Neuroanatomical Evidence That Kisspeptin Directly Regulates Isotocin and Vasotocin Neurons

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Abstract

Neuropeptide kisspeptin has been suggested to be an essential central regulator of reproduction in response to changes in serum gonadal steroid concentrations. However, in spite of wide kisspeptin receptor distribution in the brain, especially in the preoptic area and hypothalamus, the research focus has mostly been confined to the kisspeptin regulation on GnRH neurons. Here, by using medaka whose kisspeptin (kiss1) neurons have been clearly demonstrated to be regulated by sex steroids, we analyzed the anatomical distribution of kisspeptin receptors Gpr54-1 and Gpr54-2. Because the both receptors were shown to be activated by kisspeptins (Kiss1 and Kiss2), we analyzed the anatomical distribution of the both receptors by in situ hybridization. They were mainly expressed in the ventral telencephalon, preoptic area, and hypothalamus, which have been suggested to be involved in homeostatic functions including reproduction. First, we found gpr54-2 mRNA expression in nucleus preopticus pars magnocellularis and demonstrated that vasotocin and isotocin (Vasopressin and Oxytocin ortholog, respectively) neurons express gpr54-2 by dual in situ hybridization. Given that kisspeptin administration increases serum oxytocin and vasopressin concentration in mammals, the present finding are likely to be vertebrate-wide phenomenon, although direct regulation has not yet been demonstrated in mammals. We then analyzed co-expression of kisspeptin receptors in three types of GnRH neurons. It was clearly demonstrated that gpr54-expressing cells were located adjacent to GnRH1 neurons, although they were not GnRH1 neurons themselves. In contrast, there was no gpr54-expressing cell in the vicinities of neuropeptidergic GnRH2 or GnRH3 neurons. From these results, we suggest that medaka kisspeptin neurons directly regulate some behavioral and neuroendocrine functions via vasotocin/isotocin neurons, whereas they do not regulate hypophysiotropic GnRH1 neurons at least in a direct manner. Thus, direct kisspeptin regulation of GnRH1 neurons proposed in mammals may not be the universal feature of vertebrate kisspeptin system in general.

Introduction

Recent studies of human hypogonadotropic hypogonadism and knockout mice for the kisspeptin gene Kiss1 and the receptor gene Gpr54 have revealed that the kisspeptin/GPR54 system is essential for the mammalian reproductive functions [1–5]. Subsequently, in vitro electrophysiological experiments using GnRH-GFP transgenic mice showed that kisspeptin has a persistent depolarizing effect on the GnRH neurons, which are supposed to be the basis for facilitation of release of GnRH [6–10]. Moreover, kisspeptin is also reported to facilitate gonadotropin release in vivo as well. Thus, kisspeptin system has been attracting much attention of neuroendocrinologists. However, limited number of studies have assessed the function of kisspeptin-GPR54 system in non-mammalian species. In addition, little is known about the physiological role(s) of kisspeptin-GPR54 system in non-reproductive functions even in mammals.

Therefore, for the basis of full understanding of the general physiological roles of kisspeptins in vertebrate brains, we examined distribution of kisspeptin receptor gpr54-1 and gpr54-2 in the brain of medaka. Medaka is a small teleost fish that is widely used as a model animal because of many advantages, such as availability of genome database and easy access to various genetic tools. Because of the lack of knowledge on kisspeptin functions in non-mammalian vertebrates, such a model animal should go a long way towards the understandings of both evolution and general functions of the kisspeptin systems in vertebrates. In the present study, our gpr54 in situ hybridization study has shown that kisspeptin receptors are expressed by neurons in the preoptic area and hypothalamus, which are supposed to be involved in the regulation of homeostasis and instinctive behaviors. After careful preliminary experiments for identification of kisspeptin receptor expressing neurons, we demonstrated the co-expression of kisspeptin receptor mRNA in magnocellular vasotocin and isotocin neurons for the first time in vertebrates. Oxytocin (isotocin in teleosts) and vasopressin (vasotocin in teleosts) neurons are well-known to be involved in the regulation of reproduction-related and other behaviors in many vertebrate species [reviewed in [11,12]]. As in mammals, teleost isotocin and vasotocin neurons are suggested to be involved in the regulation of social behaviors.

Citation: Kanda S, Akazome Y, Mitani Y, Okubo K, Oka Y (2013) Neuroanatomical Evidence That Kisspeptin Directly Regulates Isotocin and Vasotocin Neurons. PLoS ONE 8(4): e62776. doi:10.1371/journal.pone.0062776

Editor: Hubert Vaudry, University of Rouen, France

Received August 26, 2012; Accepted March 26, 2013; Published April 25, 2013

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Funding: This work was supported by Grants-in-Aid for the Promotion of Science (JSPS) (20-10112) to SK, JSPS (20247005), Ministry of Education, Culture, Sports, Science, and Technology (2012012), and the Program for Promotion of Basic Research Activities for Innovative Biosciences Project from Bio-oriented Technology Research Advancement Institution of Japan to YO. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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including sex behaviors and aggressive behavior, although the effects vary among species [13]. Therefore, together with the fact that medaka kisspeptin neurons increase their kiss1 mRNA expression in the breeding state, kisspeptin may mediate information regarding the breeding state to the isopecin and vasotocin neurons, which leads to the state-dependent behavioral modulation. In addition to these novel findings, we examined the possible co-expression of kisspeptin receptors in three functionally distinct GnRH systems. Recent studies have shown that most vertebrates have one or two extra-hypothalamic GnRH neuronal systems, whose cell bodies are localized in the midbrain tegmentum (TEG-GnRH2 neurons) and the terminal nerve (TN-GnRH3 neurons) in addition to the conventional hypophysiotropic GnRH (GnRH1) system, and all three GnRH systems are well developed in teleosts [14,15]. Our double in situ hybridization study showed anatomical evidence to suggest the lack of direct kisspeptin regulation and leaves the possibility of indirect regulation via neighboring interneurons on hypophysiotropic GnRH1 neurons, whereas kisspeptins do not have such regulation on neuromodulatory GnRH2 and 3 neurons.

Materials and Methods

Animals

Male and female d-R strain medaka (Oryzias latipes; teleost fish) were maintained under a 14 h light/10 h dark photoperiod at a temperature of 27°C. The fish were fed twice daily with live brine shrimp and flake food. The animals were maintained and used in accordance with the guidelines of the Physiological Society of Japan and the University of Tokyo for the Use and Care of Experimental Animals. We used only anamniotes, which do not require any permission by the University of Tokyo for the Use and Care of Experimental Animals.

Luciferase Assays

The decapetide of medaka Kiss1 (Kiss1 [10]: YNLNSFLRKY-NH2, believed to be the core peptide sequence for its physiological function), pentadecapeptide of Kiss1 (Kiss1 [15]: pED-LSSYNLNSFLRKY-NH2) and dodecapeptide of medaka Kiss2 (Kiss2 [12]: SKFNYNPPLRKY-NH2) were synthesized (Sigma-Aldrich Japan, Tokyo, Japan; Scrum, Tokyo, Japan; Bonac Corporation, Kurume, Japan, respectively).

The cDNA clones containing full-length open reading frames of gpr54-1 and gpr54-2 were subcloned into the expression vector pCMV3.1 (Invitrogen). COS-7 cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% foetal bovine serum. One day before transfection, the cells were seeded into 24-well plates. The plasmid DNAs (100 ng/10 μl) were transfected into monolayer culture cells with either Lipofectamine LTX (Invitrogen). The cells were maintained in a serum-free medium (2.5 ng/well; Promega, Madison, WI), using Lipofectamine LTX containing the Renilla luciferase reporter gene, and pRL-CMV containing the Renilla luciferase reporter gene assay was performed using a Dual-Glo Luciferase Assay System (Promega) with Lumat LB9507 (EB & G Berthold, Bad Wildbad, Germany).

In situ Hybridization

For the in situ hybridization analysis, we used sexually mature male and female medaka pairs that had oviposited fertilized eggs on the day of the fixation. The medaka was deeply anesthetized with MS-222 (Sigma, St. Louis, MO, USA) and perfused with 4% paraformaldehyde in 0.05 M phosphate buffered saline (PBS) from the comus anteriosus. The brain was postfixed with the same fixative at least 1 h at 4°C. They were then embedded in 5% agarose (Sigma Type IX) solution containing 20% sucrose and were quickly frozen in n-hexane (−160°C). Complete serial frontal sections were cut on a cryostat at 20 μm, and dried at room temperature (RT) for at least 2 h. To detect mRNA, we prepared a gene-specific digoxigenin (DIG)-labeled probe and performed nonradioactive in situ hybridization based on the methods as previously reported [16]. The sections were hybridized with 100 ng/ml DIG-labelled antisenese cRNA probes (kiss1, position 21-365, AB272755; gpr54-1, position 1–1101 of ENSORLT0000002103, chromosome 9, 4480521-4500733; gpr54-2, position 6–1125 of ENSORLTT0000002192, chromosome 17, 29834753-29839926; for gk2g3, vaso, and vasotocin, probes were synthesized based on the previous report [17]).

The sections were observed under the light microscope. For the coexpression of kisspeptin receptors in three functionally distinct GnRH systems. Recent studies have shown that most vertebrates have one or two extra-hypothalamic GnRH neuronal systems, whose cell bodies are localized in the midbrain tegmentum (TEG-GnRH2 neurons) and the terminal nerve (TN-GnRH3 neurons) in addition to the conventional hypophysiotropic GnRH (GnRH1) system, and all three GnRH systems are well developed in teleosts [14,15]. Our double in situ hybridization study showed anatomical evidence to suggest the lack of direct kisspeptin regulation and leaves the possibility of indirect regulation via neighboring interneurons on hypophysiotropic GnRH1 neurons, whereas kisspeptins do not have such regulation on neuromodulatory GnRH2 and 3 neurons.

Results

Both Kiss1 and Kiss2 Activate Both Subtypes of gpr54

To assess GPR54 stimulating activity of Kiss1 and Kiss2, we carried out a luciferase reporter assay for the two types of Gpr54 receptors in medaka, Gpr54-1 and Gpr54-2. The luciferase assay showed that both Kiss1 and Kiss2 significantly activate Gpr54-1 as well as Gpr54-2 (Fig. 1). To examine the signal transduction pathway for each type of medaka Gpr54, SRE- or CRE-driven luciferase reporter gene assay was performed using COS-7 cells. For SRE-luc reporter system,
the post-receptor signaling pathway of Gpr54-2 was similarly and significantly activated by both Kiss1 and Kiss2 (Fig. 1B), whereas Gpr54-1 was only slightly activated by Kiss1 but not by Kiss2 (Fig. 1A). For CRE-luc reporter system, both Gpr54-1 and Gpr54-2 were activated by both peptides, although Kiss2 showed a higher potency than Kiss1 (Fig. 1C and 1D). In the present analysis, the CRE-driven luciferase activity in Gpr54-2 expressing cells showed the strongest dose-dependent response to Kiss1/Kiss2 application (Fig. 1D).

Two Subtypes of Kisspeptin Receptors are Expressed Mainly in the Ventral Telencephalon, Preoptic Area, Habenula, and Hypothalamus

In situ hybridization of two subtypes of kisspeptin receptors, gpr54-1 and gpr54-2, was performed. Although the cDNA sequences of the two types of receptors are similar (61%), we could specifically label neurons that expressed gpr54-1 (n = 3 for male, n = 8 for female) and neurons that expressed gpr54-2 (n = 3 for male, n = 6 for female) as separate populations (Fig. 2, 3).

In situ hybridization of gpr54-1 (Fig. 2) showed that the expression of gpr54-1 mRNA is restricted in the preoptic area,

Figure 1. Luciferase assays for the activation of two types of receptors, Gpr54-1 and Gpr54-2, by the ligands, Kiss1 and Kiss2. Medaka gpr54-1 (A, C) or gpr54-2 (B, D) cDNA was transfected to COS-7 cells with SRE-luc or CRE-luc vector. Various concentrations of medaka Kiss1 and Kiss2 were applied to the culture medium, and the luciferase activity was measured. The results are indicated as mean ± SEM, each of which was conducted in triplicates. The data are expressed as the ratio of changes in luciferase activity over the control renilla luciferase activity.

doi:10.1371/journal.pone.0062776.g001
nucleus preopticus pars magnocellularis (POm) and nucleus preopticus pars parvocellularis (POp) (Fig. 2A; Fig. 4D), area ventralis telencephali pars dorsalis/supracommissuralis/posterior (Vd/Vs/Vp), and habenula (Fig. 2B; Fig. 4E), where autocrine/paracrine regulation by kisspeptin is suggested in zebrafish [22,23]. In the preoptic area, large cells in the ventralmost area expressed gpr54-1, while both large and small cells in the dorsomedial area expressed gpr54-1. On the other hand, gpr54-2 mRNA showed broader distribution in the brain (Fig. 3), in the boundary between the telencephalon and the alfactory bulb (Fig. 3A; Vd/Vs/Vp (Fig. 3B–D; Fig. 4B–D), POA (Fig. 3E; Fig. 4C), POm (Fig. 3F; Fig. 4D), nucleus diffusus tori lateralis (NDTL; Fig. 3G; Fig. 4F), nucleus posterioris periventricularis (NPPv; Fig. 3H; Fig. 4F), NVT (Fig. 3I; Fig. 4F), NRI (Fig. 3J; Fig. 4H,1), and corpus mammillare (CM; Fig. 4I). There was no significant sex difference in the expression pattern of kisspeptin receptors throughout the brain. The distribution of kisspeptin receptor mRNA-expressing neurons in the medaka brain is schematically summarized in Fig. 4.

**Isotocin and Vasotocin Neurons Express Kisspeptin Receptors**

We found large cells expressing gpr54-2 mRNA in POm. Accordingly, we performed dual in situ hybridization to examine if they are isotocin and/or vasotocin neurons. We demonstrated that isotocin (Fig. 5A–C) as well as vasotocin neurons (Fig. 5D–F) express gpr54-2. In contrast, no isotocin (Fig. 5G–I) or vasotocin neurons (Fig. 5J–L) expressed gpr54-1. The percentage of co-localization was as follows. Vasotocin neurons; Female LD, 31±4% (n = 7), SD, 59±4% (n = 5); Male LD, 52±6% (n = 5); Male SD, 49±6% (n = 3); Isotocin neurons; Female LD, 19±1% (n = 3), SD, 18±1% (n = 2); Male LD, 15±3% (n = 4); SD17±2% (n = 3).

**Kisspeptin Receptors Are Expressed in Proximity to GnRH1 Neurons**

As described above, the gpr54-1 and gpr54-2 mRNA-expressing neurons were mainly localized in the ventral telencephalon, POA, habenula, and hypothalamus. Especially, we found that both gpr54-1 and gpr54-2 were expressed in POA surrounding the GnRH1 neurons (Fig. 6A–C; approximately 50 to 150 cells), but not in the regions surrounding the TEG-GnRH2 neurons (Fig. 6D–F) or TN-GnRH3 neurons (Fig. 6G–H).

Since the gpr54-1- and gpr54-2-expressing neurons were localized in the proximity of the POA GnRH1 neurons, dual in situ hybridization combining either one of the two subtypes of kisspeptin receptor (gpr54-1 or gpr54-2) mRNAs and gnrh1 mRNA was performed (Fig. 7; n = 4 for male, n = 5 for female). However, at the single neuron level, neither gpr54-1 nor gpr54-2 mRNA was detected in the GnRH1 neurons themselves; they were abundantly expressed in the non-GnRH1 neurons surrounding the GnRH1 neurons (for gpr54-1, Fig. 7A–C; for gpr54-2, Fig. 7D–F). In contrast to the situation in the GnRH1 neurons, kisspeptin receptor was not expressed in proximity of neuromodulatory GnRH2 (Fig. 6D–F) or GnRH3 (Fig. 6G–I) neurons.

**Discussion**

In the present study, we analyzed the distribution of kisspeptin receptors in a teleost medaka, and found the first evidence for kisspeptin’s direct regulation on magnocellular isotocin and vasotocin neurons in vertebrates. The kisspeptin receptors in the medaka brain showed characteristic distribution in that most of the neurons expressing gpr54 mRNA were localized in the areas that have been suggested to be involved in the homeostatic regulations including reproduction and reproductive behaviors. We will mainly discuss below possible functions of kisspeptins and their receptors in non-mammalian vertebrates and its relevance to the vertebrate species in general.

**Gpr54-1 and Gpr54-2 Are the Intrinsic Receptors for Both Kiss1 and Kiss2 in Medaka**

Our luciferase assay has shown that both Kiss1 and Kiss2 activate both Gpr54-1 and Gpr54-2 signaling pathways. This result is consistent with the previous studies using zebrafish, African clawed frog, and goldfish [18,20,24]. In the present study, Kiss1 and Kiss2 activated Gpr54-2 CRE signaling to a similar extent (Fig. 1B), which has also been reported in zebrafish and goldfish [20,24]. On the other hand, Kiss2 activated Gpr54-2 CRE signaling more potently than Kiss1 (Fig. 1D). Although the Gpr54-1-expressing COS-7 cells showed milder activation than Gpr54-2-expressing cells, they showed a clear dose dependent activation by Kiss1 and/or Kiss2 (Fig. 1A, C). Thus, it is concluded that both Gpr54-1 and Gpr54-2 are the intrinsic receptors for both Kiss1 and Kiss2 in medaka. These results lead
us to perform the anatomical analysis of the distribution of both Gpr41-1 and Gpr45-2 in the medaka brain.

Kisspeptin Receptors are Densely Expressed in POA and Hypothalamus

We demonstrated that the kisspeptin receptor genes, gpr41-1 and gpr45-2 are densely expressed in certain regions of ventral telencephalon, POA, and hypothalamus as well as habenula. Among those areas, gpr41-1 was mainly expressed in ventral telencephalon, POA and habenula (Fig. 2), whereas gpr45-2 was more widely expressed in the brain (Fig. 3). Previous study in a cichlid fish suggested that they lack gpr45-1, whereas gpr45-2 mRNA is broadly expressed in the brain [25]. Moreover, in zebrafish, gpr45-2 is expressed predominantly compared to gpr41-1 [23]. Phylogenetically, in teleosts, many species lack gpr41-1, while gpr45-2 is conserved throughout all the species analyzed to date. On the other hand, gpr45-2 is lost in placental mammals [26,27]. Taken together, our results and previous studies suggest that Gpr41-1 system may predominantly function in tetrapods, whereas Gpr45-2 system may do so in teleost brains.

As will be discussed in the next section, it is suggested that the kisspeptin receptors in POA are primarily involved in the control of release activities of hypophysiotropic hormones such as GnRH. In addition, previous brain lesion and stimulation studies in teleosts suggested that Vv, Vd/Vs/Vp, and POA are involved in the control of sexual behavior [28,29]. In the hime salmon brain, electrical stimulation of these specific areas immediately evoked sexual behaviors, suggesting that these regions may function as an important part of the neural circuit for sexual behavior. In the goldfish, it has been shown that male sexual behaviors were severely impaired after bilateral lesions confined to the area ventrals telencephali pars supracommissurals and/or posterior parts of the area ventrals telencephali pars ventrals (Vs-pVv) and the nucleus preopticus periventricularis (NPP) [29], which appear to overlap with the gpr45-1- and gpr45-2-expressing neurons in medaka.

Evidence for the Direct Regulation of Kisspeptin Neurons on Isotocin and Vasotocin Neurons

We demonstrated clearly that vasotocin and isotocin neurons express gpr45-2. In mammals, some studies have shown that Kiss1 is involved in the control of release of oxytocin or vasopressin, although Kiss1’s site of action has not been clarified [30–32]. Some studies have also shown that vasotocin/vasopressin and isotocin/oxytocin neurons are involved in the control of social behaviors such as aggression and reproduction [33–36]. Given that gpr45-2 can be activated by both Kiss1 and Kiss2, it is suggested that the isotocin and vasotocin neurons expressing gpr45-2 are regulated by both Kiss1 and Kiss2 neurons. Previously, it has been reported by immunohistochemistry that medaka Kiss1 neurons project to POm [37]. Interestingly, medaka Kiss1 neurons change their kiss1 expression levels according to the breeding states [16]. Although there is no report of Kiss2 neuronal projection in medaka, Kiss2 neurons in zebrafish are reported to project to the preoptic area [23]. Recently, POA Kiss2 neurons in goldfish, which belongs to the same Cyprinidae, were reported to show steroid dependent expression of kiss2 mRNA [27,38]. Thus, in teleosts, it is suggested that kisspeptin (Kiss1 and Kiss2) neurons project to isotocin/vasotocin neurons and show characteristic seasonal variations in their gene expression. Interestingly, in halfspotted goby the number and the size of arginine vasotocin and GnRH immunoreactive cells have been shown to be correlated with seasonal reproductivity [39]. Therefore, we propose that the Kiss1 and/or Kiss2 neurons may convey important information about the reproductive/gonadal states to the neural networks responsible for some reproductive and other behaviors that are regulated by vasotocin/isotocin neurons.

Relationships between Kiss1 and Three GnRH Neuronal Systems

There are general agreements as to the concept that the POA GnRH1 neurons are hypophysiotropic, and the extrahypophysial GnRH2 and GnRH3 neurons are non-hypophysiotropic and neuromodulatory in nature [15,40,41]. This principle appears to be widely conserved throughout vertebrate species including mammals [42–45].

In the present study, we demonstrated the anatomical relationships between the kisspeptin neurons and three different types of GnRH neurons for the first time in vertebrates by examining whether the kisspeptin receptors are localized to the GnRH neurons by using dual in situ hybridization.

We have clearly shown here that numerous neurons close to the GnRH1 neurons, but not the GnRH1 neurons themselves, expressed gpr45-1 or gpr45-2 (Figs. 6 and 7; also see below) in medaka. It was recently demonstrated in a cichlid fish (Astatotilapia burtoni) by in situ hybridization that kisspeptin receptor is expressed in GnRH3 neurons but not in GnRH2 neurons or GnRH1 neurons [25], which clearly shows that the adjacent neurons, but not GnRH1 neurons themselves, express kisspeptin receptors; the authors also stated that the receptor expression was much heavier in such non-GnRH1 neurons in the POA. This situation in teleosts is unique, because kisspeptin’s main function in mammals have been supposed to be the regulation of GnRH1 peptide release through Gpr54 on GnRH1 neurons [46], and will be discussed in the next section. On the other hand, Kiss1 system does not appear to have such a pathway for the neuromodulatory GnRH2 neurons. There may be some species variations as to whether the GnRH3 neurons express kisspeptin receptors or not. Zhao and Wayne reported on changes in medaka GnRH3 firing frequency after the application of Kiss1 through some interneurons, which is consistent with the results of the present study, indicating that GnRH3 neurons in medaka do not express gpr54 mRNA [47].

Possible Pathway for the Indirect Kisspeptin Regulation of GnRH1 Neurons via Interneurons in POA

Our present dual in situ hybridization study clearly indicated that gpr54-expressing cells are located closely adjacent to the hypophysiotropic GnRH1 neurons but they are not the GnRH1 neurons themselves (Fig. 7). In mammals, kisspeptin is supposed to be an essential regulator of GnRH neuron activity. On the other hand, in teleosts, not many, but a few studies reported that kisspeptin up-regulates the expression of LHβ and FSHβ in
zebrafish [48] and promotes LH secretion in goldfish [24] and sea bass [49,50]. However, the direct action of kisspeptin on the pituitary was denied in goldfish [24]. We also examined possible direct action of Kiss1 (10) on LHβ and FSHβ mRNA expression of the isolated medaka pituitary in culture. The Kiss1 (10) peptide turned out to have no direct pituitary effect, while

Figure 4. Schematic illustration of the distribution of kisspeptin receptors in medaka brain. gpr54-1- and gpr54-2-expressing neurons are mainly localized in the ventral telencephalon, preoptic area, and hypothalamus, suggesting their functions in homeostatic and behavioral regulations. Note that gpr54-1 is also expressed in habenula but not in NIP, which is innervated by habenular neurons, suggesting the autocrine/paracrine regulation of habenular neurons by kisspeptins. Tel, telencephalon; TeO, optic tectum; ca, anterior commissure; Cb, cerebellum; Hy, hypothalamus; Hb, habenula; fr, fasciculus retroflexus; NIP, interpeduncular nucleus. doi:10.1371/journal.pone.0062776.g004
Figure 5. Dual fluorescence in situ hybridization showing that isotocin and vasotocin neurons express gpr54-2. Isotocin neurons (A; green) and vasotocin neurons (D; green) express gpr54-2 (B, E; magenta). Merged photographs are shown in C and F. On the other hand, neurons that do not express isotocin or vasotocin (green) express gpr54-1 (magenta) mRNA (G–I, and J–L, respectively). Scale bar: 50 μm.

doi:10.1371/journal.pone.0062776.g005
GnRH, as a positive control, potently enhanced their expressions in the same condition (data not shown). Thus, it may suggest that medaka Kiss1 indirectly regulates LH and FSH secretion probably via kisspeptin receptor-expressing interneurons in the POA in the vicinity of GnRH1 neurons, if the upregulation by kisspeptin of gonadotropin release (reported in some teleosts [24,48,50,51]) is common to teleost species. It should be noted that in recent studies of mammalian kisspeptin neurons, Pielecka-Fortuna et al [6,52] reported on the indirect kisspeptin regulation of GnRH neurons via interneurons, in addition to the direct regulation [7–9]. Therefore, such indirect kisspeptin regulation of GnRH neurons may be rather widely conserved in vertebrate species.

In the present study, we could not specify the identity of the neurons expressing kisspeptin receptors in the proximity of GnRH1 neurons from various technical reasons. The future identification of these neurons (the transmitter candidate and the anatomical and physiological nature) should be critical for understanding the mechanism of HPG axis regulation by kisspeptins.

In summary, by performing a systematic in situ hybridization analysis on the overall distribution of two types of kisspeptin receptors throughout the brain, we found evidence to show that kisspeptin neurons directly regulate isotocin and vasotocin neurons via Gpr54 for the first time in vertebrates. On the other hand, all three GnRH neuronal population were shown to lack the expression of kisspeptin receptors in medaka. Among them, however, neurons in proximity to GnRH1 neurons were shown to express gpr54-1 or gpr54-2 mRNA, leaving a possibility that kisspeptin indirectly regulates the GnRH1 system. Anyway, the expression of kisspeptin receptor in isotocin and vasotocin neurons suggests the following new functions of kisspeptin systems; the gonadal sex steroid-sensitive kisspeptin neurons (Kiss1 neurons in medaka) may alter reproduction-related behaviors by affecting isotocin and vasotocin neurons in accordance with the breeding states. Because isotocin and vasotocin neurons are homologous to mammalian oxytocin and vasopressin neurons, similar regulation may also exist in mammalian oxytocin and vasopressin neurons. Thus, the elucidation of regulatory mechanisms of teleost kisspeptin neurons on vasotocin and isotocin neurons may open
the new era of research of kisspeptin neurons’ physiological functions in vertebrates.

Acknowledgments

We thank Dr. Naoyuki Yamamoto (Nagoya University) for helpful advice and discussion. We are also grateful to Ms. Kiyoko Kataoka (The University of Tokyo) for technical assistance. We also thank Ms. Miho Kyokuwa and Hisako Kohno for their excellent care of the fish used in this study.

Author Contributions

Conceived and designed the experiments: SK YO. Performed the experiments: SK YA YM KO. Analyzed the data: SK YA YM KO YO. Wrote the paper: SK YO.

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