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Review article

Evolution of the regulatory mechanisms for the hypothalamic-pituitary-gonadal axis in vertebrates–hypothesis from a comparative view

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ABSTRACT

Reproduction is regulated by the hypothalamic-pituitary-gonadal (HPG) axis in vertebrates. In addition to wealth of knowledge in mammals, recent studies in non-mammalian species, especially teleosts, have provided evidence that some of the components in the HPG axis are conserved in bony vertebrates. On the other hand, from the comparisons of the recent accumulating knowledge between mammals and teleosts, unique characteristics of the regulatory system in each group have been unveiled. A hypophysiotropic neurotransmitter/hormone, gonadotropin releasing hormone (GnRH), pituitary gonadotropins, follicle stimulating hormone (FSH), and luteinizing hormone (LH) were proven to be common important elements of the HPG axis in teleosts and mammals, although the roles of each vary. Conversely, there are some modulators of GnRH or gonadotropins that are not common to all vertebrates. In this review, I will introduce the mechanism for HPG axis regulation in mammals and teleosts, and describe their evolution from a hypothetical common ancestor.

1. Central regulatory mechanism of reproduction in mammals

1.1. GnRH has an essential role in both folliculogenesis and ovulation in mammals

The hypothalamus and pituitary play pivotal roles in the regulation of gonads in vertebrates. In mammals, gonadotropin releasing hormone (GnRH), which is synthesized in GnRH neurons, is released to the hypophyseal portal vessel. GnRH acts on gonadotrophs in the pituitary and stimulates gonadotropin release, luteinizing hormone (LH), and follicle stimulating hormone (FSH). During folliculogenesis, FSH plays an important role in the beginning, then pulsatile LH stimulates folliculogenesis in the later phase. GnRH, FSH, and LH are all essential for folliculogenesis because their knockout (KO)/natural mutants result in immature gonads and infertility in both males and females (Abel et al., 2000; Cattanach et al., 1977; Kumar et al., 1997; Ma et al., 2004).

For the regulation of GnRH and gonadotropin release, negative and positive feedback regulation by gonadal steroids are important, which are extensively studied in females (Herbison, 2014). Gonadal sex steroids, mainly estrogen, suppress the frequency of LH pulses and FSH release during folliculogenesis, which in turn prevents excess maturation of follicles (negative feedback). After the follicles are fully developed, a high concentration of serum estrogen produced by the follicles induces an increase in LH release. It is called an LH surge and triggers final oocyte maturation and ovulation.

1.2. Kisspeptin neurons are the key mediator of steroid feedback in mammals

Although such strong relationships among GnRH, FSH, LH, and gonadal steroids have been suggested, the regulatory mechanism of GnRH neurons by gonadal steroids had been unclear because GnRH neurons do not express estrogen receptor alpha, which forms the essential pathway from gonad to the hypothalamus (Herbison and Theodosis, 1992; Shivers et al., 1983). In 2003, the neurons expressing a novel peptide kisspeptin were found to be the prime candidates for this “missing link” of the steroid feedback. Since then, kisspeptin-expressing neurons have been a topic of interest in the field of reproductive endocrinology. Extensive studies have been conducted in the last decade, which have unveiled the mechanism and roles of kisspeptin in the regulation of GnRH and gonadotropin release. More recently, kisspeptin neurons localized in the arcuate nucleus were proven to be the LH pulse generator. These kisspeptin neurons, also known as KNDy (pronounced “candy”) neurons, co-express three kinds of neuropeptides: kisspeptin, neurokinin B (NKB), and dynorphin (Dyn) in the arcuate nucleus (Navarro, 2012, 2013; Okamura et al., 2013). In the

Abbreviations: HPG, hypothalamic-pituitary-gonadal axis; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; KO, knockout; NKB, neurokinin B; Dyn, dynorphin; AVPV, anteroventral periventricular nucleus; POA, preoptic area; ELISA, enzyme-linked immunosorbent assay; FOM, final oocyte maturation; PMSG, pregnant mare serum gonadotropin; GPCR, G protein-coupled receptor

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network of these KNDy neurons, NKB facilitates while Dyn suppresses the firing activity of their own in an autocrine/paracrine manner, resulting in intermittent burst firing of the neurons and release of kisspeptin from their nerve terminals in the median eminence (Navarro et al., 2009; Wakabayashi et al., 2010). This intermittent kisspeptin release produces pulsatile release of GnRH from the adjacent fibers of GnRH neurons. As GnRH travels to the adenohypophysis via hypophyseal portal vessel, this GnRH pulse induces pulsatile release of LH from the pituitary (Maeda et al., 2010; Okamura et al., 2013). Actually, a recent study using optogenetics revealed that high-frequency stimulation of arcuate kisspeptin neurons increased serum LH level, while the same stimulation of channel rhodopsin expressed in KNDy neurons in kisspeptin receptor KO mice did not alter serum LH concentration (Han et al., 2015). Interestingly, expression of kisspeptin in the arcuate nucleus is negatively regulated by gonadal estrogen (Smith, 2008, 2013), which enables upregulation of LH release when the serum estrogen level is low. Thus, these KNDy neurons are suggested to have an important role in the negative feedback regulation of LH release by estrogen.

On the other hand, kisspeptin neurons in anteroventral periventricular nucleus (AVPV) or preoptic area (POA), whose kisspeptin expression is positively regulated by gonadal estrogen (Smith, 2008), are suggested to stimulate GnRH release when serum estrogen concentration is high. Thus, this population of kisspeptin neurons are suggested to play a role in positive feedback regulation of GnRH release by estrogen.

Thus, as the missing link of the steroid feedback regulation has been found, the main framework of HPG axis regulation has been proven in mammals. The scheme of HPG axis regulation in mammals is summarized in the right panel of Fig. 1.

2. Teleosts provide insights into the common regulatory mechanism of HPG axis in vertebrates when compared to mammals

Unlike the wealth of knowledge in mammals, the understanding of HPG axis regulation in non-mammalian species is ambiguous. It is a disadvantage to have minimal knowledge of non-mammalian species because we cannot tell whether the mammalian system is common to other vertebrates, or is a specialized system. Teleosts are located in a very important position in the vertebrate phylogeny and the understandings of both mammals and teleosts give us important hints toward understanding of the vertebrates in general (Kanda, 2018). Let me consider the evolution of important factors of the HPG axis. GnRH and kisspeptin are considered to have emerged before the emergence of agnathans. Pituitary hormones, and the beta subunit of LH and FSH had been duplicated before the emergence of jawed vertebrates, and before this duplication, LH, FSH, and TSH were a single hormone (Norris and Carr, 2013). Furthermore, before the emergence of jawed vertebrates, basic pathways for HPG axis regulation (such as the hypophyseal portal vessel) had been formed (Norris and Carr, 2013). Thus, important molecules for the regulation of HPG axis had been acquired before the emergence of the common ancestor of teleosts and mammals. Interestingly, however, recent studies in teleosts have suggested many differences in the functions of these molecules. In this review, by introducing studies of teleosts, which represented many differences from mammalian results, I would suggest the basic principle of regulatory mechanism for HPG axis regulation in bony vertebrates.

3. GnRH is an important regulator of gonadotropin secretion in vertebrates

Since the discovery of GnRH, quite a few studies have examined the activity of GnRH or its analogues on serum LH and ovulation by peripheral injection in fishes (Ando et al., 2004; Dickey and Swanson, 2000; Yaron et al., 2003). Overall, GnRH robustly increased serum LH level, and slightly increased serum FSH level. These experimental designs demonstrated the contributions of GnRH in controlling the reproduction in fishes. In addition to such studies, cell-targeted electrophysiology and Ca^{2+} imaging have been developed to understand the underlying mechanisms. Karigo et al. used transgenic technique to generate the medaka expressing Ca^{2+} -sensitive recombinant fluorescent protein specifically in the LH or FSH cells in the pituitary (Karigo et al., 2014). In this study, Ca^{2+} imaging using the whole brain *in vitro* preparation of these transgenic medaka demonstrated that GnRH peptide immediately increases intracellular Ca^{2+} levels in both LH and FSH cells, which strongly suggests the existence of the direct action of GnRH on LH and FSH cells. Interestingly, the stimulation of GnRH1 axons to the pituitary also induced an increase in intracellular Ca^{2+} concentration of LH cells. The effects of GnRH on LH/FSH cells were demonstrated in other Ca^{2+} -imaging studies, which used Ca^{2+} -sensitive dye instead of genetically encoded Ca^{2+} -sensitive fluorescent protein (Strandabo et al., 2016; Strandabo et al., 2013). As these studies used isolated culture of pituitary cells, GnRH was strongly suggested to act directly on LH and FSH cells. The action of GnRH on LH and FSH cells was also shown in cod by patch-clamp and Ca^{2+} imaging (Hodne et al., 2013). These studies demonstrate that GnRH peptide can evoke LH and FSH release from pituitary cells in multiple teleost species.

3.1. KO studies revealed functions of GnRH and gonadotropins

Until recently, gene-targeted knockouts could not be applied to species without established embryonic stem (ES) cell lines. The recent developments of genome editing using zinc finger nuclease (Bibikova et al., 2002), transcription activator-like effector nuclease (TALEN) (Bedell et al., 2012), and clustered regularly interspaced short palindromic repeats (CRISPR) (Jinek et al., 2012) have enabled researchers to perform site-directed mutagenesis in any species easily only if they can collect fertilized eggs. Owing to this dramatic technological development, recent studies of zebrafish and medaka have provided important information regarding the essential functions of individual neurotransmitters/hormones in reproductive functions (Kanda, 2018).

In medaka, *gnrh1* is the only GnRH gene that is expressed in hypophysiotropic neurons (Karigo and Oka, 2013). The *gnrh1* knockouts (Takahashi et al., 2016) showed a phenotype similar to that shown by *lhb* KO medaka. As described later, *lhb* KO and *gnrh1* KO medaka show deficiencies in ovulation but not in folliculogenesis, whereas *fshb* KO disrupts folliculogenesis. Thus, the GnRH-LH pathway is essential for ovulation, whereas FSH is important for folliculogenesis and it does not require GnRH regulation. Real time PCR analysis of *lhb* and *fshb* expression in *gnrh1* KO medaka (OVX + E model) also suggested the importance of GnRH for *lhb*, but not for *fshb* expression (Takahashi et al., 2016).

Surprisingly, in zebrafish, the knockout of *gnrh3* (the only GnRH that is expressed in hypophysiotropic neurons and terminal nerve neurons in this species) did not affect the fertility of either males or females (Liu et al., 2017; Spicer et al., 2016). Moreover, a double knockout of *gnrh2* and *gnrh3* also resulted in unchanged fertility in females (Marvel et al., 2018), which strongly suggests that zebrafish appear to be able to reproduce without a GnRH system. Note that axons of GnRH3 (Abraham et al., 2008) and GnRH2 neurons (Xia et al., 2014) project to the pituitary instead of those of GnRH1 neurons in zebrafish, because they have lost *gnrh1* during the evolution of ostariophysi/cypriniform lineage (Okubo and Nagahama, 2008). However, in goldfish, which belong to the same cypriniform family, GnRH peptide administration effectively induces ovulation. Thus, in cypriniforms, GnRH is important for ovulation, although it may not be essential. In fact, none of cypriniform species has naturally lost *gnrh3* gene.

Compensation from other factors may explain why *gnrh3* KO zebrafish can ovulate (Trudeau, 2018). In fish, there are many hypophysiotropic factors reported to date, although the mechanisms have not been well documented. Among these factors, strong persistent

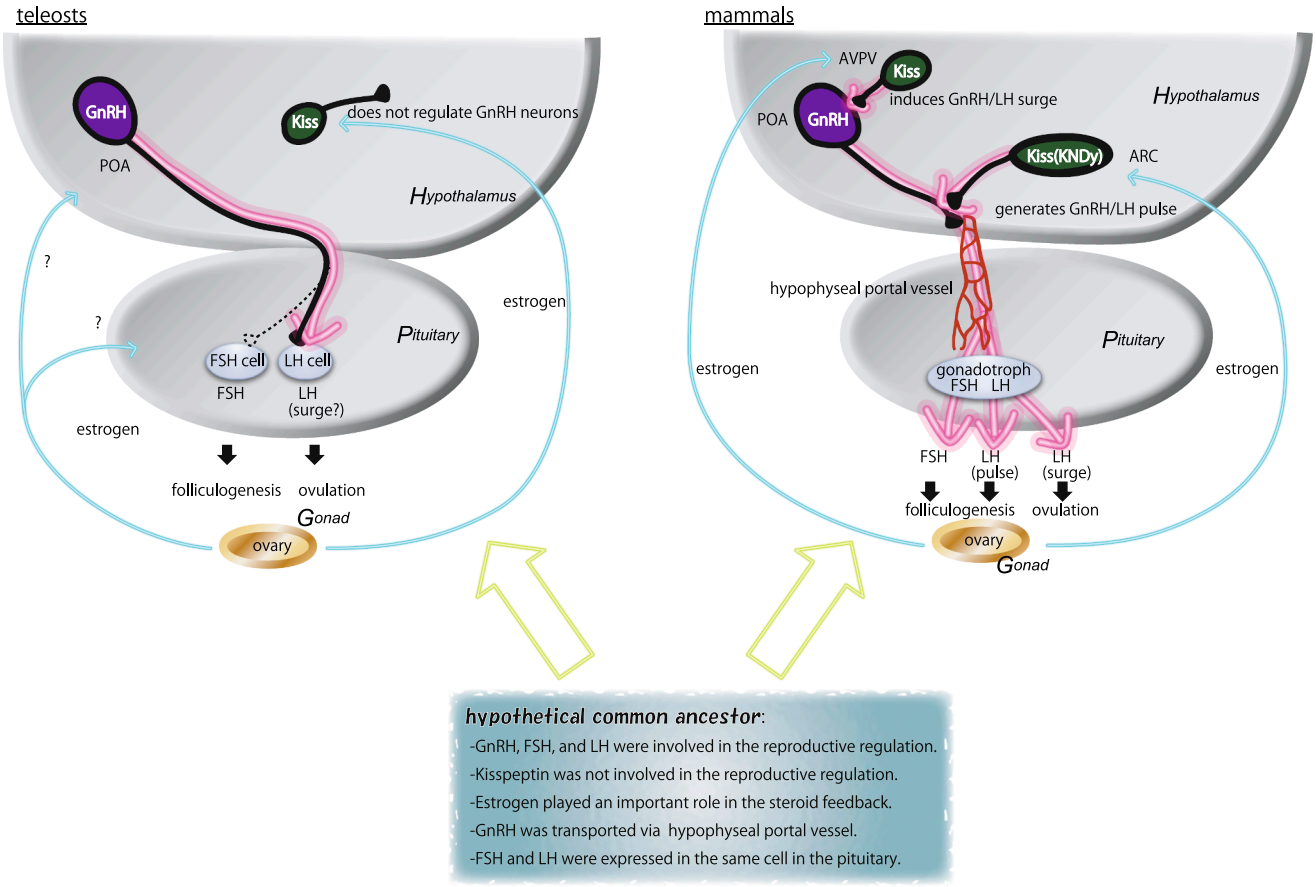

Fig. 1. Schematic illustration of HPG axis regulation in teleosts and mammals. In both mammals and teleosts, GnRH, FSH, and LH play important roles in the regulation of gonads in accordance with serum sex steroids. In teleosts, one of the gonadotropins, FSH is exclusively essential for folliculogenesis and its release does not require GnRH regulation. GnRH neurons directly project to the pituitary, and mainly regulate LH release. GnRH release is not regulated by kisspeptin, and kisspeptin is involved in functions other than HPG axis regulation. In mammals, FSH and LH are expressed in the same gonadotroph, and receive GnRH peptide through the hypophyseal portal vessel. Because of the existence of a pulse generating mechanism involving KNDy neurons, which regulate GnRH release by their neurotransmitter, kisspeptin, LH is released in a pulsatile manner. This LH pulse is essential for folliculogenesis in addition to FSH. Serum sex steroids act on estrogen receptor alpha in kisspeptin neurons and thus the steroid feedback system is formed. The main part of this drawing was reused from Kanda (2018) "Small teleosts provide hints toward understanding the evolution of the central regulatory mechanisms of reproduction" under a permission of Springer Nature publishing.

inhibition of LH release by dopamine has been well studied. In goldfish, it has been reported that intraperitoneal injections of GnRH analogue induce much higher rate of ovulation when co-injected with a dopamine D2 receptor antagonist, pimozide (Chang and Peter, 1983). Thus, disinhibition of dopaminergic effect by some factor(s) is considered to be a strong candidate for the compensatory factor in GnRH knockout zebrafish. In fact, the mixture of GnRH analogue and a dopamine D2 receptor inhibitor, domperidone, is used as a commercial drug, Ova-prim™, to induce ovulation in many fish species including goldfish.

3.2. Involvement of dopamine varies among species – hypothesis from an evolutionary view point

Interestingly, the strength of the dopamine-driven inhibition of LH release varies among teleost species. As described above, in cyprinidae fishes, LH release is strongly inhibited by dopamine. Therefore, injection of GnRH analogue alone rarely induces ovulation and necessitates simultaneous disinhibition of dopaminergic effects in goldfish. Similarly, it is also well-known that co-injection of dopamine inhibitor is necessary to induce ovulation efficiently by exogenous GnRH in eel (Vidal et al., 2004). On the other hand, in many species of acanthopterygii, GnRH alone can induce ovulation efficiently, e.g., dusky grouper (Marino et al., 2003) and chub mackerel (Shiraishi et al., 2008). In addition to this, in medaka, injections of D2 agonists, quinpirole, pergolide, and ropinirole did not inhibit spontaneous ovulation, which suggests that medaka do not have a strong intrinsic inhibition of LH release by dopamine (Mochizuki, personal communication), although further trials with various injection timings are necessary. Thus, acanthopterygian fishes show that dopamine has little involvement in ovulation while GnRH plays a much more important role.

Interestingly, GnRH analogues alone could induce ovulation in Siberian sturgeon (Williot et al., 2002), which is a non-teleost actinopterygian species. Moreover, another non-teleost actinopterygii, gar, did not require disinhibition of dopamine for artificial induction of ovulation (Alfaro et al., 2008). From these reports, it can be hypothesized that dopamine inhibition originated early in teleost lineage and lost its importance during evolution. However, the number of species examined for the involvement of dopamine disinhibition in ovulation is limited. Further knowledge of dopamine's role in multiple species, including results of histological and physiological studies, may improve the understanding of evolutionary changes in the mechanism of HPG axis regulation in teleosts.

Because of the strength of dopamine inhibition of LH release in ostariophysi, it is natural that disinhibition of dopamine may be one of the strongest candidates for the trigger of ovulation in GnRH knockout zebrafish. It is still unclear whether the role of GnRH is not strong in cypriniform or if it is specific to laboratory-bred zebrafish. Further knockout studies using another cypriniform fish may give us the answer.

3.3. Roles of FSH and LH in teleosts

In mammals, as their names imply, FSH and LH were initially identified as the factors that stimulate folliculogenesis and induce luteinization (formation of the corpus luteum, which is formed after ovulation), respectively. Later, LH and its regulation by GnRH were proven to be essential not only for ovulation. Pulsatile release of LH was found to be required also for folliculogenesis, and deficiency of this LH pulse causes infertility in mammals. Therefore, functions and regulations of LH have been extensively studied. There have been some difficulties in the study of gonadotropic hormones, especially with respect to their structure, since they belong to the glycoprotein family, which cannot be synthesized by bacterial expression system. Furthermore, because each gonadotropin consists of a common alpha subunit and a subtype specific-beta subunit, it has not been easy to produce specific antibodies for radioimmunoassay and Enzyme-Linked Immunosorbent Assay (ELISA). As a solution, researchers have synthesized recombinant protein using insect cell lines and mammalian cell lines (Kazeto et al., 2008; Ogiwara et al., 2013). For the synthesis of recombinant proteins, a single protein containing both alpha and beta subunits connected by a linker can be used as a gonadotropin instead of generating the hetero dimer (Hayakawa et al., 2008). These approaches, in combination with a reporter assay in culture cells, have clarified whether LH and FSH activate LH or FSH receptors. Interestingly, many promiscuous situations arise regarding the LH/FSH and LHR/FSHR relationship. For instance, in zebrafish and medaka, LH can activate both the LH and FSH receptor (Ogiwara et al., 2013; So et al., 2005). On the other hand, FSH activates the FSH receptor, and both LH and FSH can activate LH receptor in amago salmon (Oba et al., 1999a,b). The promiscuity of this relationship varies among species.

A unique approach for producing specific gonadotropin antibodies has recently been achieved. Nyuji et al. and Okuzawa et al. made chimeric recombinant protein of glycoprotein, containing alpha subunit of rabbit and each gonadotropin beta subunit of a fish of interest (Nyuji et al., 2016; Okuzawa et al., 2016). These recombinant proteins were used to immunize rabbits. For the immunized rabbits, the alpha subunit was the same as their intrinsic alpha subunit, and they do not produce antibodies against it, while they produce antibodies against the beta subunit. For this reason, although the three-dimensional structure of the recombinant gonadotropin is close to that of the native protein, antibodies are only produced against the fish-type beta subunit by rabbits. Including such improvement of methodology, protein assays and functional analyses, have suggested that FSH works during the development of oocytes, and LH is released after folliculogenesis. In fact, LH was demonstrated to induce final oocyte maturation (FOM) *in vitro* in red seabream (Kagawa et al., 1998).

In addition to these results, recent studies of TALEN and CRISPR knockout have shown the essentiality of FSH and LH in the process of folliculogenesis, and FOM and ovulation, respectively, in medaka and zebrafish (Chu et al., 2014; Takahashi et al., 2016; Zhang et al., 2015a; Zhang et al., 2015b). In both species, knockout of either hormone severely affected fertility of females but not of males.

In female medaka, *fshb* knockouts result in disruption of folliculogenesis. On the other hand, unlike mammals, knockout of either *gnrh1* or *lhb* did not affect folliculogenesis in medaka. However, they were still infertile because they did not ovulate. A single injection of pregnant mare serum gonadotropin (PMSG), which is an agonist for the LH receptor in medaka (Ogiwara et al., 2013), could rescue the infertility of both *gnrh1* and *lhb* knockouts. The infertility of these knockouts is due to the deficiency of FOM and ovulation, rather than long-term folliculogenesis. Thus, in medaka, FSH is responsible for folliculogenesis, and GnRH and LH are required for the process of ovulation (Takahashi et al., 2016).

In zebrafish, it has been reported that knockouts of LH receptor and FSH receptor show the same results as those of LH and FSH in medaka, respectively (Chu et al., 2014), which indicates that the FSH system is essential for folliculogenesis and that the LH system is essential for ovulation. The discrepancy that *fshb* KO but not *fshr* KO females could reproduce (Zhang et al., 2015a) can be explained by the fact that intrinsic LH can activate FSH receptor in zebrafish, as described above.

Interestingly, in medaka although LH can activate the FSH receptor, FSH knockout causes disruption of folliculogenesis, which is close to the phenotype of FSH receptor knockout (Murozumi et al., 2014). This is probably because LH is positively regulated by gonadal estrogen in medaka (Kanda et al., 2011), and immature gonad with low estrogen production fails to induce LH release sufficient for the induction of folliculogenesis via FSH receptor activation. On the other hand, in goldfish, which belongs to the same family of zebrafish, cypriniform, LH is negatively regulated by gonadal estrogen (Kobayashi et al., 2000). It is possible in immature zebrafish that LH are released in negative feedback manner, which replaces FSH function during folliculogenesis. Anyway, the consistent conclusions in the phylogenetically distant two species, zebrafish and medaka (Betancur et al., 2013), strongly suggest a general rule widely applicable to teleosts that FSH system is required for folliculogenesis and LH system for ovulation.

Interestingly, it is well known that LH and FSH are expressed in a single gonadotrophic cell in mammals. On the other hand, in teleosts, they are expressed in different cells in general (Kanda et al., 2011; Miranda et al., 2001; Nozaki et al., 1990; Yaron et al., 2003; Yaron et al., 2001). Because *lhb* and *fshb*, which are essential beta-subunit genes of LH and FSH, respectively, were likely duplicated from a single *gthb* gene before the emergence of gnathostome, the situation in mammals (co-expression) should be the original form. Recent studies have suggested that a certain event during the evolution of actinopterygian linage have separated LH and FSH expression into distinct cellular populations in the pituitary. Previous analysis of the syntenic relationship among gonadotropin genes in vertebrates showed that *lhb* but not *fshb* genes in teleosts are located on a locus different from that for any other gonadotropin genes in tetrapods and hypothetical common ancestral bony vertebrates (Kanda et al., 2011). Thus, it may be suggested that rearrangement of the *lhb* locus caused the differential expression of *lhb* gene in the pituitary during evolution of actinopterygii. Although it is unclear whether teleost-specific 3R whole genome duplication is involved, future studies using ancient fishes may suggest the specific evolutionary event that caused differential expression of *lhb*.

4. Function of kisspeptin in vertebrates; kisspeptin is not involved in the HPG axis regulation in teleosts in principle

Since the importance of kisspeptin in the HPG axis regulation was first reported in mammals, kisspeptin has been attracting much attention, and extensive studies have been performed in the early 2000s not only in mammals but also in non-mammalian species. From various lines of evidence carried out in mammals, there is a general consensus that kisspeptin directly stimulates GnRH neurons, which express kisspeptin receptors, and increases serum LH. Earlier comparative studies in non-mammalian vertebrates have simply surmised that this mammalian scheme is also applicable to non-mammals. However, later prudent studies including electrophysiological and morphological analyses that analyzed the functional connections between kisspeptin and GnRH neurons have denied the possible pathway for kisspeptin regulation on GnRH neurons and have modified the hypothesis (Nakajo et al., 2018). It now seems to be a general consensus that kisspeptin is not involved in the regulation of GnRH neurons in most teleost species.

In the injection studies, some altered serum LH concentration while others did not (Felip et al., 2009; Kim et al., 2014; Li et al., 2009; Nakajo et al., 2018; Ohga et al., 2014). These conflicting results in injection studies may have partly arisen from pharmacological side effects of kisspeptin administrations. Kisspeptin belongs to the large family of RF amide peptides, which play various roles and sometimes shows promiscuous relationships among ligands and receptors even within the

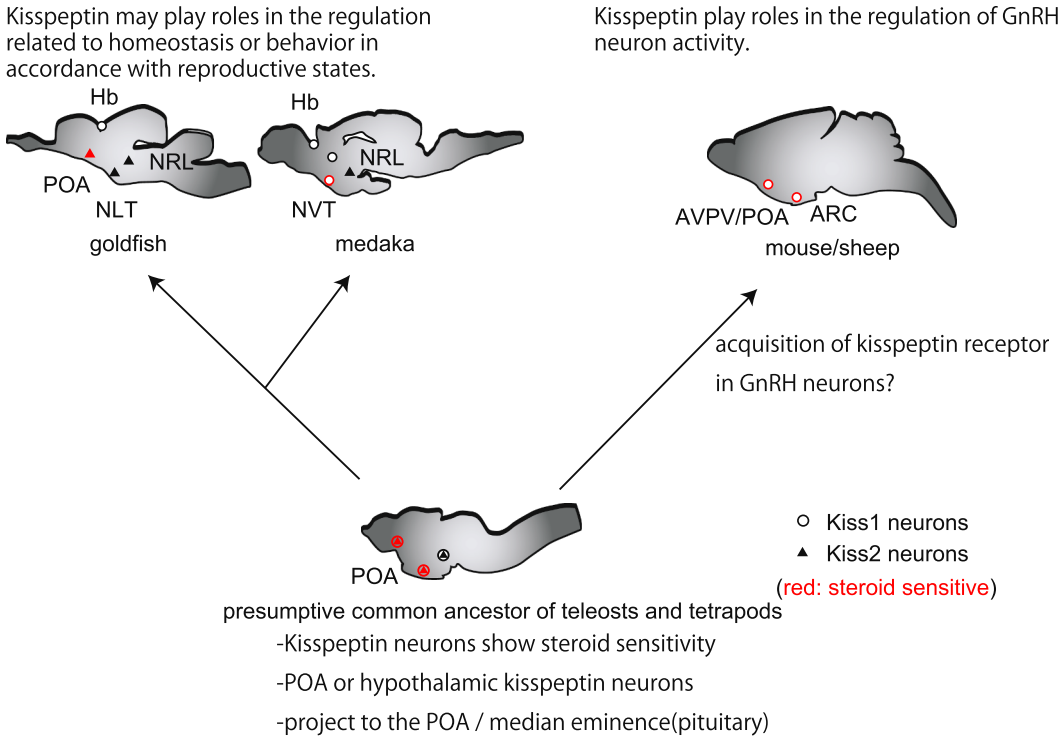

Fig. 2. Evolution of steroid sensitive kisspeptin neurons in vertebrates. Steroid sensitivity is conserved between teleosts and mammals. Judging from the steroid sensitivity and the localization, Kiss2 neurons in goldfish and Kiss1 neurons in medaka may be equivalent to Kiss1 neurons in AVPV/POA and the arcuate nucleus in mammals. Because of the absence of kisspeptin receptors in GnRH neurons, kisspeptin cannot work as a regulator of GnRH neurons in teleosts. During the evolution of tetrapods, GnRH neurons likely began to express kisspeptin receptor, and the mammalian-type steroid feedback system was formed as kisspeptin neurons showed steroid sensitivity and GnRH neurons showed axonal projection to the pituitary via the median eminence.

same ligand and receptor families (Oishi et al., 2011). In this case, high dose injection of exogenous kisspeptin may cause the activation of other receptors such as RFRP/GnIH receptors. However, kisspeptin administration experiments included in our recent paper (Nakajo et al., 2018) in which we cautiously controlled the experimental conditions and replicated parameters of kisspeptin administrations of one of the reports in goldfish demonstrating that kisspeptin administration increase serum LH (Li et al., 2009), failed to obtain similar results. Thus, we should be really cautious in interpreting the results of the earlier reports of simple exogenous kisspeptin administrations. It should be noted that recent double in situ hybridization studies in some teleost species (Escobar et al., 2013; Grone et al., 2010; Kanda et al., 2013) denied the possibility that GnRH neurons express kisspeptin receptors, which was once proposed based on an RT-PCR study of laser-microdissected brain tissue (Parhar et al., 2004). Our recent deep sequencing analysis of gpr54-expressing neurons also failed to detect *gnrh* expressions in these cells (Nakajo et al., 2018, Supplemental material). Therefore, from the results of the cellular anatomical and molecular studies above, kisspeptin neurons cannot regulate GnRH neurons directly, unlike in mammals. Another study using patch-clamp and Ca^{2+} imaging also clearly denied the possible regulation of kisspeptin on GnRH1 neurons or LH cells (Nakajo et al., 2018). From these studies, it should be concluded that kisspeptin is not an essential regulator of the HPG axis in teleosts. Although kisspeptin peptide may act at some process(es) in the HPG axis under certain specific conditions in some teleosts, the mechanism of such kisspeptin action(s) that results in increased serum LH should be considered to be rather pharmacological and quite different from mechanisms of kisspeptin actions in mammals in which kisspeptin neurons directly stimulate GnRH neurons.

On the other hand, recent studies have demonstrated that dopamine, isotocin, vasotocin, somatostatin, and neuropeptide B neurons express kisspeptin receptors in medaka and/or seabass (Escobar et al., 2013; Kanda et al., 2013; Nakajo et al., 2018). Thus, kisspeptin neurons may play rather general roles in the regulation of homeostasis or behavior. Given the fact that avian species have lost all kisspeptin-related genes (Kanda and Oka, 2013; Pasquier et al., 2014), it is possible that the use of kisspeptin as a potent regulator of GnRH neurons is an unique feature of mammals acquired during evolution. In the next section, I will introduce this possibility and discuss the evolution of kisspeptin and the GnRH system in vertebrates.

4.1. Evolution of kisspeptin functions

Although kisspeptin does not regulate GnRH neurons in teleosts, kisspeptin neurons clearly show steroid sensitivity as follows. In medaka, *kiss1* neurons in the hypothalamus reduces their *kiss1* expression as well as their firing activity in the absence of gonadal steroids (Hasebe et al., 2014; Kanda et al., 2008; Mitani et al., 2010). In goldfish, it was demonstrated that *kiss2* neurons in the POA upregulate their *kiss2* expression in accordance with the elevation of serum estrogen level (Kanda et al., 2012). This is very similar to the situation in mammals in that their *Kiss1* expression of Kiss1 neurons in the POA are upregulated by gonadal estrogen, while those in the arcuate nucleus are attenuated by gonadal estrogen (Smith, 2013). Because such upregulation and downregulation of the gene can be altered by the components of co-expressed transcription factors (Li et al., 2007), the fact that kisspeptin neurons in both teleosts and mammals show steroid sensitivity is important, regardless of the direction. It strongly suggests that kisspeptin neurons in the common ancestor of mammals and teleosts should have shown steroid sensitivity (Kanda and Oka, 2012).

Moreover, Kiss1 neurons in medaka and Kiss2 neurons in zebrafish were demonstrated to project their fibers to the POA, where GnRH neurons are localized (Hasebe et al., 2014; Servili et al., 2011). Thus, in hypothetical common ancestor of teleosts and mammals, all components for the basic backbone of kisspeptin-mediated steroid feedback regulation on GnRH neurons might have been acquired except for

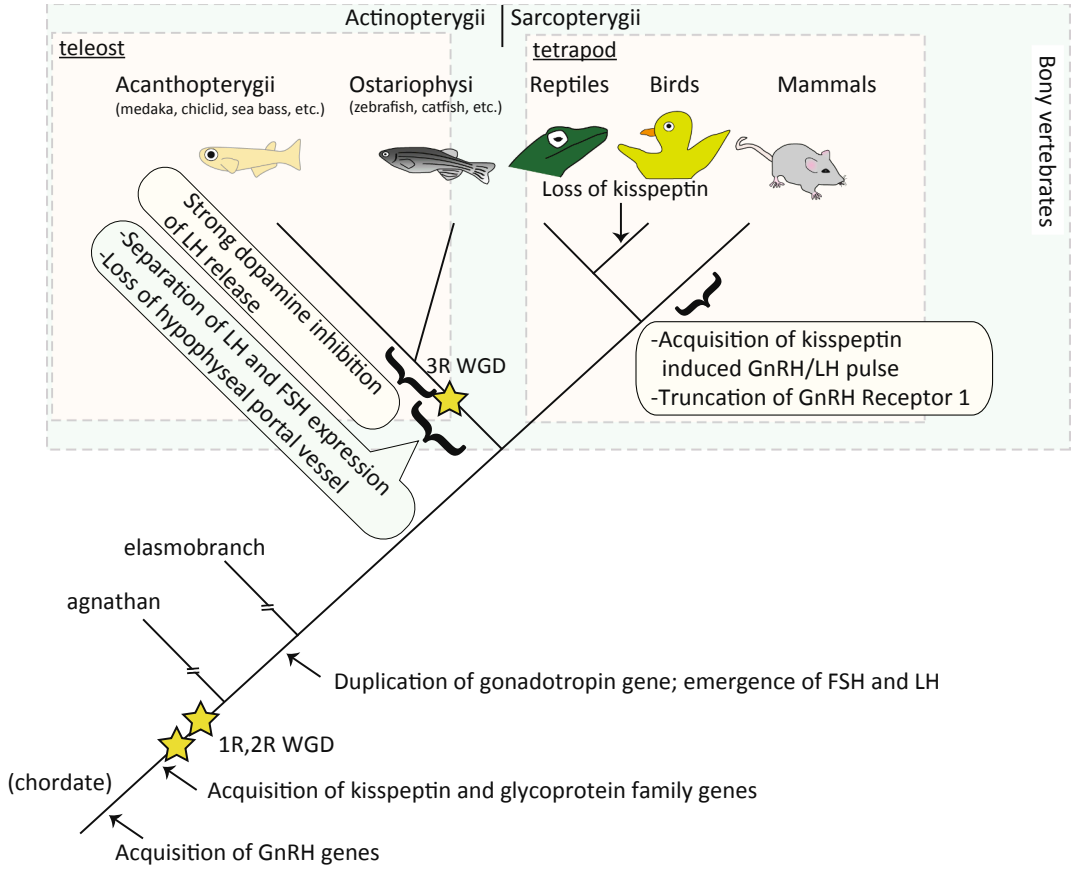

Fig. 3. Evolution of HPG axis regulation in vertebrates. Before the emergence of bony vertebrates, all genes for possible regulators, GnRH, kisspeptin, FSH, and LH had been acquired. In teleost lineage, around 3R whole genome duplication, LH and FSH started to express in separate cells. Moreover, the hypophyseal portal vessel was lost and GnRH neurons projected directly to the pituitary instead. Interestingly, strong inhibition of LH release by dopamine is observed in teleost species that diverged earlier. In tetrapod lineage, GnRH induced LH pulse, which is generated by kisspeptin neurons, and truncation of GnRH receptor1 occurred.

kisspeptin receptor expression in GnRH neurons (Fig. 2).

In contrast to the well-documented LH pulse, which plays an essential role in folliculogenesis in mammals (see "1.2 *Kisspeptin neurons are the key mediator of steroid feedback in mammals*"), there are only a few reports on the observation of serum LH fluctuation in birds and fish (Bacon and Long, 1996; Zohar et al., 1986). Given that LH is not required for folliculogenesis in teleosts (Murozumi et al., 2014; Takahashi et al., 2016; Zhang et al., 2015a), such fluctuation of LH levels should not be required for folliculogenesis. Therefore, the fluctuation of LH levels in non-mammals, if it exists, may not be generated by the same mechanism or have the same role as mammalian LH pulse.

4.2. Coincidence of GnRH receptor truncation, LH pulse, and kisspeptin regulation on GnRH neurons in mammals

From the following evidence, it suggests that LH pulse may have been specifically acquired in mammals. One reason is the fact that there are only a few reports of LH fluctuations in non-mammalian vertebrates as described above. Moreover, there is a mammalian-specific phenomenon probably related to the LH pulse, which is the truncation of GnRH receptor. Normal G protein-coupled receptors (GPCR) contain seven transmembrane regions, internal and external loops, and N-terminal and C-terminal tails. After the activation of G protein, some amino acid residues in the C-terminal tail are phosphorylated, which causes recruitment of β-arrestin and internalization by endocytosis. This endocytosis of the GPCR enables the recycling of receptors. However, mammalian type 1 GnRH receptor is an exception (McArdle et al., 1999). It lacks a C-terminal tail, and thus cannot undergo endocytosis (Hislop et al., 2000). There are some studies of this phenomenon that examined the effects of long exposure to GnRH on mammalian and non-mammalian type GnRH receptors. One study reported in type I mammalian GnRH receptor that pre-exposure to GnRH peptide for more than 10 min causes drastic reduction in Ca^{2+} response to the following GnRH peptide stimulation, suggesting an irregularly strong desensitization occurring in the mammalian GnRH receptor, which lacks C-terminal tail (McArdle et al., 1996). Interestingly, GnRH "agonists" are clinically used to reduce gonadotropin-induced sex steroid levels for the treatment of tumors that progress in a sex steroid-dependent manner. GnRH agonists lead to dysfunction of GnRH receptors, which drastically reduces the effect of intrinsic GnRH (Santen and Bourguignon, 1987). On the other hand, it cannot occur to GnRH receptors in non-mammalian species because they have normal C-terminal tails. From my preliminary data in an isolated pituitary culture of medaka, persistent exposure to GnRH for 48 h resulted in a ~10 fold increase in *lhb* expression, which suggests that in non-mammalian species, those which have intact GnRH receptors do not require pulsatile GnRH release for their activation. As shown in the Ca^{2+} imaging study of mammalian GnRH receptor described above, long exposure to GnRH can cause dysfunction of GnRH receptor unlike the cases in teleost studies. It is highly possible that the pulsatile release of GnRH observed in mammals is important to prevent dysfunction of GnRH receptor. In other words, such pulsatile stimulation by GnRH is not required for the activation of normal GPCRs, which includes GnRH receptors of all non-mammalian species.

From these lines of evidence, I speculate that the importance of LH pulse may be a mammalian-specific phenomenon. In addition to this, zebrafish and medaka have been reported to lack KNDy neurons, which generates pulsatile release of GnRH/LH (Ogawa et al., 2012; Zempo

et al., 2012). Kisspeptin and NKB are expressed in separate cells in both species, which suggests that teleosts lack LH pulse generator.

Given that platypus have a mammalian type truncated GnRH receptor (data not shown), the truncation of the GnRH receptor should have occurred before the emergence of the basal mammalian group of monotremes. Although it is still unclear whether the truncation or use of kisspeptin for LH pulse generation occurred first, both of these changes should have occurred around the time of emergence of mammals. Lastly, it should be noted that this evolutionary working hypothesized mostly depends on results of mammals and teleosts (Fig. 3). Further studies using a greater variety of non-mammalian species will further provide footprints of the evolution of the HPG axis regulation, which will give us hints toward understanding the common and diversified mechanisms in vertebrates.

4.3. Principle and the meaning of diversity of HPG axis regulation will be understood in the light of evolution

There are more mechanisms to be investigated in order to understand the HPG axis regulation in vertebrates. In the steroid feedback system, gonadal steroids, mainly estrogen, plays important roles in vertebrates. In mammals, estrogen receptor alpha (Esr1) is considered essential, and recent studies have strongly suggested that Esr1 expressed in Kiss1 neurons plays an important role in the tuning of LH pulse frequency to avoid excess development of gonads. In other words, Esr1 is essential for negative feedback regulation of gonadotropin release. However, at least in teleosts, kisspeptin and LH pulse are not involved in folliculogenesis, while FSH is important. Given big differences between mammals and teleosts reviewed in this paper, other working hypotheses are also necessary for understanding the mechanism of steroid feedback in vertebrates in general. Interestingly, recent studies have indicated that knockouts of *esr1* do not lead to infertility in either medaka or zebrafish (Lu et al., 2017; Tohyama et al., 2017). More recently, KO medaka for all subtypes of ERs have been generated and *fshb* expression was examined after ovariectomy. Detailed analyses of this mechanism are currently underway (Kayo et al., unpublished).

Dobzhansky stated that “nothing in biology makes sense except in the light of evolution” (Dobzhansky, 1973). In the near future, I hope that “species differences” in the HPG axis regulation mechanisms of vertebrates will be changed to the principles and diversity in the light of evolution.

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