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Evolutionary insights into the steroid sensitive *kiss1* and *kiss2* neurons in the vertebrate brain

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Kisspeptin was originally found as a peptide product of *Kiss1* gene and is now supposed to be an essential central regulator of reproduction in mammals. However, there is now a growing body of evidence to suggest that *kiss2*, the paralogous gene for *kiss1*, evolved in parallel during vertebrate lineage, and the *kiss2* product also activates the GPR54 (kisspeptin receptor) signaling pathways. Therefore, it is now widely accepted that both *kiss1* and *kiss2* are the kisspeptin genes. Interestingly, either *kiss1* or *kiss2* or both have been lost during evolution in many vertebrate species, and the functional significance of *kiss1* or *kiss2* for the central regulation of reproduction is suggested to vary according to the species. Here, we argue that the steroid sensitivity of the *kiss1* or *kiss2* neurons has been well conserved during evolution among tetrapods and teleosts, and thus it may be the key to understanding the functional homologies of certain populations of kisspeptin (*kiss1* or *kiss2*) neurons among different species of vertebrates. In the present review, we will first introduce recent advances in the study of steroid sensitive *kiss1* and *kiss2* systems in vertebrates and effects of peptide administrations *in vivo*. By comparing the similarities and differences between *kiss1* and *kiss2* of neuronal localization and sensitivity to gonadal steroids in various tetrapods and teleosts, we discuss the evolution of kisspeptin neuronal systems after gene duplication of ancestral kisspeptin genes to give rise to *kiss1* and *kiss2*.

Keywords: kisspeptin, *kiss1*, *kiss2*, evolution, estrogen, steroid feedback, GnRH

INTRODUCTION

Recent studies have shown that kisspeptin plays an essential role in reproductive functions in mammals. Kisspeptin attracts particular attention, since previous reports have shown that the lack of kisspeptin receptors gene, *GPR54*, in both mice and humans, or of the ligand gene (*Kiss1*) in mice results in reproductive dysfunctions. More interestingly, the kisspeptin neurons have been shown to express estrogen receptor alpha (ERα), while the GnRH neurons express kisspeptin receptors, and kisspeptin depolarizes GnRH1 neurons in mice. These results suggest that they are not only involved in the sex steroid feedback but also are possible candidate for the "missing link" in the gonadotropin feedback control (Tena-Sempere, 2005; Smith, 2008).

On the other hand, fewer studies exist on the non-mammalian kisspeptin, and somewhat contradictory results among different species appear to confuse general conclusions about the functions of kisspeptin, especially in teleosts. This may be because of the fact that not a few studies in teleost kisspeptin have used pharmacological methods such as intracerebroventricular (ICV) or peripheral administration of kisspeptins. We should consider that the physiological functions of each kisspeptin neuron population may not be assessed only by such analyses, because the pharmacological administration may activate unexpected signaling pathways apart from the actual kisspeptin neuron networks. Therefore, the detailed information on the anatomy of the axonal projections and on the physiology and distribution of receptors are necessary before the administration studies. On the other hand, the kisspeptin neurons in certain brain nuclei show steroid sensitivity in all the animal species thus far examined across teleosts and tetrapods. Therefore, we may be able to correlate the functional properties of the different populations of kisspeptin neurons in various species by using the sex steroid sensitivity as more physiological criteria.

On the other hand, it has been generally accepted in evolutionary biology that genes duplicated from a single gene in the ancestral vertebrate undergo sub-functionalization, neo-functionalization, or non-functionalization (Ohno, 1970). Recent literature on the kisspeptin of non-mammalian species suggests that the *kiss1* and *kiss2* systems may have undergone such evolutionary processes. To understand the parallel evolution of these genes in the kisspeptin neuronal systems, we here propose that the steroid sensitivity helps to identify the functionally equivalent neuronal populations among different species, because the steroid sensitivity appears to be the evolutionarily well conserved feature of certain populations of the kisspeptin neurons. As will be argued below, the non-mammalian kisspeptin systems show a wealth of diversity of gene expression (*kiss1* and/or *kiss2*) pattern in the brain and dynamic changes in expression according to the sex steroid milieu. Therefore, in spite of some kind of confusion in the kisspeptin studies of non-mammalian species, the biological study of *kiss1* and *kiss2*, and of neurons that express these genes will provide us interesting insights into the general features of the kisspeptin systems in vertebrates. In addition to be an interesting model to understand the general evolutionary mechanisms of paralogous

genes, the study of kisspeptin systems in non-mammalian vertebrates may lead us to find novel functions of kisspeptins, which may have been overlooked in previous studies using limited groups of mammalian species, which have globally lost *Kiss2*.

In the present review, we will introduce recent findings about *kiss1* and *kiss2* in vertebrates, mainly mammals and teleosts, and discuss their functions from various aspects, including cellular localizations, steroid sensitivity, and receptor distributions. Although there are a small number of studies that have been performed in non-mammalian tetrapods and vertebrates that emerged prior to the divergence of tetrapod from teleosts, we propose some general hypotheses about the evolution of *kiss1* and *kiss2* in the vertebrate lineage by comparing the two distinct groups, teleosts and tetrapods (see **Table 1** for nomenclature of kisspeptin genes and peptides)[1].

KISS1 AND KISS2

KISS1 AND *KISS2* ARE THE SISTER GENES: WHOLE GENOME DUPLICATION IN THE ANCESTRAL VERTEBRATE

In 2008, *kiss1* gene was isolated in non-mammalian species (Kanda et al., 2008; van Aerle et al., 2008). Then in 2009, *kiss2* was cloned from teleosts (Kitahashi et al., 2009) and amphibians (Lee et al., 2009) as the gene responsible for the peptide that showed a similar amino acid sequence to Kiss1. Because some ligand–receptor interaction studies showed that both Kiss2 and Kiss1 activate the kisspeptin receptor signaling pathways in *Xenopus tropicalis*, zebrafish (Lee et al., 2009), bullfrog (Moon et al., 2009), and goldfish (Li et al., 2009), Kiss2 has been recognized as one of the "kisspeptin" peptides.

Felip et al. (2009) performed a sophisticated synteny analysis of *kiss1* and *kiss2* genes in vertebrate species. In their report, it was strongly suggested that *kiss1* and *kiss2* are duplicated together with some surrounding genes such as *golt1a/b*, *plekha5/6*, *pik3c2b/cg*, and *etnk1/2*. They also discovered that only one co-ortholog for each pair was found in a chordate (*Ciona*), suggesting that these genes including *kiss1*/*kiss2* resulted from a gene duplication event that occurred at least "after" the divergence of urochordates and vertebrates.

Concerning the duplication of genes in general, it is strongly suggested that the common ancestors of the present vertebrates underwent whole genome duplication (WGD) twice (2R hypothesis; Ohno, 1970) that is evidenced by results of recent genome sequencing in vertebrate and urochordate species (Putnam et al., 2008). In addition, the ancestors of teleosts are suggested to have undergone one additional WGD [third-round (3R)-WGD; reviewed in Sato and Nishida, 2010]. Here, because amphibians and lampreys have both *kiss1* and *kiss2*, these homologs are considered to have duplicated in the ancestral vertebrate before the emergence of lamprey, as suggested by Felip et al. (2009). During evolution, some species such as the puffer fish, stickleback (Felip et al., 2009; Kitahashi et al., 2009; Li et al., 2009; Shahjahan et al., 2010; Yang et al., 2010), and some perciform fish (Felip et al., 2009; Mechaly et al., 2011) seem to have lost the *kiss1* gene. On the other hand, in tetrapods, most mammals have lost *Kiss2* during evolution. Because the platypus possesses both *Kiss1* and *Kiss2*, the loss of *Kiss2* must have occurred at least after the divergence of monotreme and other mammals. On the other hand, because the opossum is reported to lack *Kiss2* in its genome database (Felip et al., 2009), we may predict that the loss of *Kiss2* in the mammalian lineage occurred before the divergence of marsupials and placentarians (**Figure 1**).

Recent studies that are based on the genome sequence data suggested that most of the duplicated genes are subsequently lost rapidly after duplication (Brunet et al., 2006; Sato et al., 2009). Thus, for the teleost specific 3R-WGD, it is suggested that both *kiss1* and *kiss2* duplicated once again to give rise to four genes, and two of them were likely lost immediately in the early teleost lineage.

Taken together, this conservative organization of loci that contain *kiss1*/*kiss2* observed widely in vertebrates strongly suggest that *kiss1* and *kiss2* genes were duplicated at the locus level, and as Um et al. (2010) suggested, this duplication probably occurred in two rounds of WGD (1R-WGD and 2R-WGD; reviewed in Sato and Nishida, 2010) event (**Figure 1**).

KISS1 AND KISS2 ACTIVATE KISSPEPTIN RECEPTOR, GPR54

After the identification of Kiss1 and Kiss2, several ligand–receptor interaction studies have shown that both Kiss1 and Kiss2 activate the kisspeptin receptor signaling pathways in goldfish (Li et al., 2009), zebrafish, *Xenopus* (Lee et al., 2009), orange spotted grouper (Shi et al., 2010), and bullfrog (Moon et al., 2009), suggesting that Kiss1 and Kiss2 bind to the same kisspeptin receptor in vertebrates. Although the activation of the PKC or the cAMP pathway by Kiss1 and Kiss2 is slightly different in each species, it is generally accepted that both Kiss1 and Kiss2 are ligands for GPR54 in vertebrates. Thus, both Kiss1 and Kiss2, the peptide products of sister genes, can function as kisspeptins.

The kisspeptin receptor in mammals has been referred to either as GPR54 (Seminara et al., 2003) or Kiss1r (Gottsch et al., 2009). In many species studied so far, it has often been shown that more than one ligand and more than one receptor for kisspeptin bind to one another promiscuously (Lee et al., 2009; Li et al., 2009). Since recent studies also show the promiscuity of kisspeptin and other RF amide peptides (Lyubimov et al., 2010), it may be also possible that certain peptides other than Kiss1 and Kiss2 activate the kisspeptin receptor signaling pathways. Thus, although we once proposed a systematic nomenclature for kisspeptin receptor (Akazome et al., 2010), we refer to the kisspeptin receptors as GPR54-1 and 2 as proposed in Lee et al. (2009) in the present review.

[1]According to the Zebrafish Information Network, ZFIN; http://zfin.org/zf info/nomen.html, we will italicize gene names, such as *kiss1* and *kiss2*, and Romanize protein and peptide name, such as Kiss1 and Kiss2 (for details, see **Table 1**). We will call the receptor for kisspeptins as "GPR54" in the present review. This is because a recent study has reported on the promiscuous nature of ligands and receptors for RF amide families, including kisspeptin (Lyubimov et al., 2010), and the terms, kissr or Kissr, may lead to misunderstandings about the ligand receptor relationships.

Table 1 | Terminology of gene and protein names for kisspeptin.

	Primates	Non-primate mammals	Non-mammalian vertebrates
gene/mRNA	*KISS1*	*Kiss1*	*kiss1*
Protein	KISS1	Kiss1	Kiss1

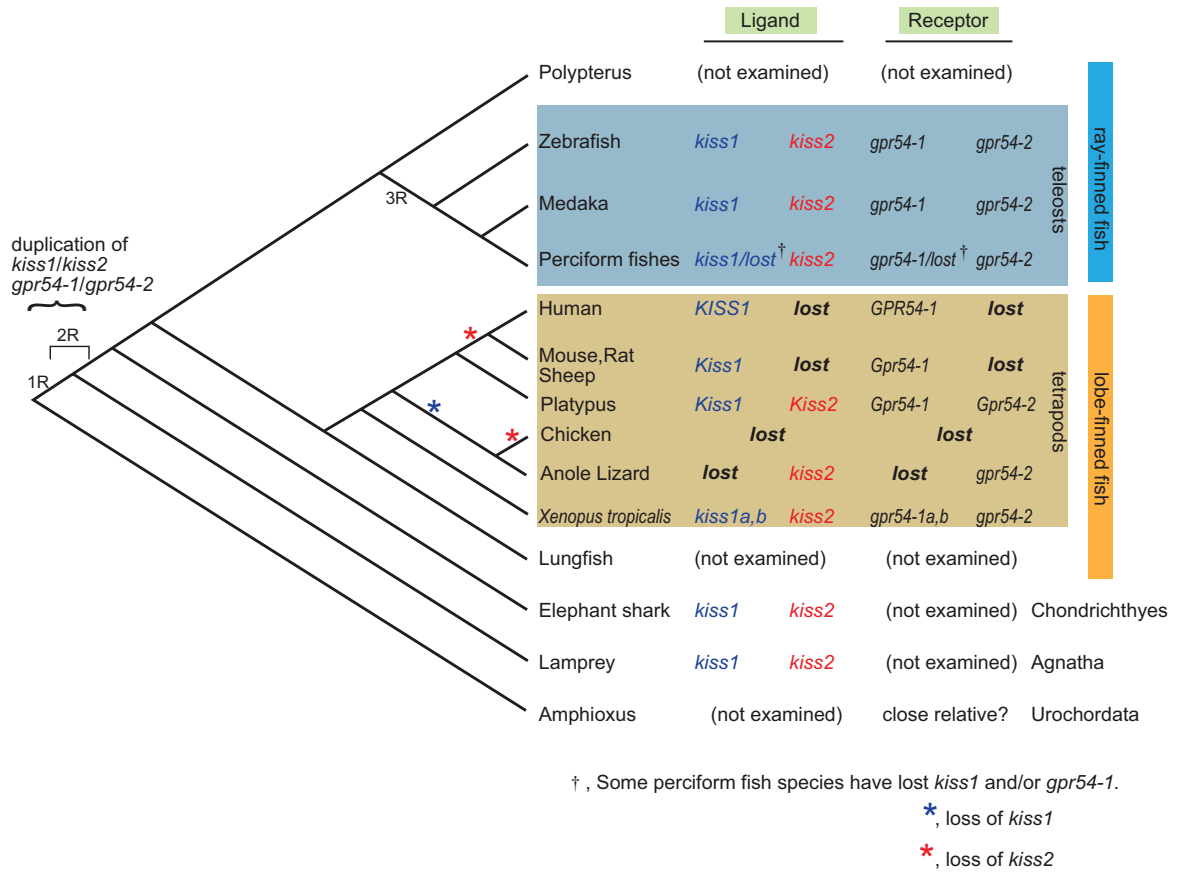

FIGURE 1 | Summary of the subtypes of ligand and receptor genes for kisspeptin systems in the genome along the vertebrate lineage. *kiss1* and *kiss2* are supposed to be duplicated before the emergence of lamprey, and probably due to the whole genome duplication of the ancestral vertebrate. It is supposed that *kiss2* and *gpr54-2* were lost in marsupial and placental mammals after the divergence from the monotreme during mammalian evolution. It should be noted that some teleost species have lost *gpr54-1*, but no teleosts have lost *gpr54-2*, suggesting the significance of *gpr54-2* in teleosts, which is opposite to the case in the mammalian lineage. It is also consistent with the higher level of expression of *gpr54-2* compared to that of *gpr54-1* in teleost brains. The loss of *kiss1* (blue) or *kiss2* (red) is indicated by asterisks.

KISSPEPTIN ADMINISTRATION STUDY OF KISSPEPTIN FUNCTIONS

In mammals, many studies have shown that peripheral or ICV injection of kisspeptin evokes LH secretion in rodents (Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Navarro et al., 2004, 2005a,b), sheep (Messager et al., 2005), monkey (Shahab et al., 2005; Plant et al., 2006; Seminara et al., 2006), and human (Dhillo et al., 2005). Moreover, it has been reported that the kisspeptin stimulates GnRH neurons both directly and indirectly via interneurons (Han et al., 2005; Dumalska et al., 2008; Liu et al., 2008; Pielecka-Fortuna et al., 2008; Zhang et al., 2008; Pielecka-Fortuna and Moenter, 2010). However, the number of studies in animals that possess both Kiss1 and Kiss2 is limited. In such animals, there is no evidence for direct kisspeptin GnRH regulation on GnRH release, and the results of peripheral administration of Kiss1 and Kiss2 decapeptides are not consistent with one another. Administration of the core decapeptide of Kiss2, but not of Kiss1, significantly increased LHβ and FSHβ subunit mRNA expression in the zebrafish pituitary (Kitahashi et al., 2009). Kiss2 also induced LH and FSH secretion in sea bass with higher potency than Kiss1 decapeptide (Felip et al., 2009). In contrast, intraperitoneal administration of Kiss2 peptide in goldfish did not increase serum LH levels, although the administration of Kiss1 peptide did (Li et al., 2009). Thus, there appears to be a difference amongst species in the relative potencies of Kiss1 and Kiss2 for facilitating LH and FSH secretion and in the time of LH/FSH rise after administration of kisspeptins. Therefore, the induction of LH/FSH secretion by kisspeptins (Kiss1 or Kiss2) in non-mammalian vertebrates needs more experimental evidence to be accepted as a general notion. Moreover, because peripheral administration does not reflect the actual axonal projection and the receptor distribution of kisspeptin neurons in the brain, cellular-level studies, such as electrophysiology, are necessary to determine the action sites of kisspeptins and the possible interactions between kisspeptin and GnRH neurons. Therefore, electrophysiological examination of effects of either Kiss1 or Kiss2 on GnRH neurons in species that possess both genes is important for the understanding of such peptidergic systems that arose from the gene duplication.

INVOLVEMENT OF KISSPEPTIN NEURONS IN THE STEROID FEEDBACK SYSTEM

In the rodent anteroventral periventricular nucleus (AVPV) and sheep preoptic area (POA), *kiss1* mRNA expression in Kiss1 neurons are upregulated, whereas those in the arcuate nucleus (ARC) are downregulated, by gonadal steroids (Irwig et al., 2004;Smith et al., 2005a,b, 2007, 2008; Revel et al., 2006; Adachi et al., 2007; Ansel et al., 2010). Although there is a discrepancy that ewe ARC Kiss1 show higher expression in the breeding season in spite of the negative regulation of gonadal steroids (Estrada et al., 2006; Smith, 2009), it is generally accepted that AVPV/POA Kiss1 neurons are positively regulated, and ARC Kiss1 neurons are negatively regulated by the gonadal steroids. These properties are widely recognized in mammalian species.

In mice, it has been shown that GnRH neurons do not express ERα (reviewed in Herbison and Pape, 2001), which are essential for normal reproductive functions (Couse et al., 2003; Dorling et al., 2003; Wintermantel et al., 2006). Thus, Herbison concludes that estrogen acts indirectly on GnRH neurons to bring about their activation (Herbison, 2008). This missing link in mice was found to be explained by steroid sensitive kisspeptin neurons, because kisspeptin directly depolarizes GnRH neurons in mice (Han et al., 2005; Dumalska et al., 2008; Liu et al., 2008; Pielecka-Fortuna et al., 2008; Zhang et al., 2008; Pielecka-Fortuna and Moenter, 2010), which is expected to facilitate firing activities and then GnRH release. Thus, the role of kisspeptin neurons in steroid feedback is one of the most interesting topics in the study of kisspeptin neuron systems.

In non-mammalian vertebrates, the localization of *kiss1*/*kiss2* neurons is reported in medaka (Kanda et al., 2008; Mitani et al., 2010), zebrafish (Kitahashi et al., 2009), *Xenopus* (Lee et al., 2009), seabream (Shimizu et al., 2012), and puffer fish (Kanda et al., 2010). Among them, medaka and goldfish are the only species whose steroid sensitivity has been examined experimentally. Therefore, recent data shown by us in medaka will be described below. In addition, we will also describe results in the goldfish, which gives us insights into the evolutionary aspects of *kiss1*/*kiss2* genes.

GONADAL STEROIDS UPREGULATE *KISS1* EXPRESSION IN MEDAKA NVT NEURONS

In situ hybridization studies have shown the localization of *kiss1* and *kiss2* neurons in the medaka brain (Kanda et al., 2008; Kitahashi et al., 2009; Mitani et al., 2010). The *kiss1* neurons are localized in the hypothalamic nuclei, nucleus ventralis tuberis (NVT) and nucleus posterioris periventricularis (NPPv), as well as in an extrahypothalamic nucleus, habenula. On the other hand, the *kiss2* neurons are localized in nucleus recessus lateralis (NRL). Among these *kiss1* and *kiss2* neurons, only the *kiss1* neurons in NVT show prominent steroid sensitivity in their kisspeptin gene expression. Ovariectomy (OVX) dramatically reduced *kiss1* expression in NVT neurons, which were recovered by subsequent estrogen replacement. Because our unpublished data showed that 11 ketotestosterone, a non-aromatizable androgen, did not recover this decrease at all, the steroid feedback activity seems to be mediated by estrogen receptor. It was also shown that NVT *kiss1* neurons are the only kisspeptin neuron population that shows expressional variations according to the breeding states. Moreover, double insitu hybridization analysis has shown that *kiss1* neurons in NVT express ERα. In addition to ERα, NVT *kiss1* neurons in medaka express ERβ as well (Mitani et al., 2010), which is similar to *kiss1* neurons in mice (Smith et al., 2005a).

Interestingly, in addition to steroid sensitivity, NVT *kiss1* neurons also show sexual dimorphism in number (Kanda et al., 2008). In contrast to the sexual dimorphism found in rodents, in which females have more AVPV *Kiss1* neurons than males (Clarkson and Herbison, 2006; Smith et al., 2006; Adachi et al., 2007; Kauffman et al., 2007), male medaka show significantly more NVT *kiss1* neurons than females. Comparative analyses in much more vertebrate species should be necessary for understanding the organization of sexual dimorphism and the functional significance of male- or female-predominant expressions.

POA *KISS2* NEURONS IN GOLDFISH SHOW STEROID SENSITIVITY LIKE POA *KISS1* NEURONS IN MAMMALS

In zebrafish, localization of *kiss1* and *kiss2* neurons was analyzed by *in situ* hybridization (Kitahashi et al., 2009; Servili et al., 2011). The *kiss1* neurons in the zebrafish brain are distributed in the habenula and periventricular hypothalamus, while the *kiss2* neurons are distributed in the posterior tuberal nucleus, the periventricular hypothalamic nucleus, and parvocellular preoptic nucleus (Kitahashi et al., 2009; Servili et al., 2011). In the juvenile zebrafish, it was demonstrated that estradiol administration increases the mRNA expression of *kiss1*, *kiss2*, and *gpr54-2* in the brain (Servili et al., 2011). Among them, Servili et al. focused on *kiss2* neurons in the dorsal hypothalamus (Hd), caudal hypothalamus (Hc), and anterior tuberal nucleus (ATN), and demonstrated that all of them showed higher *kiss2* expression after estradiol administration in juvenile fish. Because it may not be physiological to administrate estrogen to juveniles, it may be rather difficult to interpret these results. In spite of this, it is intriguing to investigate the homologous relationships of these neurons to the medaka steroid sensitive *kiss1* neurons in NVT and steroid insensitive *kiss2* neurons in NRL. However, as the hypothalamic structures vary even among teleosts, and no clear experimental evidence for the nucleus-specific steroid sensitivity in adults has been shown in any fish except medaka, further examination of the effects of gonadal steroids on the teleost kisspeptin neurons using ovariectomy should be necessary.

Therefore, we recently performed *kiss1* and *kiss2* *in situ* hybridization in the goldfish, because the goldfish is rather easily amenable to ovariectomy, and it belongs to the same Cypriniformes as the zebrafish. We found a prominent expression of *kiss2* in POA, unlike results in zebrafish, and found that the POA *kiss2* neurons show clear steroid sensitivity (Kanda et al., 2012). In the adult goldfish in the breeding condition, the *kiss1* neurons are localized in the habenula, whereas the *kiss2* neurons are located in nucleus lateralis tuberis (NLT), NRL, and POA. Among these neurons, the POA *kiss2* neurons decreased in number after OVX, and the reduction was recovered by estrogen implant. It strongly suggests that POA *kiss2* neurons are upregulated by ovarian estrogen, which is similar to the AVPV/POA *kiss1* neurons in mammals. The discussion on the homology and the evolutionary hypothesis derived there from will be described in detail later in this review.

PUFFER FISH, WHICH POSSESS ONLY *KISS2*, SHOW *KISS2* EXPRESSION IN POA AND HYPOTHALAMUS

From the genome database analysis, puffer fish are supposed to possess only *kiss2* expressing neurons, because they have lost *kiss1* at the genome level. Shahjahan et al. (2010) took advantage of the seasonally breeding grass puffer and showed changes in the expression levels of *kiss2*/*gpr54-2* genes together with the gonadosomatic index (GSI) during the spawning period. Here, because of the absence of *kiss1* neurons, the *kiss2* neurons in certain brain area are supposed to subserve the kisspeptin functions in this species.

Recently, we analyzed the localization of *kiss2* neurons in juvenile green puffer fish by *in situ* hybridization and found that they are expressed in the hypothalamic nucleus NRL and the POA (Kanda et al., 2010), which is similar to the results in the zebrafish (Kitahashi et al., 2009; Servili et al., 2011). Unfortunately, it is technically difficult to raise green puffer to breeding conditions, and future studies using puffer fish that are capable of breeding to full maturity in aquarium tanks will be interesting.

In addition to this observation, it was recently shown that *kiss2* expression is increased during the pre-spawning season (late spermatogenesis stage in male, and early vitellogenesis stage in female) in club mackerel (Selvaraj et al., 2010). Further expression analyses in some teleost species may lead us to find some general expressional variations of kisspeptin genes in the seasonal breeders.

SPECIES DIFFERENCE IN THE FUNCTIONS OF THE SISTER GENES, *KISS1* AND *KISS2*

There are many species differences in the functions of *kiss1* and *kiss2* neurons in vertebrates. The most extreme example lies between *kiss1*-lacking puffer fish and *KISS2*-lacking human. Even within the teleost species that express both *kiss1* and *kiss2*, there are obvious species differences. For example, in medaka, only the NVT *kiss1* neurons show steroid sensitivity, whereas there is no such *kiss1* neuron in the hypothalamus of zebrafish, and, instead, many *kiss2* neurons are localized in the hypothalamus.

Medaka *kiss2* neurons are localized in NRL, where *kiss2* neurons are also localized in zebrafish. Although Servili et al. (2011) proposed a possibility of functional similarity between the steroid sensitive medaka NVT *kiss1* neurons and some of the zebrafish *kiss2* neurons in the ventral hypothalamus, the zebrafish *kiss2* neurons appear to contain neurons equivalent to the medaka *kiss2* neurons and some other populations of neurons. It should be interesting to search for experimental evidence for such homologies. *In situ* hybridization and immunohistochemistry using some other fish species may give us clues to further understanding of the functional homology and evolution of these sister gene-expressing neuron systems. Experimental analysis on the effects of gonadal steroids by gonadectomy in various non-mammalian species should be very helpful to discuss true functional or morphological homologies, because the steroid sensitivity well characterizes the property of each nucleus, but such studies have been performed only in a small number of species such as medaka and goldfish.

EVOLUTION OF *KISS1* AND *KISS2* NEURONS IN EACH NUCLEUS – A WORKING HYPOTHESIS

As described above, *kiss1* and *kiss2* in the present vertebrate species are suggested to be the sister genes, which originate from the gene duplication event in the ancestral vertebrate. Furthermore, it is highly possible that they were duplicated during the genome-wide duplication events. Because these sister genes possess family genes in their loci, they are considered to have duplicated at least at the locus level, regardless of whether the WGD event made these sister genes or not. Thus, just after the duplication, *kiss1* and *kiss2* must have had completely the same sequence in their open reading frame as well as the regulatory sequence, and they must have been co-expressed in the same location at first. During evolution, one or even both of them was silenced in some species, and their location of expression and function diverged. Moreover, as seen between medaka and zebrafish/goldfish, the general functions or relative functional importance of *kiss1* and *kiss2* for the central regulation of reproduction are different among species; their functions are considered to have diverged among species during evolution (Kanda et al., 2012).

Interestingly, the inversion of the importance of *kiss1* and *kiss2* for the central regulation of reproduction occur rather commonly among different species (see the previous section). On the other hand, this phenomenon never occurred during the evolution of the hypophysiotropic GnRH system; the Cyprinids and Salmonids have lost *gnrh1*, but this lack seems to have been functionally compensated by the remaining genes (Okubo and Nagahama, 2008). We suppose that the functional conservativeness of the GnRH systems may be due to the fact that loss of the *gnrh* function, especially the hypophysiotropic one, would lead to severe reproductive dysfunctions (Cattanach et al., 1977) or to a failure of normal sexual maturation (Wu et al., 2006).

We have been routinely performing in situ hybridization of *kiss1* and *kiss2* genes (Kanda et al., 2008; Mitani et al., 2010) and find it more difficult to detect them compared to *gnrh2* or *gnrh3* (Gopinath et al., 2004; Okubo et al., 2006; Palevitch et al., 2007), which empirically suggests the lower level of expression of *kiss1*/*kiss2*, especially during the developmental stages. Moreover, the reported lack of *gpr54* expression in the hypophysiotropic GnRH1 neurons in some teleosts (Grone et al., 2010; and our unpublished observation) suggests that the physiological functions and their mechanisms are somewhat more diverged in the vertebrate *kiss1*/*kiss2* systems, compared to the rather conservative GnRH systems. Thus, unlike the GnRH systems, in which the inversion of physiological functions between the hypothalamic hypophysiotropic (GnRH1) and extrahypothalamic neuromodulatory GnRH systems (GnRH2/GnRH3) has never been reported, the *kiss1* and *kiss2* systems are the ones having rather promiscuous ligand–receptor relationships and are supposed to be more adaptive during evolution; they appear to have avoided extreme selection pressure. Thus, the understanding of the evolution of *kiss1* and *kiss2* neuron systems may lead to a model for the study of general evolutionary mechanism of peptidergic neurons in the absence of strong selection pressure.

EVOLUTION OF STEROID SENSITIVE KISSPEPTIN NEURONS IN POA/AVPV

The distribution of *kiss1* and *kiss2* neurons in representative animal species and in the presumptive common ancestral animals are shown in **Figure 2**. It has been already suggested that POA Kiss1 neurons in sheep, AVPV Kiss1 neurons in rodents, and PeN Kiss1 neurons in pig are homologous in mammals (Smith, 2009; Tomikawa et al., 2010). These forebrain kisspeptin neurons in mammals, which show steroid sensitivity, are Kiss1 neurons, although they have not been examined in monotreme, which have both *Kiss1* and *Kiss2* genes. On the other hand, the goldfish steroid sensitive POA kisspeptin neurons express *kiss2* mRNA (Kanda et al., 2012). This may sound a little bit strange at first sight. However, the results in *Xenopus laevis* may give us a hint for the evolutionary mechanism. The *Xenopus* POA kisspeptin neurons express *kiss2* mRNA (Lee et al., 2009). Thus, all the vertebrate forebrain populations of kisspeptin neurons studied so far, except for mammals, are Kiss2 neurons. We therefore hypothesize that both ancestral teleosts and ancestral tetrapods expressed *kiss2* in POA. We further hypothesize that in mammals *Kiss1* is expressed in the neurons that are homologous to *Xenopus* POA *kiss2* neurons; this conversion of *Kiss2* to *Kiss1* may have occurred because of the loss of *Kiss2* after the divergence of the ancestor of the present mammals from the monotreme. Thus, we assume that, during mammalian evolution, the loss of *Kiss2* gene triggered the expression of *Kiss1* in mammalian POA. An alternative possibility is that *Kiss1* neurons emerged in the POA before they lost *Kiss2*.

However, the overlapped functions tend to be lost rapidly during evolution in general. We suppose that the loss of *Kiss2* triggered the expression of *Kiss1* in the same neurons because of the similar regulatory sequence between *Kiss1* and *Kiss2*. In other words, the loss of *Kiss2* might have canceled the expressional inhibition of *Kiss1* in POA kisspeptin neurons. Such a phenomenon is called the "genetic robustness" and has been only examined in the duplicated genes of *C. elegans* and yeast (Gu et al., 2003; Conant and Wagner, 2004), or has been observed in human genetic disease (Hsiao and Vitkup, 2008). In these studies, it was shown that the duplicated genes, which have a similar copy as the sister genes, tend to cause genetic disease less frequently compared to the singleton genes. Thus, it is supposed that closely related genes, such as sister genes, often compensate for the gene loss, and we suppose that *Kiss2* gene loss and emergence of *Kiss1* neurons in mammalian lineage coincided with each other. To our knowledge, there has been no report on the occurrence of such "genetic robustness" in the highly complicated neuronal systems of vertebrates. Although it is difficult to use monotreme for experimental use, it is intriguing to study the localization of monotreme *Kiss1* and *Kiss2* neurons. Thus, the evolutionary studies of *kiss1* and *kiss2* genes should serve as a good model system for the study of evolution of sub-functionalized sister genes in the central nervous system in general.

STEROID SENSITIVE AND INSENSITIVE HYPOTHALAMIC KISSPEPTIN NEURONS

In mammals, the hypothalamic Kiss1 neurons in ARC are steroid sensitive as described above. Because OVX increases the *Kiss1*

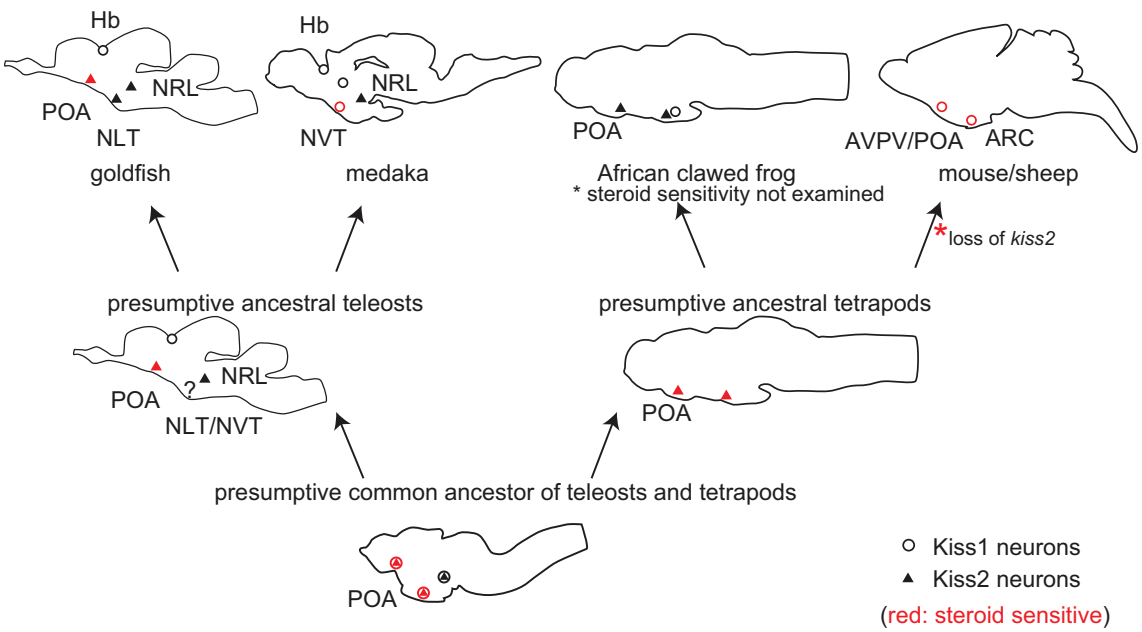

FIGURE 2 | Schematic illustrations for the distribution of *kiss1* and *kiss2* neurons in vertebrate brains, including some hypotheses. Open circles indicate *kiss1*, and filled triangles indicate *kiss2* neurons. Circles/triangles in red are *kiss1*/*kiss2* neurons that are steroid sensitive. Because *kiss1* and *kiss2* are duplicated sister genes, they are considered to have been co-expressed in the same neurons in the common ancestor of teleosts and tetrapods. Considering the fact that both amphibians and teleosts express *kiss2* in POA, the ancestral teleosts and ancestral tetrapods are supposed to have expressed *kiss2*. Because *Kiss2* was lost in the mammalian lineage, we hypothesize that *Kiss1* began to be expressed where *Kiss2* used to be expressed, to compensate for the loss of *Kiss2* during mammalian evolution.

expression, and estrogen or testosterone nullifies this increase in ARC, the ARC Kiss1 neurons are considered to be negatively regulated by ovarian steroids.

In species other than mammals, the only report about the gonadal steroid regulation on the hypothalamic kisspeptin neurons is on medaka Kiss1 neurons (Kanda et al., 2008; Mitani et al., 2010). Here, unlike the mammalian ARC Kiss1 neurons, their expression is enhanced, instead of inhibited, by ovarian steroids. Thus, one may argue against the homology of these nuclei in teleosts and mammals. We should note, however, that positive or negative regulation can be rather easily inverted by the composition of co-expressed transcription factors, such as Sp1 and Sp3 (Li et al., 2007). Thus, the difference in positive/negative regulation itself should not disprove the homology. Moreover, NVT also contains some other types of neurons involved in the homeostatic regulation like the mammalian ARC, supporting the evidence for the possible homology of these nuclei. However, because the teleosts lack the median eminence, the characteristic projection of ARC Kiss1 neurons to the median eminence, which is usually observed in mammals, cannot be observed. Instead, the medaka NVT Kiss1 neurons are shown to project to the POA. Experimental analysis of steroid sensitivity and the axonal projections of the hypothalamic Kiss1 neurons in other animals are necessary for the understanding of the evolutionary origin and possible homology of the hypothalamic Kiss1 neurons in other animals are necessary for the understanding of the evolutionary origin and possible homology of the hypothalamic Kiss1 neurons.

In medaka, gene expression of the NRL *kiss2* neurons was shown to be independent of breeding conditions), and these *kiss2* neurons were not shown to express ERα (Mitani et al., 2010). Thus, the neurons that are homologous to the steroid insensitive teleost hypothalamic (NRL) *kiss2* neurons may be absent in mammals. Further comparative studies among various species of teleosts and amphibians may solve the problem of whether steroid insensitive hypothalamic *kiss2* neurons are specific to the teleosts or just lost in mammals.

TELEOST-SPECIFIC EXPRESSION OF *KISS1* IN HABENULA

The kisspeptin neurons in the habenula have been reported only in teleosts (Kitahashi et al., 2009; Mitani et al., 2010; Servili et al., 2011), and they express *kiss1* in all those species. The projection of habenular neurons have been well studied by classical neuroanatomical experiments as well as by using recent molecular genetic techniques (Aizawa et al., 2005; Gamse et al., 2005). Consistent with results of these studies of habenular projections, the habenular Kiss1 neurons in zebrafish (Servili et al., 2011) and medaka (Kanda et al., unpublished data) appears to project to the interpeduncular nucleus (IPN) via fasciculus retroflexus. As *gpr54-2*, one subtype of *gpr54* genes in teleosts, is reported to be expressed in IPN, and *gpr54-1*, another subtype, is co-expressed in habenula in zebrafish, Servili et al. (2011) suggested the existence of the autocrine regulation of habenula (via *gpr54-1*) and the target neurons in IPN (via *gpr54-2*). Because there is no other report on the existence of *kiss1* neurons in the tetrapod habenula, it remains to be known whether the habenular *kiss1* was originally expressed in the ancestral vertebrate and subsequently lost in the tetrapod lineage, or solely began to be expressed in the teleost lineage. In order to answer this question, expression analysis of *kiss1* in phylogenetically significant species such as lungfish and polypterus are ongoing in our laboratory.

CONCLUSION

In this review article, we reviewed recent findings of kisspeptin neurons in vertebrates, by comparing studies in mammalian species and non-mammalian species, mainly teleosts. To date, only a handful of studies have shown kisspeptin neurons' functional significance in the central regulation of reproduction in species other than mammals. Because kisspeptin receptor *gpr54-2* as well as *gpr54-1* are expressed in POA and hypothalamus, but not in GnRH1 neurons in some species, kisspeptin's novel function other than the central regulation of reproduction (Kadokawa et al., 2008; Szawka et al., 2010; Yang et al., 2010; Luque et al., 2011) will also be an interesting topic in the future studies.

As reviewed above, the steroid sensitivity of kisspeptin neurons has been reported in a wide variety of species including non-mammalian vertebrates. Although the mechanisms are not well studied in the non-mammalian species, the steroid sensing feature and the related functions are highly conserved throughout vertebrates. Although the natural selection of functions of paralogous *kiss1* and *kiss2* genes are complicated, the study of evolutionary process of these sister genes may give clues to understand the evolution of the central nervous system after genome duplication in general. Here, the kisspeptin neuron system may be not only the regulator of reproductive/homeostatic functions in vertebrates but also the pioneer toward further understanding of the evolution of the central nervous system functions.

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