kisspeptin (metastin) provoked phosphorylation of Akt, p42/44 MAPK, and PKC. Specific blockers of phosphatidylinositol-3-kinase and MAPK did not alter kisspeptin (metastin)-mediated metastasis inhibition (Stathatos *et al.* 2005). Different effects on MAPK activation have also been obtained with pancreatic cancer cell lines expressing endogenous KissR1 (Masui *et al.* 2004). The authors chose the AsPC-1 cell line for its high KiSS1 and low KissR1 expression level, and the PANC-1 cell line for its low KiSS1 and high KissR1 expression level. Kisspeptin (metastin) was able to inhibit cell migration in PANC-1, but not in AsPC-1. ERK1 phosphorylation was activated in both cell lines by kisspeptin treatment, while an increase in p38 phosphorylation was only observed in PANC-1 cells. Becker *et al.* (2005) established a recombinant expression system in the human MDA-MB-435S cell line, originating from a metastatic ductal breast carcinoma, in order to investigate the intracellular mechanisms involved in the reduction of their metastatic potential. The transcription of all microarray-identified genes was abolished by the use of inhibitors of PLC, PKC, and p42/44 MAP kinases (Becker *et al.* 2005).

Some other *in vitro* studies were then conducted in a physiologically relevant context in terms of control of




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reproductive function, using cultured hypothalamic explants (Castellano *et al.* 2006), hypothalamic slices (Choe *et al.* 2013), rat pituitary cells (Gutiérrez-Pascual *et al.* 2007), baboon pituitary cells (Luque *et al.* 2011) and the LβT2 pituitary gonadotrope cell line (Witham *et al.* 2013). Castellano *et al.* (2006) explored the signaling cascades involved in kisspeptin-induced GNRH secretion by means of pharmacological blockade using rat hypothalamic explants. A PLC inhibitor totally suppressed kisspeptin-induced the GNRH secretion, whereas an adenylate cyclase (AC) blocker failed to modify the GNRH response to kisspeptin-10 (Castellano *et al.* 2006). In addition, the ability of kisspeptin to induce GNRH secretion was abrogated after depletion of intracellular $Ca^{2+}$ stores by thapsigargin, while blockade of extracellular $Ca^{2+}$ entry by cadmium did not affect the GNRH secretory response to kisspeptin (Castellano *et al.* 2006). Treatments with blockers of ERK1/2 and p38 kinases were able to totally prevent kisspeptin-stimulated GNRH secretion, whereas inhibition of Jun N-terminal kinase only partially reduced GNRH responses to kisspeptin. In two GNRH-secreting neuronal cell lines (GT1–7 and GN11), which express KissR, pretreatment with a ERK1/2 MAPK inhibitor and a PI3K inhibitor attenuated the effects of kisspeptin on *GNRH* mRNA expression (Novaira *et al.* 2009). Choe *et al.* (2013) used cultured hypothalamic slices prepared from transgenic mice expressing a *GNRH* promoter-driven luciferase reporter, and confirmed the involvement of PKC in kisspeptin signaling in GNRH stimulation. Using the LβT2 pituitary gonadotrope cell line transfected with KissR1, Witham *et al.* (2013) reported that PKC signaling was required for kisspeptin-mediated induction of *LHβ* and *FSHβ* gene expression, as treatment with a PKC inhibitor significantly decreased the effect of kisspeptin on *LHβ* and *FSHβ* promoters. Using cultured pituitary cells from peripubertal male and female rats, Gutiérrez-Pascual *et al.* (2007) showed that kisspeptin acts on a subset of gonadotrophs and somatotrophs by increasing cytosolic $Ca^{2+}$ levels. By means of a panel of pharmacological intracellular signaling blockers in primary baboon pituitary cell cultures, Luque *et al.* (2011) demonstrated that Kp(10) signals through PLC, PKC, MAPK, and intracellular $Ca^{2+}$ mobilization, but not through AC, protein kinase A (PKA), extracellular $Ca^{2+}$ influx, nor nitric oxide synthase, to stimulate both LH and GH release.

Following the discovery by De Roux *et al.* (2003) and Seminara *et al.* (2003) of a link between mutation of *KissR1* and the occurrence of HH in humans, clinical studies on mutated KissRs also contributed to a better understanding of kisspeptin system signaling pathways. Seminara *et al.* (2003) determined whether the identified changes in KissR1 affected the function of the receptor by measuring inositol phosphate (IP) production in COS-7 cells transfected with mutant constructs. They found that substitution of Leu148 by Ser (L148S) markedly reduced production as compared with the WT construct. As mentioned earlier (section Structure–activity relationships and evolution of kisspeptin receptors), Leu148 is highly conserved in vertebrates. In 2005, two novel missense mutations in KissR1 were found in a patient with HH, i.e., substitutions of Cys223 by Arg (C223R) and of Arg297 by Leu (R297L) (Semple *et al.* 2005). Flp-In-293 cells expressing the *C223R* mutation variant were found to exhibit profoundly impaired signaling, with low calcium mobilization, while the other mutation variant (R297L) gave rise to mild reduction in ligand-stimulated activity (Semple *et al.* 2005). Remarkably, Cys223 is highly conserved among vertebrate KissR, while large amino acid variations are observed for Arg297 (Supplementary Figure 1). This further indicates a strong correlation between evolutionary sequence conservation and key function of KissR amino acids. Tenenbaum-Rakover *et al.* (2007) reported a novel mutation in *KissR1*, a Leu substitution with Pro at residue 102 (L102P), in five patients with HH belonging to two unrelated families. The mutated receptor expressed in HEK 293 cells showed virtually no IP accumulation after kisspeptin stimulation, compared with the WT receptor (Tenenbaum-Rakover *et al.* 2007). As mentioned earlier (section Structure–activity relationships and evolution of kisspeptin receptors), Leu102 is highly conserved among vertebrates. In 2008, a *KissR1*-activating mutation (substitution of Pro386 by Arg) was discovered in a girl with idiopathic central precocious puberty (Teles *et al.* 2008). The authors found out that this mutation led to prolonged activation of intracellular signaling pathways in response to kisspeptin, i.e. more sustained elevation of IP levels and slower decrease in phosphorylation of ERK levels, as well as prolonged binding at the plasma membranes. As discussed earlier (section Structure–activity relationships and evolution of kisspeptin receptors), this C-terminal intracellular region of KissR shows large sequence variations among vertebrates, indicating possible species-specific mechanisms of intracellular signaling.

### Signaling pathways in non-mammalian vertebrates

Studies on signal transduction pathways of the kisspeptin system in non-mammalian vertebrates are still scarce.


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In 2009, using CRE-luc and SRE-luc reporter systems, which are known to discriminate the AC/PKA and PLC/PKC signaling pathways, respectively, Moon *et al.* (2009) demonstrated that in CV-1 cells transfected with bullfrog GPR54 (KissR2 in our nomenclature), kisspeptin induced SRE-driven luciferase activity but not CRE-driven luciferase activity. Pretreatment of these cells with a PKC inhibitor (to inhibit Gq-mediating signaling) markedly reduced SRE-luc activity, while pretreatment with a Rho kinase inhibitor (to inhibit $G_{12/13}$-mediating signaling) only slightly reduced SRE-luc activity. Similar to mammalian KissR1, this indicates a preferential coupling of amphibian KissR2 to the Gq protein–PKC pathway rather than to the Gs-mediated signaling pathways (Moon *et al.* 2009). In *X. tropicalis*, in which a triplicate kisspeptin system has been characterized, Lee *et al.* (2009*a*,*b*) reported that all three KissR (KissR1, KissR2, and KissR3) were activated by the three kisspeptins as determined from SRE-luc promoter activity. This indicates that all three Xenopus KissR types are signaling through the PKC pathway; however, no such comparative investigation has been reported concerning the PKA pathway.

In teleosts, in the orange-spotted grouper (Shi *et al.* 2010), which possesses a single Kiss/KissR couple (Kiss2/KissR2 in our nomenclature), Kp2(10) was able to increase SRE promoter activity, but not the activity of the CRE pathway in COS-7 cells transiently transfected with KissR2. Similarly, Nocillado *et al.* (2012) showed that Southern bluefin tuna KissR2 and yellowtail kingfish KissR2 preferentially transduced via the PKC than PKA pathways (Nocillado *et al.* 2012). In zebrafish, which possesses two Kiss (Kiss1 and Kiss2) and two KissR (Kiss1Ra and Kiss1Rb=KissR2 and KissR3 in our nomenclature, respectively), differential activation of signaling pathways has been observed using COS cells transfected with zebrafish KissR and SRE-/CRE-reporters (Biran *et al.* 2008). While Kiss1Ra (KissR2) signaled preferentially via the PKC pathway, Kiss1Rb (KissR3) was able to activate both the PKC and PKA pathways (Biran *et al.* 2008). Similar results were obtained in another cyprinid, the goldfish (Li *et al.* 2009). A recent study in a perciform, the chub mackerel, which also possesses two KissR types, has indicated that both receptors activated the PKC pathway, while only one was able to activate PKA (Ohga *et al.* 2013). Differential signaling pathways (Fig. 7), as shown in those teleost species, may have contributed to the conservation of multiple KissR in vertebrates. Future studies should aim at investigating the signaling pathways in the eel, the only teleost species possessing up to three KissR types (Pasquier *et al.* 2012*a*).

## Conclusion

Studies in various vertebrate species have revealed the existence of multiple paralogous genes coding for Kiss and KissR. This diversity was probably generated by the two whole-genome duplication events, 1R and 2R, that occurred in early vertebrates. Independent gene losses, throughout vertebrate evolution, led to the presence of various numbers and types of *Kiss* and *KissR*, in the extant vertebrate species. In particular, there was no conserved combination of a given *Kiss* type with a *KissR* type, across vertebrate evolution. The striking conservation of the biologically active ten-amino-acid C-terminal sequence of all vertebrate kisspeptins probably allowed this evolutionary flexibility in the Kiss/KissR pairs. *KISSR* mutations, responsible for HH in humans, mostly correspond to highly conserved amino acid positions among vertebrate KissR. This further highlights the key function of these amino acids. Cross talk between evolutionary and biomedical studies should lead to further understanding of the structure–activity relationships of the Kiss/KissR system.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-13-0224.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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