MOLECULAR EVOLUTION OF GPCRS

Kisspeptin/kisspeptin receptors

Jérémie Pasquier, Nédia Kamech, Anne-Gaëlle Lafont, Hubert Vaudry,
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
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

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 (Pennsylvania) 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
‘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 metatin (54 amino acid peptide: (Ohtaki et al. 2001)).

In 2003, three groups revealed the link between hypogonadotropin 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. 2009b, Pasquier et al. 2012b) as well as multiple genes encoding their receptors (from KissR1 to KissR4) (Biran et al. 2008, Felip et al. 2009, Lee et al. 2009b, Pasquier et al. 2012a). This diversity among kisspeptin systems led to studies on its evolutionary history (Akazome et al. 2010, Tsutsui et al. 2010, Pasquier et al. 2012a,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, Macrourista nimbosa, and shown to act as a cardiac stimulator in this species (Price & Greenberg 1977a,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. 2012b)).

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
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, kisspeptin1–14, and kisspeptin1–13 (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. 2009b), 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 bioactive sequence, composed by ten amino acids, Kp(10), is represented by a hatched square.

diversification 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. 2009b, Pasquier et al. 2012b, Tena-Sempere et al. 2012; Fig. 3). Phylogenetic and syntenic analyses (Lee et al. 2009b, Kim et al. 2012, Pasquier et al. 2012a,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. (2012b)). 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. 2012b).

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. 2009b). European 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
in the Indian python genome (Pasquier et al. 2014). Cheloniens 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 phylogenetic 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. 2009b). Only Kiss1 and Kiss2 transcripts have been described in Xenopus laevis and only Kiss2 in the bullfrog (Rana catesbeiana) (Lee et al. 2009b). Concerning the early sarcopterygians, the Kiss1, Kiss2, and Kiss3 genes have been predicted from the coelacanth (Latimeria chalumnae) genome (Pasquier et al. 2012b). 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. 2012b).

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. 2012b). 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. 2012b). 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. 2009b), chub mackerel (Scomber japonicus) (Selvaraj et al. 2010), striped bass (Morone saxatilis) (Zmora et al. 2012), and European eel (Pasquier et al. 2012b).

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. 2009b, Pasquier et al. 2012b) 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. 2009b).

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. 2012b). The members of these families are
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.

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
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. 2012a,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. 2012a). 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. 2012a). 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. 2009b, Pasquier et al. 2012b, Tena-Sempere et al. 2012). Phylogenetic and syntenic analyses (Lee et al. 2009b, Kim et al. 2012, Pasquier et al. 2012a,b) revealed that vertebrate species possess up to four paralogous KissR, i.e., KissR1, KissR2,
KissR3, and KissR4 genes according to the recent classification (Pasquier et al. 2012a).

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. 2012a,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, 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 synteny 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. 2009b), 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. 2009b), striped bass (M. saxatilis) (Zmora et al. 2012), chub mackerel (S. japonicus) (Ohga et al. 2013), and European eel (Anguilla anguilla) (Pasquier et al. 2012a). 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.
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. 2012a). 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. 2012a) (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. 2012a). 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. 2012a,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 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.

**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.
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, eutharian 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. 2012a,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. 2009b, 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 decapetide 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 decapetide 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. 2009a). 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).
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 Gz 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
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 carboxyamidated 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. 2009a). 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. 2009a). 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 antimicrobial 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,
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 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...
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 Ca²⁺ stores by thapsigargin, while blockade of extracellular Ca²⁺ 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 an 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 Ca²⁺ 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 Ca²⁺ mobilization, but not through AC, protein kinase A (PKA), extracellular Ca²⁺ 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.
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-mediated 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. (2009a,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. 2012a).

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

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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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