

# 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 neuromodulatory 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.

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## 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

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 isotocin 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-rR 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 decapeptide of medaka Kiss1 (Kiss1 (10): YNLNSFGLRY-NH<sub>2</sub>, believed to be the core peptide sequence for its physiological function), pentadecapeptide of Kiss1 (Kiss1 (15): pED-LSSYNLNSFGLRY-NH<sub>2</sub>) and dodecapeptide of medaka Kiss2 (Kiss2 (12): SKFNYNPFLGRF-NH<sub>2</sub>) 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 pcDNA3.1 (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/well) were transfected into monolayer culture cells with either pSRE-Luc or pCRE-Luc (100 ng/well; Clontech, Palo Alto, CA), and pRL-CMV containing the Renilla luciferase reporter gene (2.5 ng/well; Promega, Madison, WI), using Lipofectamine LTX (Invitrogen). The cells were maintained in a serum-free medium for 24 hours. After that, they were incubated with various concentrations (from 0 to 10<sup>-5</sup> M) of medaka Kiss1 or Kiss2 for six hours and then harvested and analyzed. Luciferase activity in the cell extract was measured using 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 conus arteriosus. 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 (~-60°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 antisense cRNA probes (*kiss1*, position 21-365, AB272755; *gpr54-1*, position 1-1101 of ENSORLT00000002103, chromosome 9 4480521-4500733; *gpr54-2*, position -6-1125 of ENSORLT00000022192, chromosome 17, 29854753-29839926; for *gnrh2*, *gnrh3*, *isotocin*, and *vasotocin*, probes were synthesized based on the previous report [17]) synthesized from the medaka brain cDNA using a labelling kit (Roche Molecular Biochemicals GmbH, Mannheim, Germany) overnight at 58°C. A sense RNA probe was used as a negative control (nomenclature based on Lee et al.; *gpr54-1* corresponds to *kissrb* in [18] and *kissr2* in [19] whereas *gpr54-2* corresponds to *kisslra* in [18] and *kissr4* in [19]; see [20]).

The sections were observed under the light microscope. For the nomenclature of the medaka brain nuclei, we followed the Medaka Histological Atlas (Wakamatsu et al., Medaka Histological Atlas, edited by the Editorial Board of Medaka Histological Atlas of NBRP Medaka, [http://www.shigen.nig.ac.jp/medaka/medaka\\_atlas](http://www.shigen.nig.ac.jp/medaka/medaka_atlas)) throughout this study.

### Dual in situ Hybridization for Isotocin/Vasotocin/*gnrh* and *gpr54-1/gpr54-2*

For the dual *in situ* hybridization study, we used a mixture of fluorescein-labelled *gnrh1* (position 1-428, NM001104699) or *vasotocin* (position, 1-807, AB691137) or *isotocin* (position 1-739, AB691138) probe (specificities were confirmed by the presence or absence of signals using the antisense or sense probes) and the DIG-labeled *gpr54-1/gpr54-2* probe (described above). Dual in situ hybridization signals were visualized as previously described [21] using an HNPP fluorescence detection kit (Roche) or Fast-Red substrate kit (abcam, Cambridge, UK) according to the manufacturer's instruction. We used long day-conditioned (LD, 14 hour light 10 hour dark; breeding condition) and short day-conditioned (SD, 10 hour light 14 hour dark; non-breeding condition) medaka for the analysis of the percentages of colocalization. The fluorescence was observed under confocal laser-scanning microscope LSM-710 (Carl Zeiss, Oberkochen, Germany). For GnRH2 and 3 neurons, because in situ hybridization using adjacent sections proved that cells near GnRH2 or GnRH3 neurons did not express *gpr54-1* or *gpr54-2* mRNA, we did not perform the double labeling and showed adjacent sections instead?

## Results

### Both Kiss1 and Kiss2 Activate Both Subtypes of *gpr54*

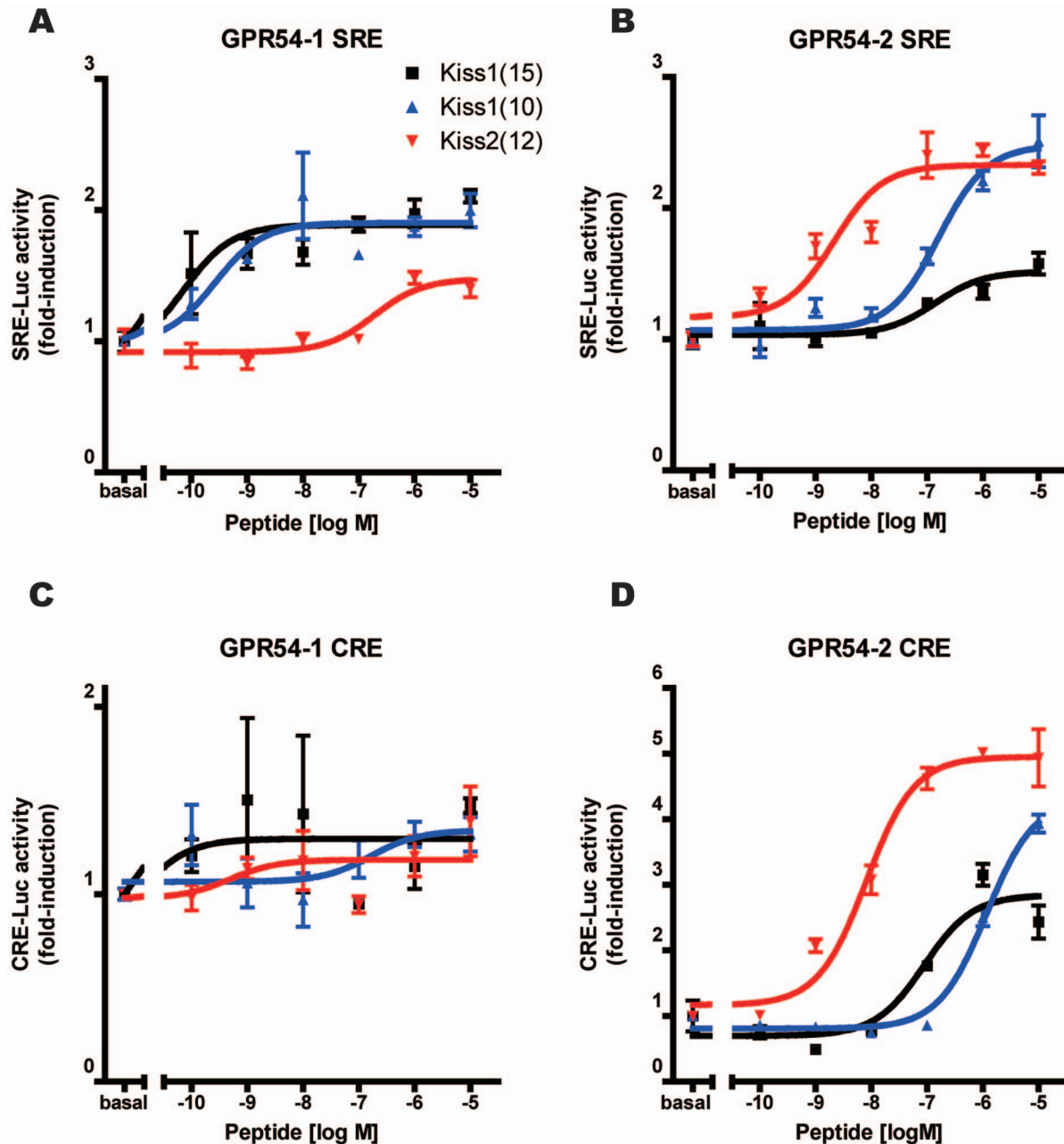
To assess GPR54 stimulating activity of Kiss1 and Kiss2, we carried out a luciferase reporter assay for the two types of Gpr54 receptors in medaka, Gpr54-1 and Gpr54-2. The luciferase assay showed that both Kiss1 and Kiss2 significantly activate Gpr54-1 as well as Gpr54-2 (Fig. 1). To examine the signal 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).

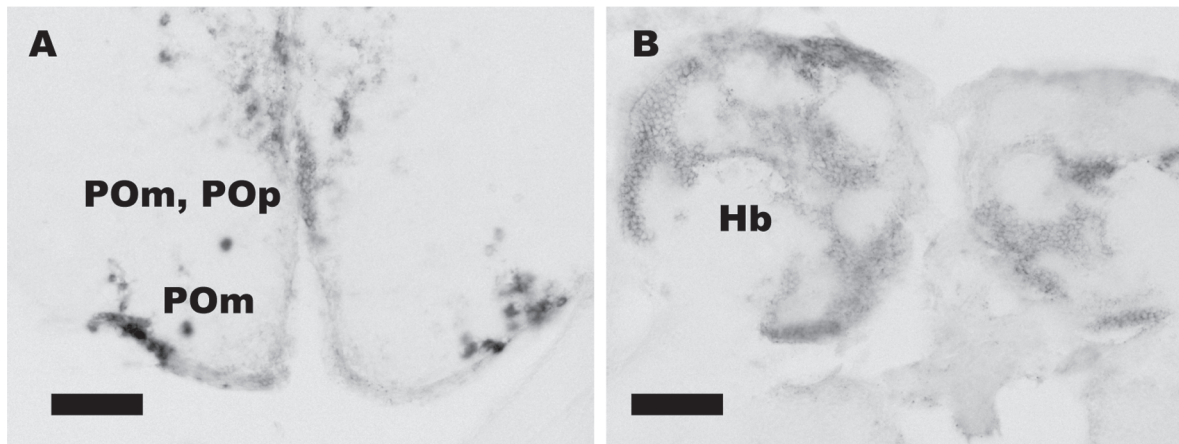
## Two Subtypes of Kisspeptin Receptors are Expressed Mainly in the Ventral Telencephalon, Preoptic Area, Habenula, and Hypothalamus

*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  $\pm$  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



**Figure 2. DIG-labelled *in situ* hybridization of *gpr54-1* shows localization of *gpr54-1* mRNA positive cells.** *gpr54-1* mRNA positive neurons are localized in POp, POm (A), Vd/Vs/Vp (not shown), and habenula (Hb; B). Scale bars: 50  $\mu$ m. doi:10.1371/journal.pone.0062776.g002

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 olfactory 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), NRL (Fig. 3J; Fig. 4H,I), 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 colocalization was as follows. Vasotocin neurons; Female LD,  $31 \pm 4\%$  ( $n = 7$ ), SD,  $59 \pm 4\%$  ( $n = 5$ ), Male LD,  $52 \pm 6\%$  ( $n = 5$ ), Male SD,  $49 \pm 6\%$  ( $n = 3$ ): Isotocin neurons; Female LD,  $19 \pm 1\%$  ( $n = 3$ ), SD,  $18 \pm 1\%$  ( $n = 2$ ), Male LD,  $15 \pm 3\%$  ( $n = 4$ ), SD  $17 \pm 2\%$  ( $n = 3$ ).

### 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).

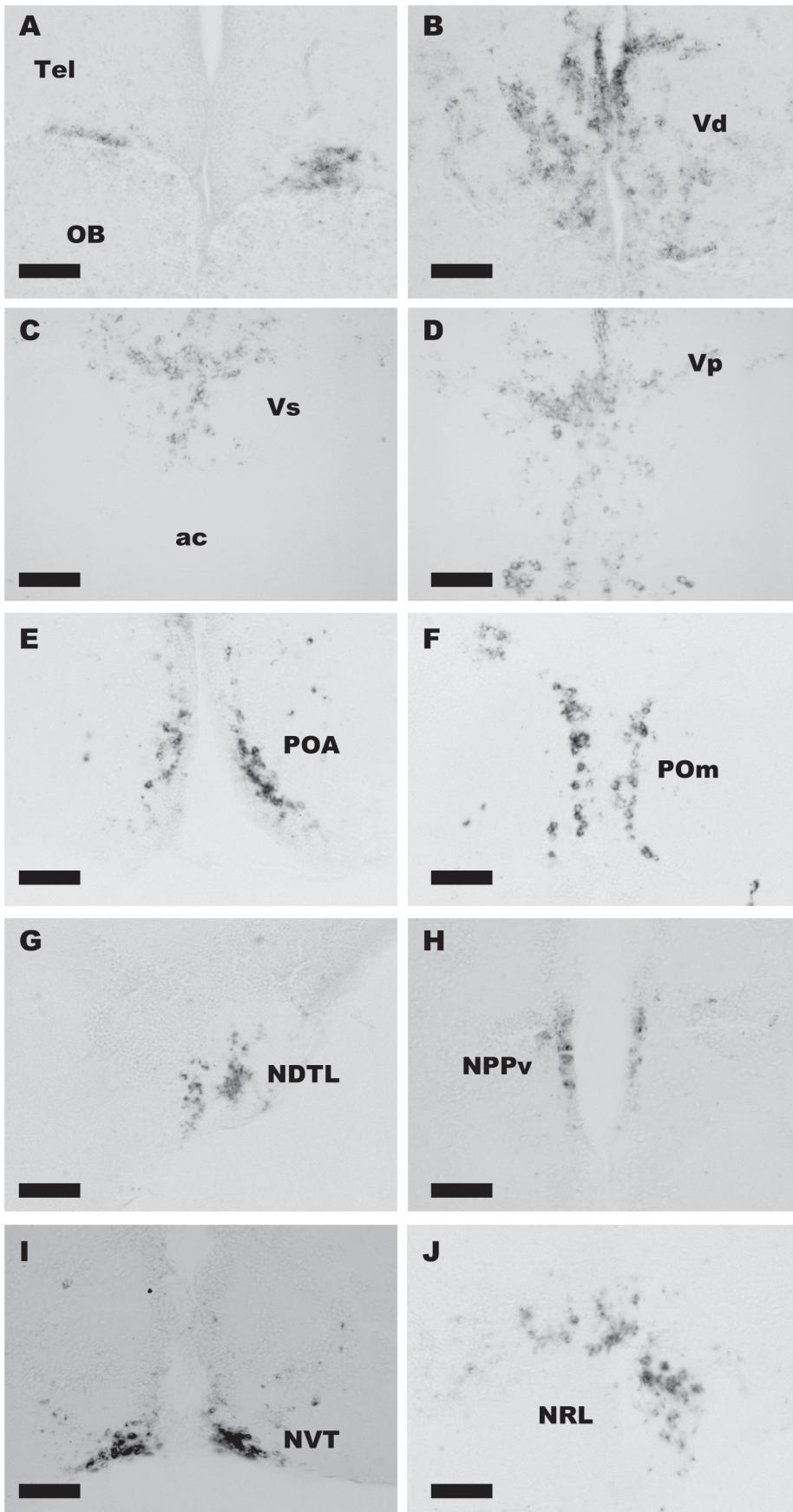
Since the *gpr54-1*- and *gpr54-2*-expressing neurons were localized in the proximity of the POA GnRH1 neurons, dual *in situ* hybridization combining either one of the two subtypes of kisspeptin receptor (*gpr54-1* or *gpr54-2*) mRNAs and *gnrh1* mRNA was performed (Fig. 7;  $n = 4$  for male,  $n = 5$  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 SRE 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



**Figure 3. DIG-labelled *in situ* hybridization of *gpr54-2* shows localization of *gpr54-2* mRNA positive cells.** *gpr54-2* mRNA positive neurons are localized in the boundary between telencephalon (Tel) and olfactory bulb (OB; A), area ventralis telencephali pars dorsalis/supracommissuralis/posterior (Vd;B/Vs;C/Vp;D), area preoptica (POA; E), nucleus preopticus pars magnocellularis (POm; F), nucleus diffusus tori lateralis (NDTL; G), nucleus posterioris periventricularis (NPPv; H), nucleus ventralis tuberis (NVT; I), nucleus recessus lateralis (NRL; J), and corpus mammillare (CM, not shown). Scale bars: 50  $\mu$ m.  
doi:10.1371/journal.pone.0062776.g003

us to perform the anatomical analysis of the distribution of both Gpr54-1 and Gpr54-2 in the medaka brain.

### Kisspeptin Receptors are Densely Expressed in POA and Hypothalamus

We demonstrated that the kisspeptin receptor genes, *gpr54-1* and *gpr54-2* are densely expressed in certain regions of ventral telencephalon, POA, and hypothalamus as well as habenula. Among those areas, *gpr54-1* was mainly expressed in ventral telencephalon, POA and habenula (Fig. 2), whereas *gpr54-2* was more widely expressed in the brain (Fig. 3). Previous study in a cichlid fish suggested that they lack *gpr54-1*, whereas *gpr54-2* mRNA is broadly expressed in the brain [25]. Moreover, in zebrafish, *gpr54-2* is expressed predominantly compared to *gpr54-1* [23]. Phylogenetically, in teleosts, many species lack *gpr54-1*, while *gpr54-2* is conserved throughout all the species analyzed to date. On the other hand, *gpr54-2* is lost in placental mammals [26,27]. Taken together, our results and previous studies suggest that Gpr54-1 system may predominantly function in tetrapods, whereas Gpr54-2 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 ventralis telencephali pars supracommissuralis and/or posterior parts of the area ventralis telencephali pars ventralis (Vs-pVv) and the nucleus preopticus periventricularis (NPP) [29], which appear to overlap with the *gpr54-1*- and *gpr54-2*-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 *gpr54-2*. 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 *gpr54-2* can be activated by both Kiss1 and Kiss2, it is suggested that the isotocin and vasotocin neurons expressing *gpr54-2* 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

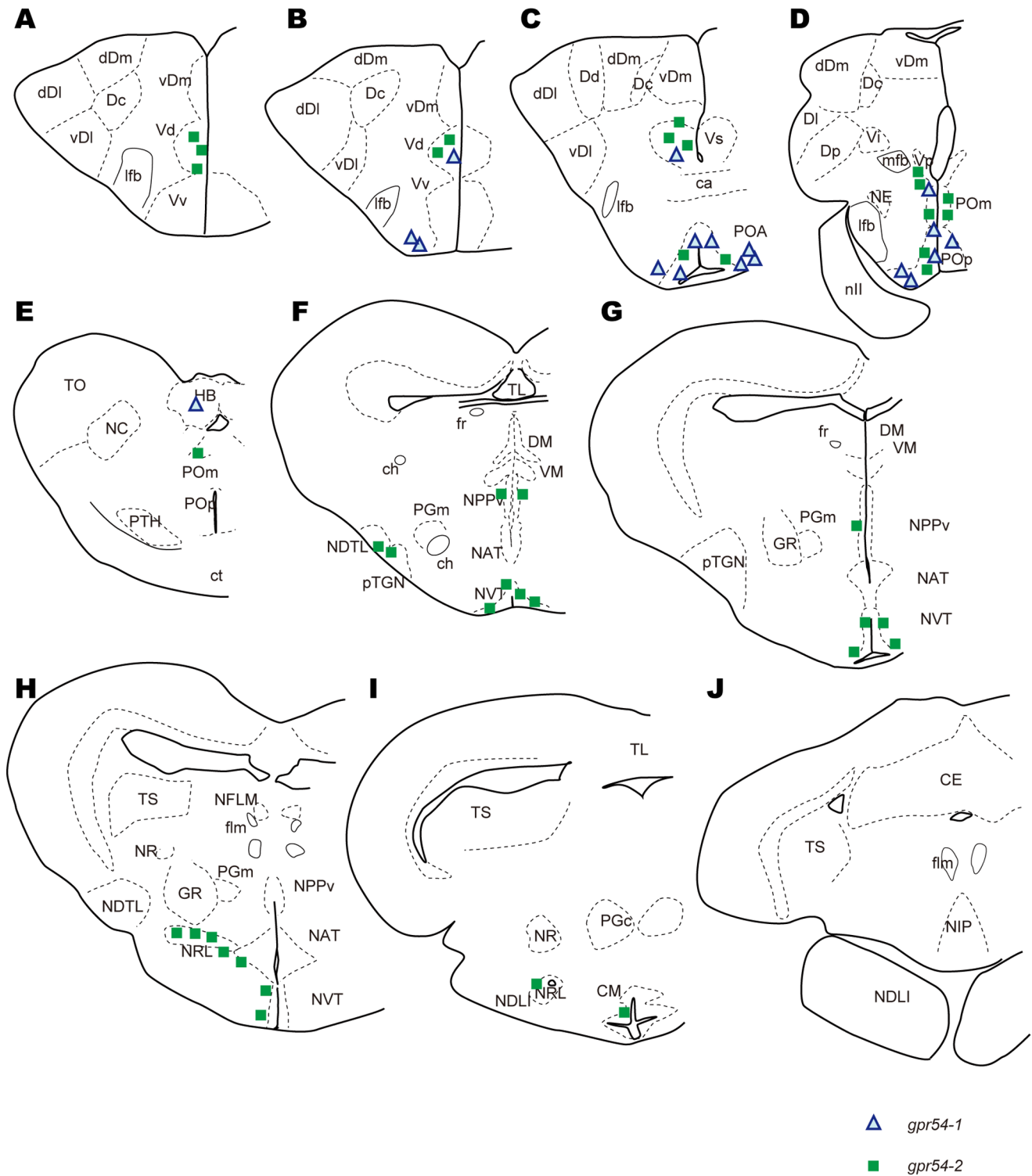
There are general agreements as to the concept that the POA GnRH1 neurons are hypophysiotropic, and the extrahypothalamic GnRH2 and GnRH3 neurons are non-hypophysiotropic and neuromodulatory in nature [15,40,41]. This principle appears to be widely conserved throughout vertebrate species including mammals [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 *gpr54-1* or *gpr54-2* (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 (reviewed in [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

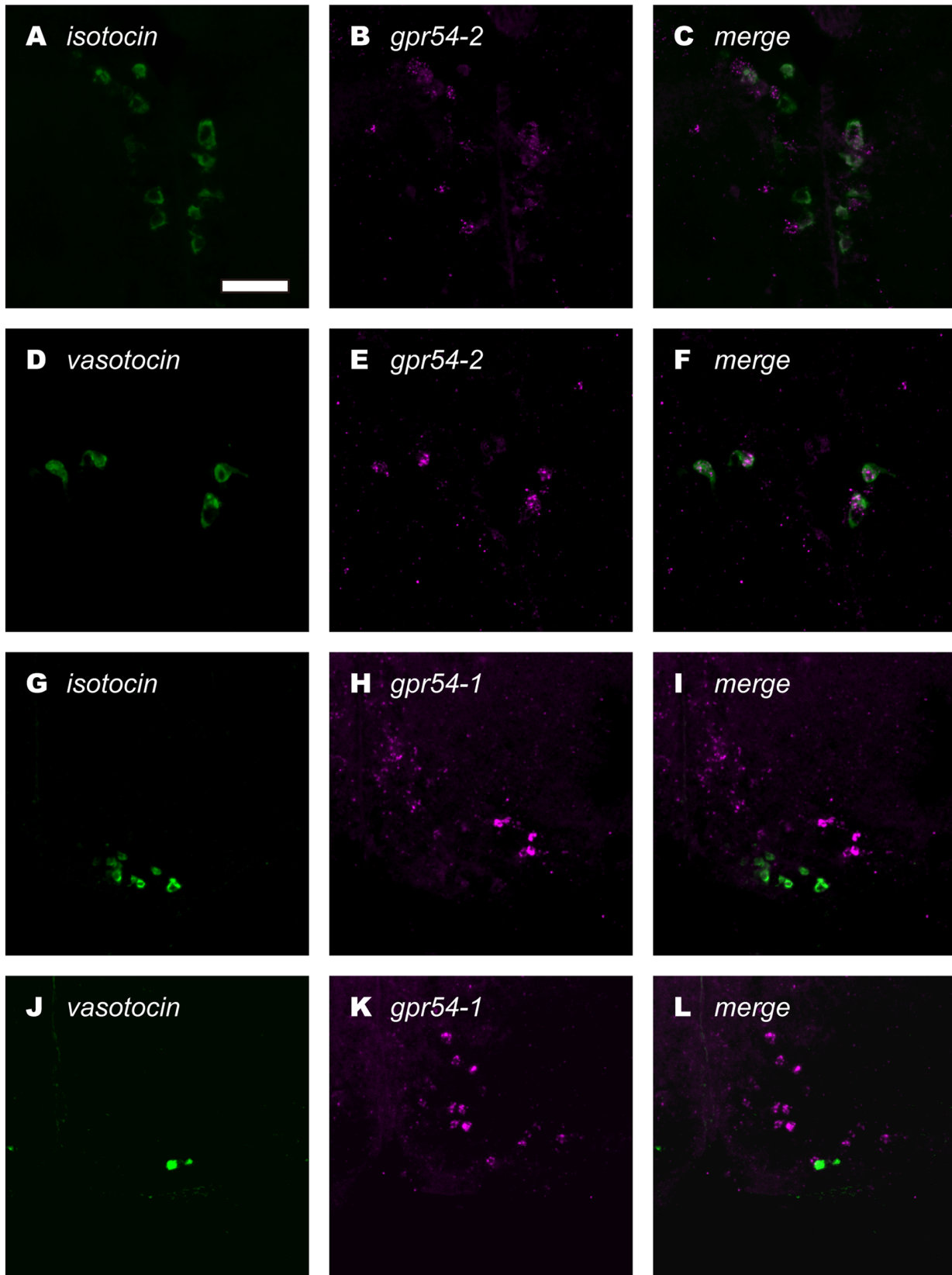
Our present dual *in situ* hybridization study clearly indicated that *gpr54*-expressing cells are located closely adjacent to the hypophysiotropic GnRH1 neurons but they are not the GnRH1 neurons themselves (Fig. 7). In mammals, kisspeptin is supposed to be an essential regulator of GnRH neuron activity. On the other hand, in teleosts, not many, but a few studies reported that kisspeptin up-regulates the expression of LH $\beta$  and FSH $\beta$  in



**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

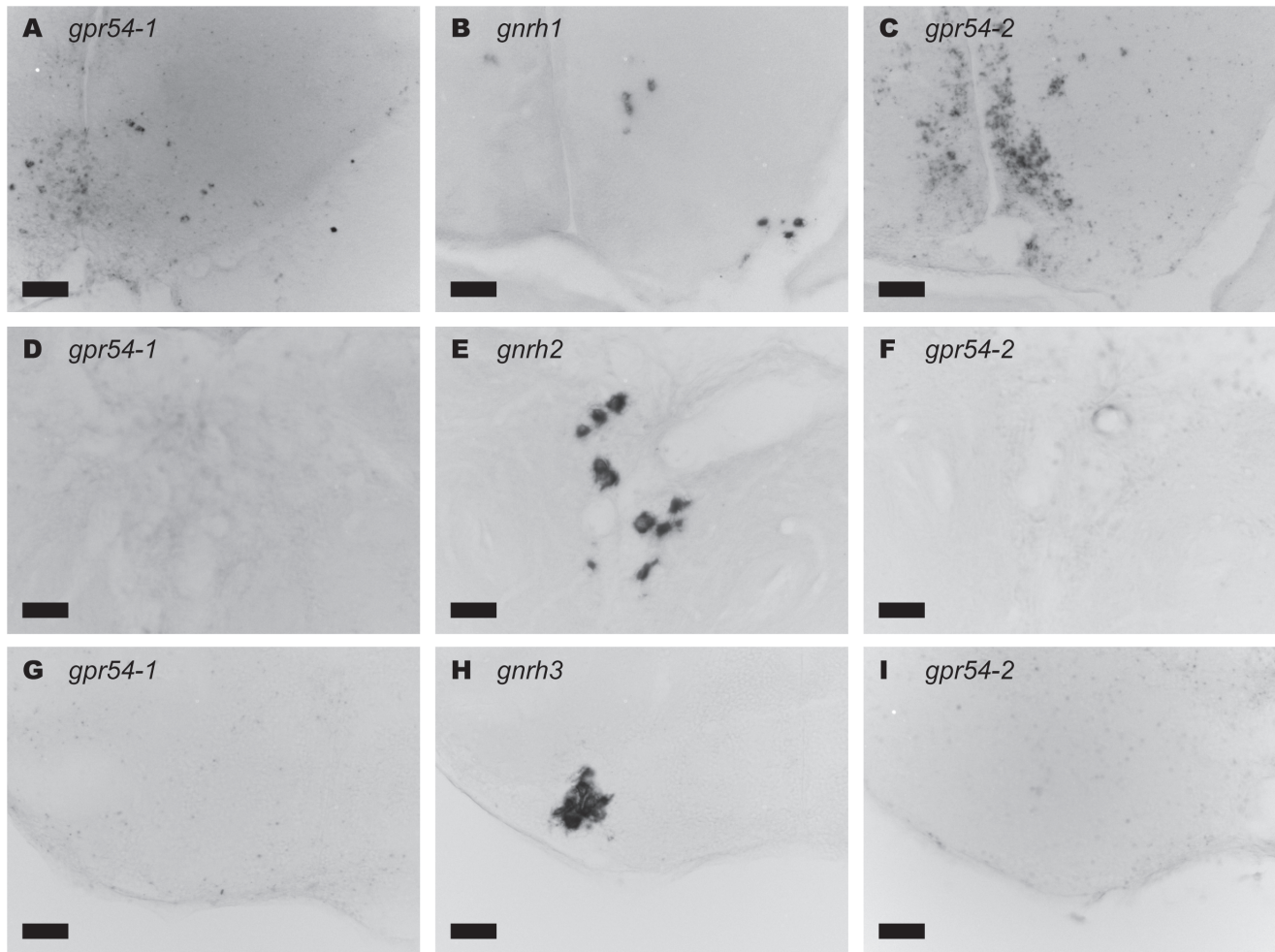
zebrafish [48] and promotes LH secretion in goldfish [24] and sea bass [49,50]. However, the direct action of kisspeptin on the pituitary was denied in goldfish [24]. We also examined possible

direct action of Kiss1(10) on LH $\beta$  and FSH $\beta$  mRNA expression of the isolated medaka pituitary in culture. The Kiss1(10) peptide turned out to have no direct pituitary effect, while



**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  $\mu$ m.  
doi:10.1371/journal.pone.0062776.g005



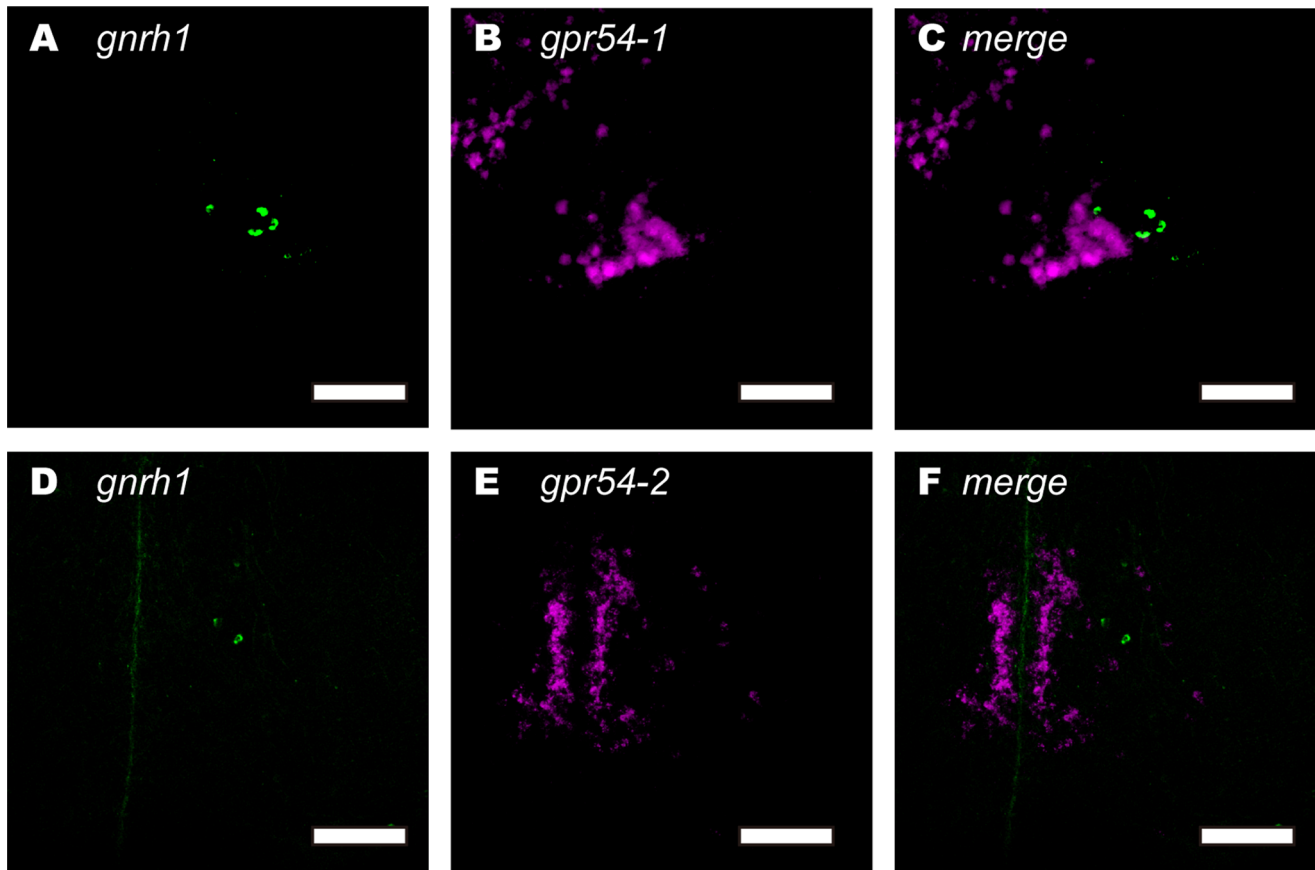


**Figure 6. DIG-labelled *in situ* hybridization of the brain regions surrounding the three types of GnRH neurons (B: *gnrh1*, E: *gnrh2*, H: *gnrh3*), showing the presence or absence of expressions for the kisspeptin receptors, *gpr54-1* or *gpr54-2*, in these regions.** The results for *gpr54-1* mRNA signals (A, D, G) and *gpr54-2* mRNA signals (C, F, I) show that the *gpr54-1*- and *gpr54-2*-expressing neurons are localized in the POA surrounding the GnRH1 neurons (A–C), but not in the midbrain tegmentum surrounding the GnRH2 neurons (D–F) or the ventral telencephalic area surrounding the GnRH3 neurons (G–I). Scale bars: 50  $\mu$ m.  
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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



**Figure 7. Dual fluorescence *in situ* hybridization showing that kisspeptin receptors (*gpr54-1* and *-2*) are highly expressed in the neurons (magenta) adjacent to the GnRH1 neurons (green) in the POA.** *gpr54-1* mRNA (B) is expressed by neurons in proximity to the ventrolateral group of GnRH1 neurons in the POA (A; merged photo in C). *gpr54-2* mRNA (E) is expressed in neurons near the dorsomedial group of GnRH1 neurons in the POA (D; merged photo in F). Scale bar: 50  $\mu$ m. doi:10.1371/journal.pone.0062776.g007

the new era of research of kisspeptin neurons' physiological functions in vertebrates.

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### 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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