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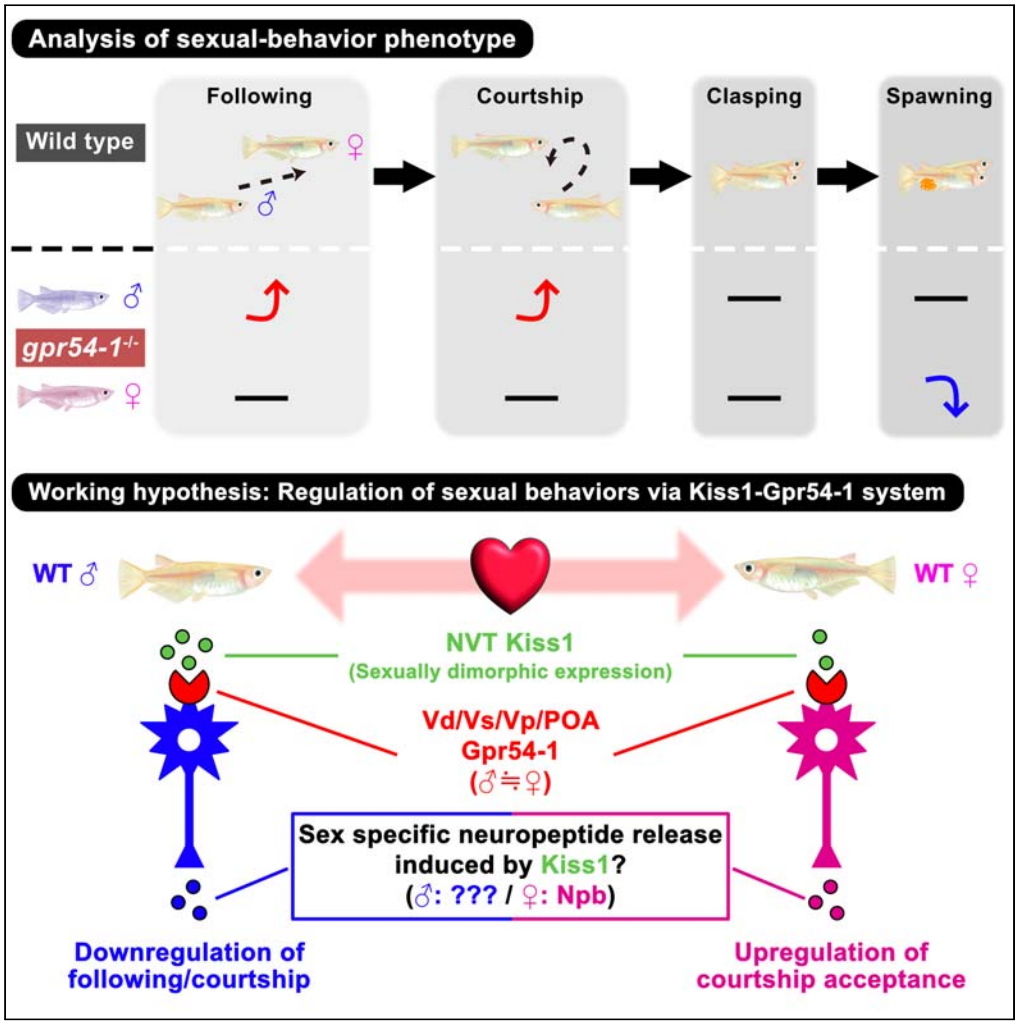

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Highlights

The knockout pairs of *gpr54-1* spawn fewer number of eggs and show delayed spawning

The knockout pairs of *gpr54-2* spawn normally

The Kiss1-Gpr54-1 system may be involved in upregulation of female spawning

The Kiss1-Gpr54-1 system may be involved in downregulation of male motivation

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Involvement of the kisspeptin system in regulation of sexual behaviors in medaka

Mikoto Nakajo,[1,4,*] Shinji Kanda,[2] and Yoshitaka Oka[3,*]

SUMMARY

In mammals, kisspeptin (Kiss1) neurons are generally considered as a sex steroid-dependent key regulator of hypothalamic-pituitary-gonadal (HPG) axis. In contrast, previous studies in non-mammalian species, especially in teleosts, propose that Kiss1 is not directly involved in the HPG axis regulation, which suggests some sex-steroid-dependent functions of kisspeptin(s) other than the HPG axis regulation in non-mammals.

Here, we used knockout (KO) medaka of kisspeptin receptor-coding genes (*gpr54-1* and *gpr54-2*) and examined possible roles of kisspeptin in the regulation of sexual behaviors. We found that the KO pairs of *gpr54-1*, but not *gpr54-2*, spawned fewer eggs and exhibited delayed spawning than wild type pairs. Detailed behavior analysis suggested that the KO females are responsible for the delayed spawning and that the KO males showed hyper-motivation for courtship. Taken together, the present finding suggests that one of the reproductive-state-dependent functions of the Kiss1 may be the control of successful sexual behaviors.

INTRODUCTION

In vertebrates, reproduction, one of the most essential biological functions for the generation of offspring, requires synchronous regulation of gonadal maturation and sexual behaviors. As a pivotal component of reproduction in mammalian species, a neuropeptide kisspeptin plays a crucial role in the regulation of hypothalamic-pituitary-gonadal (HPG) axis in a sex steroid-dependent manner.[1–4] In contrast, there is a growing body of evidence to suggest that kisspeptin does not play an important role in the HPG axis regulation in teleosts from the genetical, histological, and physiological approaches.[5–9] It has been shown that most teleost species have two paralogues of kisspeptin-coding genes, *kiss1/kiss2*, and two paralogues of their receptors, *gpr54-1/gpr54-2*.[10,11] Our previous study suggested that Kiss1 peptide possesses higher affinity to Gpr54-1 than to Gpr54-2, and Kiss2 does so to Gpr54-2 than to Gpr54-1.[6] By physiological analyses including patch clamp and Ca^{2+} imaging, we confirmed that kisspeptin peptide application does not affect neuronal activity or hormonal secretion that is involved in the HPG axis regulation.[6] We have also shown clearly that all the knockout (KO) lines of medaka (*Oryzias latipes*) in which either one of the kisspeptin-receptor genes or both of *kiss1* and *kiss2* were knocked out were fertile and they showed normal gonadal morphology with mature follicles/spermatozoa, and normal expression levels of genes related to the HPG axis regulation, which lead us to conclude that kisspeptin is dispensable for the HPG axis regulation including gonadal maturation in teleosts.[6] On the other hand, because at least either Kiss1-Gpr54-1 or Kiss2-Gpr54-2 system is conserved in most vertebrates except avian species, the kisspeptin neuronal system is considered to be involved in some important functions other than the HPG axis regulation.[10,12,13]

Sex steroids are generally suggested to play pivotal roles in signaling gonadal status to the brain in vertebrates.[14–18] Neurons that receive sex steroids, such as the mammalian kisspeptin neurons, have been suggested to regulate various reproduction-related functions such as ovulation and sexual behaviors.[17–20] It should be noted that kisspeptin neurons have been reported to be highly sensitive to sex steroids in both mammals and teleosts in spite of the fact that kisspeptins are dispensable for the HPG axis regulation in teleosts.[4,21–29] However, the functions of sex steroid-sensitive kisspeptin neurons in non-mammalian species still remain unknown. Given the sex steroid sensitivity of hypothalamic Kiss1 neurons in medaka[11,26,27,30,31] and the importance of sex steroid signaling in the regulation of sexual behaviors in vertebrates,[17–20,32] we hypothesized that the unknown kisspeptin function(s) may be reproductive state-dependent ones, such as sexual behaviors, which have not been examined in detail using the KO fish.

Therefore, we here focused on the possible kisspeptin neuronal regulation on sexual behaviors in medaka. Medaka is a small teleost that has many experimental advantages because its genome database is almost fully available for various genetic manipulations. It also shows

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stereotypical sequence of sexual behaviors that is necessary for successful spawning[33–35] and regularly spawns every morning under appropriate breeding conditions. In addition, unlike mammals, kisspeptin in teleosts is not involved in the HPG axis regulation,[6] indicating that medaka is an advantageous model animal to search for novel and reproductive state-dependent functions such as sexual behavior in vertebrates by using a combination of genome-editing and behavior analysis.[36] In the present study, we first analyzed reproductive phenotypes of the KOs of kisspeptin-receptor genes by counting eggs spawned by the pairs of each KO line. Behavior analysis was also performed to examine possible involvement of the kisspeptin signaling in the regulation of each repertoire of sexual behavior sequence.

RESULTS

gpr54-1$^{-/-}$ pairs spawn fewer eggs

To examine whether KO medaka of kisspeptin-receptor genes show sexual behaviors (Figure 1A) normally, we first analyzed the number of spawned eggs using the fish at similar stages in Condition 1 (Figure 1B). Starting from the day when half of the wild type (WT) pairs (4 out of 8) spawned (Day 1), we counted the number of eggs spawned by all of the KO and WT pairs for more than 40 days. WT, *gpr54-1*$^{-/-}$ and *gpr54-2*$^{-/-}$ pairs started spawning at similar stages around 100 dph (day post hatch). Intriguingly, only the average number of fertilized eggs in the *gpr54-1*$^{-/-}$ pairs did not increase during ~110–140 dph (Day 10–40), while those in the WT and *gpr54-2*$^{-/-}$ steadily increased to around 8–12 eggs per day (Figure 1C). In fact, during the period shown in Figure 1C, the average number of fertilized eggs spawned by *gpr54-1*$^{-/-}$ pairs per day was significantly lower than that by WT, whereas that by *gpr54-2*$^{-/-}$ pairs was not (Figures 1C and 1D). The average number of fertilized eggs per pair during the same period also showed a significant decrease in *gpr54-1*$^{-/-}$ in comparison with WT (Figure 1E). It should be noted that the total number of spawned eggs was also significantly fewer in *gpr54-1*$^{-/-}$ pairs in comparison with WT pairs (Figures S1A–S1C). Moreover, although 2 out of 5 *gpr54-1*$^{-/-}$ pairs tended to spawn fewer fertilized eggs, the fertilization rate per pair itself was not significantly different among these 3 genotypes (Figure S1D), suggesting that these KOs are potentially as capable of spawning as WT.

gpr54-1$^{-/-}$ pairs show delayed spawning

Next, to examine whether these KO pairs show delayed successful spawning in comparison with WT, we performed sexual behavior analysis in Condition 1 (Figure 2A) and Condition 2 (Figure 2B) and observed latency to spawning of each pair. In both conditions, the percentage of pairs that reached successful spawning during the 30-min analysis period was significantly lower only in the *gpr54-1*$^{-/-}$ pairs (3 out of 18 pairs in Condition 1, 6 out of 24 pairs in Condition 2). It should be noted that ~75% of the WT pairs spawned within 5 min in Condition 2 (Figure 2B), while ~40% of them in Condition 1 (Figure 2A) did, and there is a significant difference between these conditions ($p < 0.01$, logrank test), suggesting that Condition 2 is more favorable for spawning. Nevertheless, only 25% of the *gpr54-1*$^{-/-}$ pairs spawned within the recording period even in Condition 2 (Figure 2B). Thus, the data clearly indicate that lack of Gpr54-1, but not Gpr54-2, delays successful spawning. Considering that either one of the KO medaka did not show any serious abnormality in gonadotropin-gene expression levels or gonadal maturation,[6] these data strongly suggest that the decrease in the number of spawned eggs in *gpr54-1*$^{-/-}$ pairs is probably due to some malfunctions in the process of sexual behaviors of *gpr54-1*$^{-/-}$ male and/or female rather than those in the process of fertility regulated by the HPG axis.

Detailed sexual behavior analysis suggests a defect in spawning of *gpr54-1*$^{-/-}$ pairs

To further investigate whether specific repertoires of the sexual behavior sequence are affected in *gpr54-1*$^{-/-}$ pairs, we performed sexual behavior analysis with ZebraCube using a larger sample size consisting of *gpr54-1*$^{+/+}$, *gpr54-1*$^{+/-}$, and *gpr54-1*$^{-/-}$, in combination with egg counting (Figure 3A). First, *gpr54-1*$^{-/-}$ pairs spawned significantly fewer number of fertilized eggs during the recording period in comparison with *gpr54-1*$^{+/+}$ pairs (Figure 3B). In contrast, *gpr54-1*$^{+/-}$ pairs spawned as much as *gpr54-1*$^{+/+}$. Moreover, the percentage of *gpr54-1*$^{-/-}$ pairs that reached successful spawning within 30 min (37 out of 66 pairs) was significantly lower than that of *gpr54-1*$^{+/+}$ (49 out of 60 pairs) (Figure 3C). Thus, these data confirm that the phenotype of *gpr54-1*$^{-/-}$ pairs is a significant delay of spawning and fewer fertilized eggs and that the ZebraCube protocol is also suitable for detailed sexual behavior analysis as well as the video recording described previously. Then, we analyzed the indices of each repertoire of the sexual behavior sequence prior to spawning, such as following, courtship, and clasping. We found that there was no significant difference among the genotypes in the total duration of following (Figure 4A), the number of courtships (Figures 4B, S2C, and S2D), latency to first courtship (Figure S2E), latency from first courtship to clasping (Figure 4C), and duration of clasping (Figure 4D). Interestingly, the *gpr54-1*$^{-/-}$ pairs showed significantly longer duration of following after spawning in comparison with *gpr54-1*$^{+/+}$ pairs (Figure S2B). Taken together, these data suggest that *gpr54-1*$^{-/-}$ pairs show a defect mainly in spawning act itself rather than the other behavioral repertoires prior to spawning.

The lack of Gpr54-1 causes delayed spawning and disrupts motivation in females

Finally, to further examine which sex of *gpr54-1*$^{-/-}$ is responsible for this abnormality in spawning, we swapped sexes of *gpr54-1*$^{+/+}$ pairs and *gpr54-1*$^{-/-}$ pairs and prepared new pairs consisting of *gpr54-1*$^{+/+}$ male/female and *gpr54-1*$^{-/-}$ female/male for the analysis by ZebraCube using the same protocol. The *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs spawned significantly fewer number of fertilized eggs in comparison with the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs (Figure 5A). It should be noted that the *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs spawned relatively fewer number of fertilized eggs in comparison with the *gpr54-1*$^{+/+}$ pairs (Figures 3B and 5A). In the experiments for

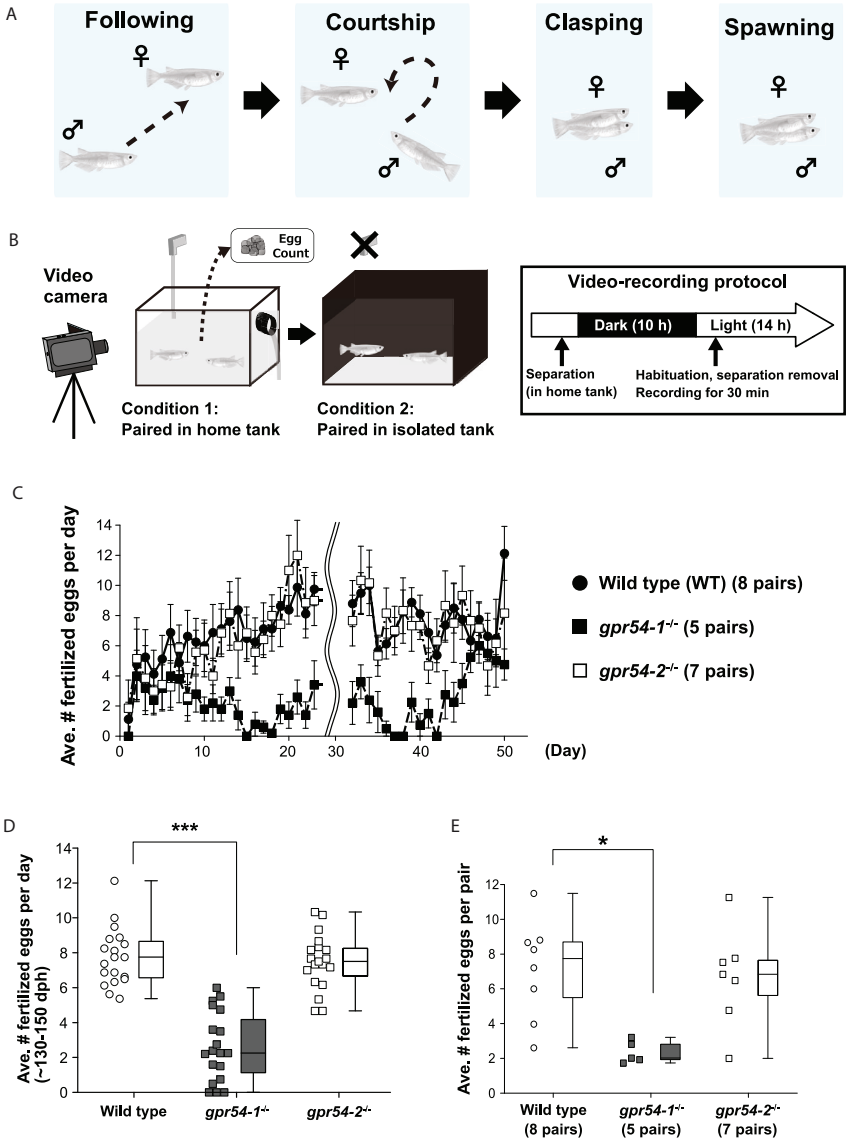

Figure 1. Egg count and sexual behavior analysis using WT, *gpr54-1*$^{-/-}$, and *gpr54-2*$^{-/-}$ pairs

(A and B) Schematic diagram of egg count and sexual behavior analysis in two types of conditions. (A), Illustration of the sexual behavior sequence of medaka. The stereotypical sequence of sexual behaviors proceeds from following to courtship, and then to clasping, and culminates in spawning. (B), Protocol of egg count and behavior analysis. The paired fish of each KO line and WT were maintained in the breeding condition during the analysis. First, egg count and sexual behavior analysis by a video camera (30 min) was performed using the pairs that spawned freely in the home tanks in the circulation system (Condition 1). Next, the same pair was used for the analysis in the isolated tanks (Condition 2).

(C) Egg count analysis shows that *gpr54-1*$^{-/-}$ pairs spawn fewer eggs, while *gpr54-2*$^{-/-}$ pairs appear normal. The line graph showing the average number of fertilized eggs spawned by mature pairs of WT (black circle, 8 pairs), *gpr54-1*$^{-/-}$ (black rectangle, 5 pairs), and *gpr54-2*$^{-/-}$ (white rectangle, 7 pairs) during approximately 100–125 and 130–150 dph. Values are shown as mean ± SEM. Although WT pairs steadily spawned every day, the *gpr54-1*$^{-/-}$ pairs spawned significantly fewer eggs during Day 10–45.

(D and E) Scatter and box-and-whisker plots showing the average number of eggs for the pairs of WT and the KOs per day (n = 19 each) (D) and per pair (E) during the period shown in C. The *gpr54-1*$^{-/-}$ pairs spawned significantly fewer eggs in comparison with WT pairs (***: $p < 0.001$, *: $p < 0.05$, Steel test). In contrast, there was no significant difference between WT and *gpr54-2*$^{-/-}$ pairs.

these figures, we used the experimental fish from the same batch. Moreover, most of the *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs showed a significant delay in successful spawning, and 11 out of 24 of the pairs failed to spawn within 30 min (*: $p < 0.05$, logrank test) (Figure 5B). In addition, the *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs exhibited a definitive delay in spawning completion in comparison with the *gpr54-1*$^{+/+}$ pairs, and the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs tended to succeed in spawning even earlier than the *gpr54-1*$^{+/+}$ pairs (Figures 3C and 5B). These data clearly indicate that *gpr54-1*$^{-/-}$ female is responsible for fewer spawned eggs and delayed spawning.

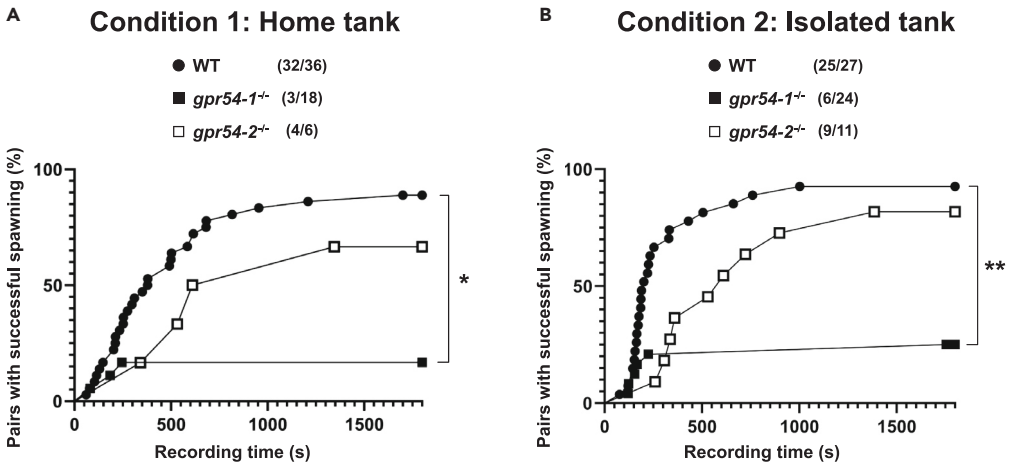

Figure 2. Sexual behavior analysis in the home tanks and the isolated tanks shows that *gpr54-1*$^{-/-}$ pairs, but not *gpr54-2*$^{-/-}$ pairs, exhibit salient delay in accomplishment of spawning

(A and B) Kaplan-Meier plots show latency of successful spawning and percentage of the pairs of each line that succeeded in spawning during the 30-min recording period in Condition 1 (A) and Condition 2 (B). The number of spawned pairs and the total number of pairs are appended to the right of each genotype symbol. Most of the *gpr54-1*$^{-/-}$ pairs (black rectangle, n = 18 and n = 24, in Condition 1 and Condition 2, respectively) failed to accomplish spawning during the 30-min recording periods, and there was a significant delay in comparison with WT pairs (black circle, n = 36, n = 27, respectively) in both conditions (**: $p < 0.01$, *: $p < 0.05$, logrank test and Kruskal-Wallis test). In contrast, there was no significant difference between WT and *gpr54-2*$^{-/-}$ pairs (white rectangle, n = 6, n = 11, respectively). Note that WT pairs spawned earlier in Condition 2 (B) than Condition 1 (A) ($p < 0.01$, logrank test), suggesting that Condition 2 is more favorable for mating, although *gpr54-1*$^{-/-}$ pairs often failed to spawn in both conditions.

Furthermore, detailed sexual behavior analysis using these pairs showed that the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs exhibited significantly longer duration of following (Figure 6A, S3A, and S3B) and a larger total number of courtships after spawning period in comparison with the *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs (Figures 6B, S3C, and S3D). Considering that the *gpr54-1*$^{+/+}$ pairs showed relatively longer total duration of following in comparison with the *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs, and shorter duration in comparison with the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs (compare Figures 4A and 6A), it is suggested that motivation for the sexual behavior of *gpr54-1*$^{-/-}$ males was hyper-activated and that of *gpr54-1*$^{-/-}$ females may be less activated. In contrast, there was no significant difference between these two groups in latency to first courtship (Figure S3E) or latency from first courtship to clasping (Figure 6C) or clasping duration (Figure 6D), suggesting that clasping action itself is not affected by the lack of Gpr54-1.

Female medaka normally exhibits spawning once a day and refuses male courtship and clasping after successful spawning. In fact, we did not observe any second spawning of the same pair in one day, at least in our behavior analysis protocol. Thus, we presume that the comparison of following and courtship during the period before and after spawning can be effective indicators of male motivation and/or female receptivity for sexual behavior. To further examine whether these behavior phenotypes in following and courtship are due to a decreased motivation of *gpr54-1*$^{-/-}$ female or an increased motivation of *gpr54-1*$^{-/-}$ male, we analyzed the change in ratio of time spent for following and courtship frequency during the period before and after spawning event (Figures 6E and 6F). As for the ratio of time spent for following, *gpr54-1*$^{+/+}$ pairs exhibited following before spawning as much as after spawning (Figure 6E). In contrast, the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs showed increased following even after spawning, and the ratio after spawning of the pairs was greater than that of the *gpr54-1*$^{+/+}$ pairs. On the other hand, the *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs showed slightly lower ratio of time spent for following after spawning, although it was not a significant difference (Figure 6E). As for the courtship frequency, the *gpr54-1*$^{+/+}$ pairs, and the *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs showed significant decrease after spawning (Figure 6F). In contrast, the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs did not show significant decrease even after spawning, and the frequency was significantly higher than that of the *gpr54-1*$^{+/+}$ pairs (Figure 6F). Consistent with these data, the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs showed the duration of following and the number of courtships after spawning period relatively longer/larger than those of the *gpr54-1*$^{+/+}$ pairs (Figures S2B, S2D, S3B, and S3D). In addition, we found that *gpr54-1*$^{-/-}$ fish tended to exhibit higher frequency of attack-like behaviors (Figures S2F and S3F). These data suggest that *gpr54-1*$^{-/-}$ males are hyper-motivated to mate so that they keep approaching females, while *gpr54-1*$^{-/-}$ females possibly become less motivated and less receptive to males.

DISCUSSION

The lack of Kiss1-Gpr54-1 system causes delayed spawning and disrupts motivation for sexual behaviors in females

Since the discovery of a kisspeptin neuron as an essential component of steroid feedback mechanism in mammals,[21–24,28] its importance in reproduction has also been examined in non-mammalian species. To date, accumulating evidence has led to a firm conclusion that kisspeptin

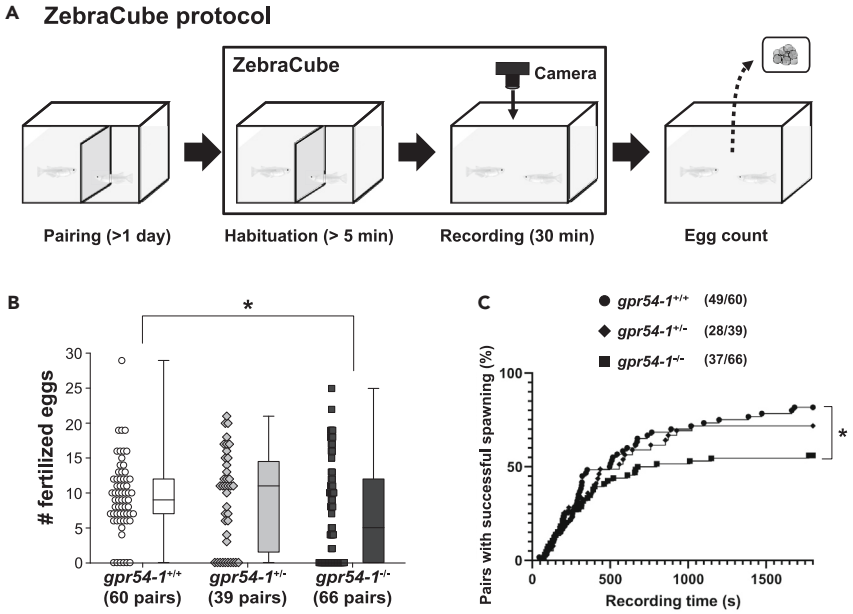

Figure 3. Detailed sexual behavior analysis using a larger batch of *gpr54-1*$^{+/+}$, *gpr54-1*$^{+/-}$, and *gpr54-1*$^{-/-}$ pairs confirms fewer spawned eggs and delayed spawning of *gpr54-1*$^{-/-}$ pairs

(A) Protocol of egg count and behavior analysis by ZebraCube. The sexually mature *gpr54-1*$^{+/+}$, *gpr54-1*$^{+/-}$, and *gpr54-1*$^{-/-}$ males and females of the same batch were paired for each genotype in isolated tanks for the analysis and were maintained for more than 1 day. The pairs were separated by transparent plates the night before the recording day. On the recording day, the separated-pair tanks were transferred into the ZebraCube chamber and were left for more than 5 min for habituation followed by recording. After recording started, the separation was removed, and the pairs were allowed to interact freely with each other during the 30-min recording period. After recording, the number of fertilized eggs in the tanks was counted.

(B) Scatter and box-and-whisker plots showing the number of eggs spawned by the pairs of each genotype (*gpr54-1*$^{+/+}$, n = 60; *gpr54-1*$^{+/-}$, n = 39; *gpr54-1*$^{-/-}$, n = 66) during the recording period. Consistent with the previous data shown in Figure 1, the *gpr54-1*$^{-/-}$ pairs in this condition spawned significantly fewer eggs in comparison with *gpr54-1*$^{+/+}$ pairs (*: $p < 0.05$, Steel test). In contrast, the *gpr54-1*$^{+/-}$ pairs spawned normally.

(C) Kaplan-Meier plot shows latency of successful spawning and percentage of the pairs of each genotype that succeeded in spawning during the 30-min recording period in ZebraCube protocol. The number of spawned pairs and the total number of pairs are appended to the right of each genotype symbol. 29 out of 66 pairs of *gpr54-1*$^{-/-}$ (black rectangle, n = 66) failed to accomplish spawning during the 30-min recording periods, and there was a significant delay in comparison with *gpr54-1*$^{+/+}$ pairs (black circle, n = 60) (*: $p < 0.05$, logrank test and Kruskal-Wallis test). In contrast, there was no significant difference between *gpr54-1*$^{+/+}$ and *gpr54-1*$^{+/-}$ pairs (black diamond, n = 39).

neuronal system in teleosts is not directly involved in the HPG axis regulation.[5–9] On the other hand, it has been generally accepted that kisspeptin neurons in various species are highly sensitive to sex steroids and drastically change their kisspeptin expression and activities in accordance with the breeding states, which may be conserved in vertebrates.[11,21–24,26–28,30,31,37] Therefore, it should be interesting to search for the sex-steroid-dependent functions of kisspeptin neuronal system other than the HPG axis regulation.

In the present study, we first demonstrated by the egg-count analysis and the sexual behavior analysis using WT and the KO of either kisspeptin-receptor genes, *gpr54-1* or *gpr54-2* (Figures 1A and 1B) that the pairs of *gpr54-1*$^{-/-}$ showed significant decrease in the total number of spawned eggs and delayed spawning, while those of *gpr54-2*$^{-/-}$ did not, in comparison with those of WT (Figure 2). Nevertheless, our previous histological data showed that gonads of *gpr54-1*$^{-/-}$ fish have normal structure in comparison with those of WT.[6] Taken together, it is reasonable to suggest that the decrease in the number of spawned eggs may be caused by some abnormalities in some repertoire(s) of the sexual behavior sequence of *gpr54-1*$^{-/-}$ instead of the defective gonadal functions, although some pathways other than the HPG axis might affect gonads, which consequently lead to the decrease in spawned eggs. Next, detailed sexual behavior analyses of the *gpr54-1*$^{-/-}$ using a larger batch including *gpr54-1*$^{+/+}$ and *gpr54-1*$^{+/-}$ (Figure 3A) confirmed the *gpr54-1*$^{-/-}$ phenotypes described previously (Figure 3) and demonstrated that the *gpr54-1*$^{-/-}$ pairs show normal sexual behavior repertoires except spawning (Figure 4). For further examination of which sex of *gpr54-1*$^{-/-}$ is more responsible for defective spawning, we swapped sexes of *gpr54-1*$^{+/+}$ and *gpr54-1*$^{-/-}$ pairs using the same protocol. Egg count analysis and behavior analysis using the sex-swapped pairs clearly showed that *gpr54-1*$^{-/-}$ female is responsible for the decrease in the number of spawned eggs and delayed spawning (Figure 5). Interestingly, we also found that *gpr54-1*$^{-/-}$ males showed following and courtship more frequently even after successful spawning, while *gpr54-1*$^{-/-}$ females often tended to escape from male's following and courtship after successful spawning (Figure 6). Consequently, successful mating of the pairs containing *gpr54-1*$^{-/-}$ females was significantly delayed (Figures 2, 3, and 5). It should be noted that *gpr54-1*$^{-/-}$ fish showed normal sexual behavior sequence and that they could normally lay fertilized eggs in *ad lib* fed breeding condition, which was applied to egg counting (Figures 1, 3, and 5). Taken together, the present study suggests that in medaka, neuronal system(s) mediated by Gpr54-1

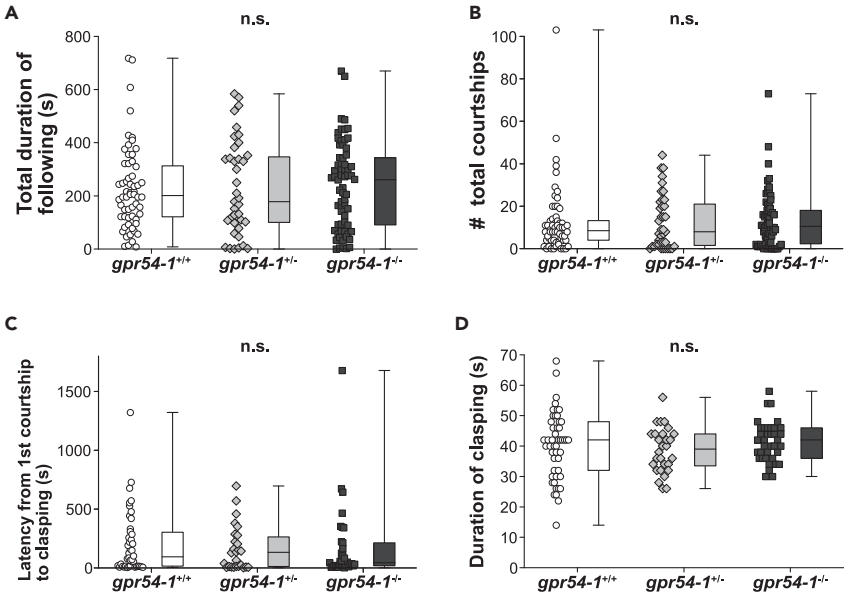

Figure 4. Detailed sexual behavior analysis by ZebraCube using *gpr54-1*$^{+/+}$ (white circle, n = 60), *gpr54-1*$^{+/-}$ (gray diamond, n = 39), and *gpr54-1*$^{-/-}$ (black rectangle, n = 66) pairs suggests that none of the repertories of the sexual behavior sequence except spawning were affected in *gpr54-1*$^{-/-}$ pairs

Scatter and box-and-whisker plots showing the total duration of following (A), the total number of courtships (B), latency from first courtship to clasping (C), and duration of clasping (D) for each genotype pair. There was no significant difference in any index shown here among these pairs (Steel test), suggesting that the delayed spawning and fewer spawned eggs in *gpr54-1*$^{-/-}$ pairs were caused by a defect in spawning act itself, rather than the other repertories of the sexual behavior sequence.

(referred to as Kiss-Gpr54-1 system) is involved in regulating female spawning and motivation for sexual behaviors such as initiation and/or acceptance of courtship.

Independent roles of Kiss1-Gpr54-1 and Kiss2-Gpr54-2 systems

As discussed previously, the present data strongly suggest that *gpr54-1*$^{-/-}$ female shows deficiency in spawning and decrease in motivation for sexual behaviors. In contrast, *gpr54-2*$^{-/-}$ fish did not show such phenotypes. Given that medaka Kiss1 peptide has higher affinity to medaka Gpr54-1 than Kiss2 peptide, that is, Kiss1-Gpr54-1 and Kiss2-Gpr54-2 systems act independently,[8] one plausible explanation for the results may be that Kiss-Gpr54-1 but not Kiss2-Gpr54-2 system is mainly involved in regulating sexual behaviors. This is partly consistent with our previous histological study in medaka showing that distributions of *gpr54-2* mRNA were broader than those of *gpr54-1* mRNA,[8] suggesting independent action sites of Kiss1 and Kiss2, although we cannot exclude effects of other unknown ligands or neuropeptides that may crosstalk with Gpr54-1/2. Because the KOs were non-conditional ones, deficiency of Gpr54-1/2 in the whole body may cause some unexpected secondary or long-term effects. Interestingly, our previous studies also demonstrated that another neuropeptide, neuropeptide B (Npb) neurons co-express *gpr54-1* and isotocin (IT) and vasotocin (VT) (orthologous products of mammalian oxytocin (OT) and vasopressin (VP), respectively) neurons co-express *gpr54-2*, and their axons are intertwined with each other.[6,8] Therefore, given that Gpr54-1/2 are Gq-type G-protein-coupled receptors (GPCRs), and that Npb is generally an inhibitory neuropeptide because Npb receptor, Npbwr is Gi-type GPCR,[38–40] it is suggested that Kiss2-Gpr54-2 system may promote IT/VT neurons in a direct manner, while Kiss1-Gpr54-1 system may indirectly inhibit them via Npb. Thus, Kiss1 and Kiss2 effects on IT/VT neuronal system may be opposite. In addition, some previous studies suggested the involvement of IT (OT)/VT (VP) and kisspeptin in behavior regulation that is related to reproduction in both mammals and teleosts, although the mechanism is still unclear.[7,19,25,41–49] It is possible that Npb regulation on IT/VT neurons via Kiss1-Gpr54-1 system is one of the neuronal pathways affecting female sexual behaviors. Taken together, the present study suggests that Kiss1-Gpr54-1 system can be a strong candidate for an important modulator of sexual behaviors in females. Further studies will elucidate mechanisms of involvement of these neuropeptides in regulation of sexual behaviors.

NVT Kiss1 neuronal regulation via Gpr54-1 neurons modulates sexual behaviors

Since the identification of Kiss1 as an endogenous ligand of Gpr54-1, the functions of Kiss1-Gpr54-1 system have been widely addressed using various model non-mammalian animals including medaka. Previous physiological studies in medaka showed that medaka Kiss1 peptide selectively binds to medaka Gpr54-1.[8] Moreover, histological studies demonstrated that Kiss1 neurons are localized mainly in habenula (Hb), ventral tuberal nucleus (NVT), and posterior periventricular nucleus (NPPv), and only the NVT population showed estrogen sensitive *kiss1* expression,[26,31] breeding state-dependent neuronal activities, and axonal projection to ventral telencephalon (Vd/Vs/Vp/Vv), preoptic area (POA) and the pituitary.[30] On the other hand, the Hb Kiss1 neurons project to the fasciculus retroflexus and interpeduncular nucleus, suggesting

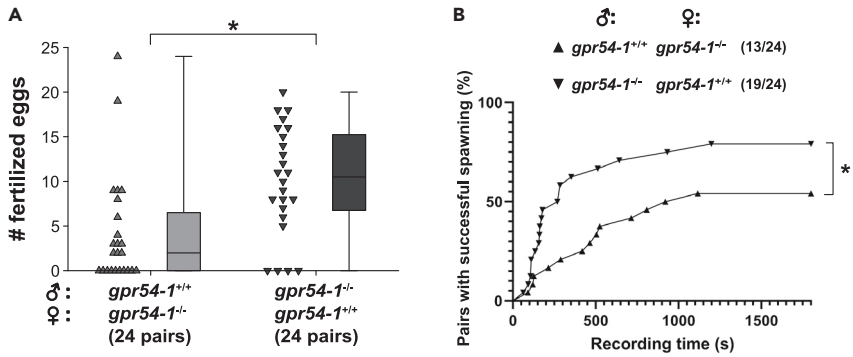

Figure 5. Detailed sexual behavior analysis by ZebraCube using the swapped pairs of *gpr54-1*$^{+/+}$ and *gpr54-1*$^{-/-}$ shows that the phenotype in spawning was caused by *gpr54-1*$^{-/-}$ female

By swapping the *gpr54-1*$^{+/+}$ and *gpr54-1*$^{-/-}$ pairs used in the analysis shown in Figures 3 and 4, the pairs of *gpr54-1*$^{+/+}$ male/female and *gpr54-1*$^{-/-}$ partner were prepared and used in the same protocol.

(A) Scatter and box-and-whisker plots showing the number of eggs spawned by the pairs of *gpr54-1*$^{+/+}$ male/female and *gpr54-1*$^{-/-}$ partner (gray triangle, *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female; dark gray inverted triangle, *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female; n = 24, respectively) during the recording period. The *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs spawned significantly smaller number of fertilized eggs in comparison with *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs (*: $p < 0.05$, Mann-Whitney *U* test).

(B) Kaplan-Meier plot shows latency of successful spawning and percentage of the swapped pairs that succeeded in spawning during the 30-min recording period. About half of the pairs of *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female (black triangle, n = 24) failed to accomplish spawning during the 30-min recording periods, and there was a significant delay in comparison with the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs (inverted black triangle, n = 24) (*: $p < 0.05$, logrank test).

that this population is not involved in regulation in Vd/Vs/Vp and POA.[30] These lines of evidence strongly suggest that the NVT Kiss1 neurons, the only estrogen-sensitive population in medaka, are most probably responsible for regulation of the *gpr54-1* expressing neurons in Vd/Vs/Vp and/or POA.[6,8] Other previous studies have shown that Vd/Vs/Vp and POA are the brain areas containing neurons that play critical roles in regulating sexual behaviors.[50–52] Taken together, under the control of NVT Kiss1 neurons, *gpr54-1* expressing neurons in these brain areas may be considered to modulate the initiation of sexual behavior patterns such as spawning and courtship. It is also possible that they control motivation for male intensity of approach and/or female acceptability, which is critical to the smooth progress of sexual behavior sequences. Thus, disruption of NVT Kiss1 signaling may have resulted in the sexual behavior phenotypes of *gpr54-1*$^{-/-}$ fish observed in the present study.

In the present study, *gpr54-1*$^{-/-}$ males showed hyper-motivation and *gpr54-1*$^{-/-}$ females showed a decrease in spawned eggs and delayed spawning as well as slightly lowered motivation. It should be noted that *kiss1* expression level in NVT is much higher in males, indicating that NVT Kiss1 neurons exhibit more salient effects in males than in females.[26] On the other hand, Npb co-expression of *gpr54-1* expressing neurons in Vs/Vp is female-specific.[6,53] Such sexual dimorphism in Kiss1-Gpr54-1 neuronal pathway may cause different phenotypes between the sexes. Notably, the previous study also demonstrated that lack of Npb function in female medaka caused disruption of female acceptability to male courtship.[54] This is consistent with the present data, suggesting that Npb neurons regulate female-specific behavior and acceptance via Kiss1-Gpr54-1 system.

Interestingly, previous studies in mammals including humans also suggest possible involvement of Kiss1 in sexual behavior and its motivation.[32,42,49,55–57] Thus, involvement of Kiss1 in regulation of sexual behavior may be a common function in vertebrates. Further detailed analyses will be necessary to reveal the mechanism of Kiss1-Gpr54-1 regulation in sexual behaviors and motivation.

Limitations of the study

In this study, we explored possible functions of the kisspeptin system in sexual behaviors using KO medaka of kisspeptin-receptor coding genes. Although we observed clear behavioral phenotypes in *gpr54-1* KO, we did not test which neuronal circuit is responsible for the behavioral difference, because these KO lines are not conditional KOs. Additionally, the present study did not use the KOs of kisspeptin (ligand)-coding genes. Therefore, although Gpr54-1 is generally considered to be a Kiss1 receptor, we could not exclude a possibility that some neuropeptides other than Kiss1 may also regulate sexual behaviors by binding to Gpr54-1.

Further work will be necessary to elucidate the regulatory mechanism of sexual behaviors by Kiss1-Gpr54-1 system in detail.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact

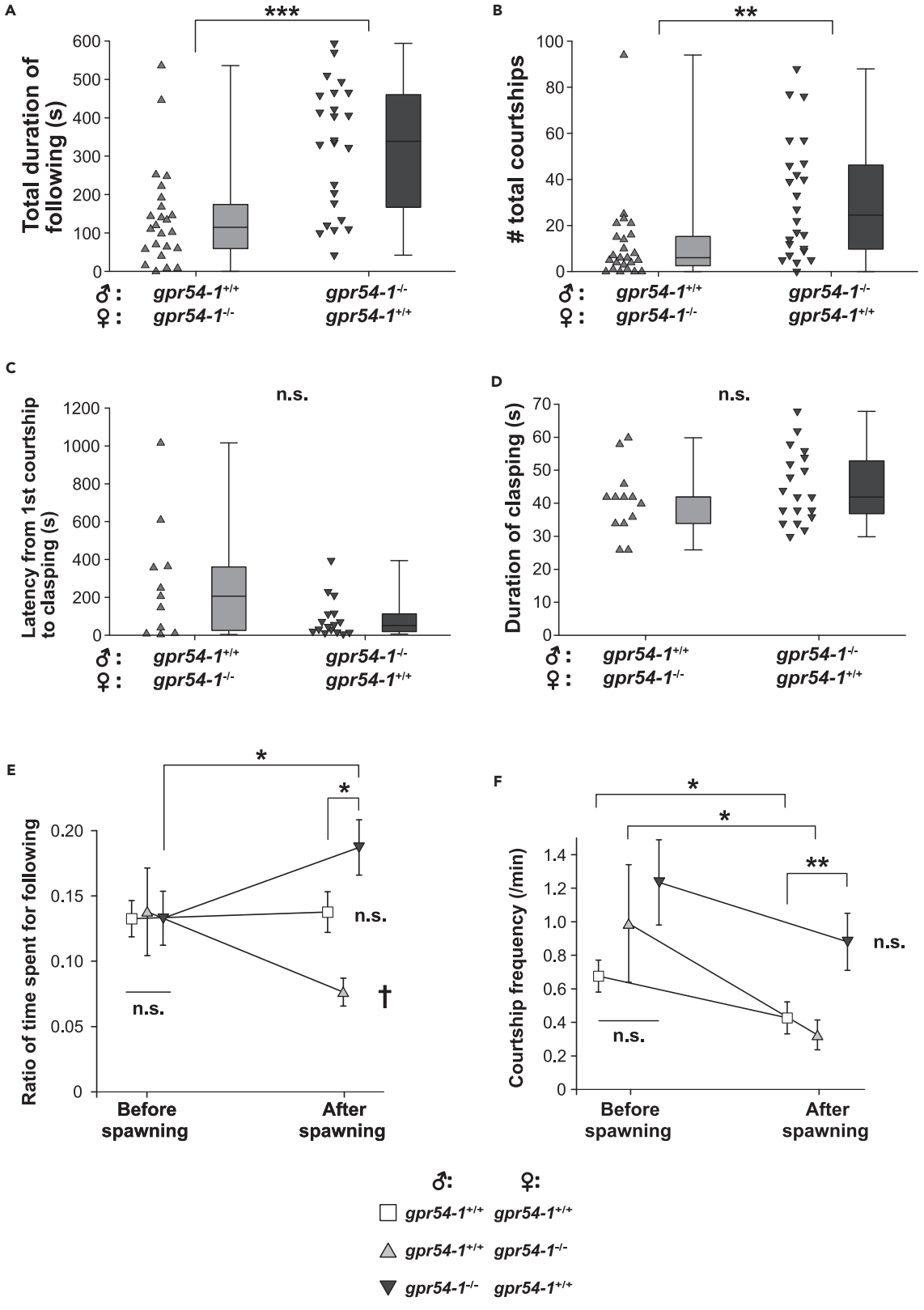
A
***
Total duration of following (s)
600
500
400
300
200
100
0
♂: gpr54-1+/+ gpr54-1-/-
♀: gpr54-1-/- gpr54-1+/+
B
**
total courtships
100
80
60
40
20
0
♂: gpr54-1+/+ gpr54-1-/-
♀: gpr54-1-/- gpr54-1+/+
C
n.s.
Latency from 1st courtship to clasping (s)
1200
1000
800
600
400
200
0
♂: gpr54-1+/+ gpr54-1-/-
♀: gpr54-1-/- gpr54-1+/+
D
n.s.
Duration of clasping (s)
70
60
50
40
30
20
10
0
♂: gpr54-1+/+ gpr54-1-/-
♀: gpr54-1-/- gpr54-1+/+
E
*
*
Ratio of time spent for following
0.20
0.15
0.10
0.05
0
n.s.
n.s.
†
Before spawning
After spawning
F
*
*
**
Courtship frequency (/min)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
n.s.
n.s.
Before spawning
After spawning
♂: ♀:
gpr54-1+/+ gpr54-1+/+
gpr54-1+/+ gpr54-1-/-
gpr54-1-/- gpr54-1+/+

Figure 6. Detailed sexual behavior analysis by ZebraCube using the swapped pairs of *gpr54-1*$^{+/+}$ and *gpr54-1*$^{-/-}$ suggests opposite phenotypes in motivation for the sexual behavior of *gpr54-1*$^{-/-}$ females and males

(A and B) Scatter and box-and-whisker plots showing the total duration of following (A), the total number of courtships (B) for each pair (gray triangle, *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female; dark gray inverted triangle, *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female; n = 24, respectively). The *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs showed significantly higher values for the both indices in comparison with *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs (***: $p < 0.001$, **: $p < 0.01$, Mann-Whitney *U* test).

(C and D) Scatter and box-and-whisker plots showing latency from first courtship to clasping (C) and duration of clasping (D) for each pair (gray triangle, *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female, n = 11, 13; dark gray inverted triangle, *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female, n = 16, 19). There was no significant difference in these indices (Mann-Whitney *U* test), suggesting that female acceptability of male courtship and clasping act itself in *gpr54-1*$^{-/-}$ were not affected.

(E and F) line graphs showing ratio of time spent for following (E) and courtship frequency (F) of the swapped pairs and *gpr54-1*$^{+/+}$ pairs during the period before and after spawning event. Values are shown as mean ± SEM. (E), The *gpr54-1*$^{+/+}$ pairs (white rectangle, n = 49) did not show significantly different change in the ratio of time spent for following before and after spawning, whereas the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs (dark gray inverted triangle, n = 19) showed significantly increased ratio after spawning (*: $p < 0.05$, Wilcoxon Signed-rank test), which is significantly higher than that of the *gpr54-1*$^{+/+}$ pairs (*: $p < 0.05$, Steel test). In contrast, the *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs (gray triangle, n = 13) showed apparently decreased ratio of time spent for following after spawning, although the difference was not significant (†: $p = 0.087$, Wilcoxon Signed-rank test). (F) The *gpr54-1*$^{+/+}$ pairs (white rectangle, n = 49) and the *gpr54-1*$^{+/+}$ male and *gpr54-1*$^{-/-}$ female pairs (gray triangle, n = 13) showed significantly decreased courtship frequency after spawning, while the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs (dark gray inverted triangle, n = 19) did not show significant decrease (*: $p < 0.05$, Wilcoxon Signed-rank test). The frequency after spawning of the *gpr54-1*$^{-/-}$ male and *gpr54-1*$^{+/+}$ female pairs was significantly higher than that of *gpr54-1*$^{+/+}$ pairs (**: $p < 0.01$, Steel test).

- Materials availability
- Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Genotyping
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  - Behavior analysis of *gpr54-1*$^{-/-}$ and *gpr54-2*$^{-/-}$
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.108971.

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AUTHOR CONTRIBUTIONS

M.N., S.K., and Y.O. designed research; M.N. performed research; M.N. analyzed data; M.N., S.K., and Y.O. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sodium Hydroxide (NaOH)	FUJIFILM Wako Pure Chemical Corporation	Cat#: 192-15985
Ethylenediaminetetraacetic acid (EDTA)	FUJIFILM Wako Pure Chemical Corporation	Cat#: BIE0729
Tris Buffered Saline (TBS) Tablets, pH7.6	Takara Bio	Cat#: T9141
Takara Ex Premier™ DNA Polymerase	Takara Bio	Cat#: RR370S
ExoSAP-IT™ PCR Product Cleanup Reagent	Thermo Fisher Scientific	Cat#: 75001.1.ML
Experimental models: Organisms/strains		
Medaka (*Oryzias latipes*)	Local dealer	N/A
Medaka (*Oryzias latipes*): KO (*gpr54-1*$^{-/-}$)	Nakajo et al.[6]	N/A
Medaka (*Oryzias latipes*): KO (*gpr54-2*$^{-/-}$)	Nakajo et al.[6]	N/A
Oligonucleotides		
Primer: gpr54-1_check_fwd.-1: TCGGATCCAGTAAACCACAACA	Nakajo et al.[6]	N/A
Primer: gpr54-1_check_rev.-1: CTGAACGCTGAAGACGAACCAT	Nakajo et al.[6]	N/A
Primer: gpr54-1_seq_fwd.-1: GTGATGCAACTTAACTGAAGGCTT	Nakajo et al.[6]	N/A
Software and algorithms		
Digital HD video camera recorder (HDR-CX420)	SONY	https://www.sony.net/
Digital HD video camera recorder (GZ-E109)	JVCKENWOOD	https://www.jvc.com/
ZebraCube	ViewPoint Life Science	https://www.viewpoint.fr/
VLC media player (Version 2.2.6)	VideoLAN	https://www.videolan.org/
Microsoft Excel macro for behavioral annotation (*Ethogramer*)	Tomihara et al.[34]	N/A
KyPlot 6.0	KyensLab	https://www.kyenslab.com/
GraphPad Prism 9.5.1	GraphPad	https://www.graphpad.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Mikoto Nakajo (mikoto.nakajo@ompu.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Male and female wild type (WT) d-rR strain of medaka (*Oryzias latipes*), the KO medaka lines of kisspeptin-receptor genes (*gpr54-1*$^{-/-}$ and *gpr54-2*$^{-/-}$)[6] and heterozygous fish of *gpr54-1* (*gpr54-1*$^{+/-}$) were used in the present study. In our previous study, both of the KO lines were confirmed to be null mutants of each targeted kisspeptin-receptor gene.[6] All fish were maintained in breeding tanks with a water circulation

system (Labreed, IWAKI Co., Ltd., Tokyo, Japan, or ZebTEC, Tecniplast, Buguggiate, Italy) under a 14 h light/ 10 h dark long-day photoperiod condition (light on: 8 A.M.-10 P.M.) at a water temperature of 28±1°C to maintain their breeding state. The fish were fed two to four times a day with live artemia (brine shrimp; Salt Creek, UT) and/or commercial flake food until they were used for all the experiments after the fish reached sexual maturity (approximately >100 dph (day post hatch)). All the fish maintenance and the experiments were conducted in accordance with the protocols approved by Committee on Animal Care and Use of the Graduate School of Science, the University of Tokyo (permission number, 15–3) and Osaka Medical and Pharmaceutical University.

METHOD DETAILS

Genotyping

For detailed analysis of *gpr54-1*$^{-/-}$, we prepared the batch of wild type (*gpr54-1*$^{+/+}$), heterozygous (*gpr54-1*$^{+/-}$), and KO fish (*gpr54-1*$^{-/-}$). The *gpr54-1*$^{+/-}$ fish were crossed and genomic DNA of their offspring fish was extracted from the caudal fin using 25 mM NaOH/0.2 mM EDTA solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). For genomic DNA extraction, we incubated clipped-fin samples in NaOH/EDTA solution at 95°C for 10 min and the samples were mixed with 40 mM Tris-HCl solution for neutralization (Takara Bio, Shiga, Japan). The amplicons that include the target region of *gpr54-1* were generated by PCR using Takara Ex Premier DNA Polymerase (Takara, Shiga, Japan) and the corresponding primers as described in our previous study.[6] After PCR reaction, the primers and dNTPs were digested by ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA) according to the respective manufacturer's instructions. The PCR Amplicons were sequenced by a commercial company (Eurofins genomics, Tokyo, Japan). We determined the genotype of each individual fish by the sequence of *gpr54-1* mutation site as shown in the previous study.[6]

Preparation of test fish and counting spawned eggs

All fish embryos for the analyses were obtained in the same period (approximately within two weeks) and maintained in shoals for about three months. Until we selected the males and females by their sexually dimorphic appearance, the fish were bred in shoals. After the fish reached sexual maturity (>100 dph), they were paired for counting spawned eggs and behavior analysis. The fertilized eggs were counted from the period when the WT pairs started sexual behavior in the morning, following video camera recordings of their sexual behaviors (HDR-CX420, Sony, Tokyo, Japan or GZ-E109, JVCKENWOOD, Yokohama, Japan) under the conditions as described below. We prepared the fish groups consisting of 4-5 individuals of each line by random selection before starting the analysis (approximately 80 dph) to average their body sizes. About two weeks later, we made the pairs from the groups (WT, 8 pairs; *gpr54-1*$^{-/-}$, 5 pairs; and *gpr54-2*$^{-/-}$, 7 pairs, respectively). Approximately one week after pairing, we observed 4 out of 8 WT pairs that spawned in one day (in approximately 100-dph stage). We defined the first observation day as "Day 1" and started continuous daily counting of all the eggs in the tanks of ~100-120 and ~130-150 dph adult pairs in the breeding system for about 40 days. The eggs were collected during the period 9 A.M.-7 P.M., and we confirmed that all the eggs were collected and removed from the tank every day. The tanks were cleaned at least once a week. As for the genotyped batch including *gpr54-1*$^{+/-}$ fish, we performed egg counting just after the recording by a ZebraCube system (ViewPoint Life Science, Leon, France) likewise.

Behavior analysis of *gpr54-1*$^{-/-}$ and *gpr54-2*$^{-/-}$

Medaka shows characteristic sexual behaviors consisting of clearly defined sequences. Mating is initiated by male's following. Then male performs courtship by turning in front of female (quick circle). If female accepts male's courtship, male holds female body by its anal fin to make their bodies close (clasping). After clasping, they spawn simultaneously (Figure 1A).[33–36] By taking advantage of this feature, we analyzed the sexual behaviors of the adult pairs of WT, *gpr54-1*$^{-/-}$, and *gpr54-2*$^{-/-}$ in two different conditions. First, we observed the pairs in the normal breeding (home) tanks to keep them as intact as possible (Condition 1, Figure 1B). We next performed a similar behavior analysis in the isolated tanks with black backgrounds to eliminate factors that may distract the fish from sexual behaviors, such as food, water flow, and dirt in their home tanks, and the presence of other fish in the adjacent tanks (Condition 2, Figure 1B). Left, right and rear sides of the isolated tanks were surrounded by black plastic or rubber plates to prevent possible disturbance by visual information around them as described above. In the behavior analysis using the same fish (170-250 dph), we separated the pairs the night (8-10 P.M.) before the behavior session by trapping the male in the handmade transparent plastic cup with small holes that allows water but not fish to pass through. On the next day (between 8 A.M. to 2 P.M.), the fish were paired again and could freely interact with each other. The video recordings (HDR-CX420 or GZ-E109) were performed for more than 30 min starting from just before the separation removal. After collecting the data under Condition 1, we next analyzed the behavior in the same way under Condition 2. In Condition 2, we transferred the pairs to the isolated tanks (approximately 22 cm x 13 cm x 13 cm), kept them separated, and left them for more than 5 min for habituation followed by recording. The tanks were filled with fresh water for breeding without water flow. To further examine the phenotypes of *gpr54-1*$^{-/-}$ with a larger sample size, we also performed a detailed analysis of sexual behavior by a ZebraCube system (ViewPoint) by comparing *gpr54-1*$^{+/+}$, *gpr54-1*$^{+/-}$, and *gpr54-1*$^{-/-}$ of a larger batch. It should be noted that ZebraCube has a recording chamber for up to eight pairing tanks with a ceiling-mounted camera, enabling us to record multiple pairs at the same time. In this ZebraCube protocol (Figure 3A), we first paired the sexually mature fish in the isolated tanks for more than 1 day. At the night before the recording, we separated the male and female with a transparent plastic plate following water change. On the recording day, we transferred the tanks into the recording chamber of ZebraCube and incubated them for more than 5 min for acclimation. A piece of white paper was placed between the tanks so that the fish in the adjacent tanks could not see each

other. After the recording started, we removed the separations to let the pairs interact and start sexual behaviors. The light/dark cycle inside the ZebraCube chamber was set the same as the breeding condition. We analyzed 30 min of the recording period after the separation removal and counted the spawned fertilized eggs of each pair after the recording. After all of the same genotype pairs were recorded, the *gpr54-1*$^{-/-}$ male/female was paired with its counterpart of the *gpr54-1*$^{+/+}$ and these swapped pairs were analyzed in the same protocol.

QUANTIFICATION AND STATISTICAL ANALYSIS

For the egg count analysis in Condition 1, we started counting from the day when 4 out of 8 pairs of WT pairs spawned (Day 1). In Condition 1, The average numbers of spawned eggs per day and during the entire counted period were used for the analysis for each line (WT, 8 pairs; *gpr54-1*$^{-/-}$, 5 pairs; and *gpr54-2*$^{-/-}$, 7 pairs). For the behavior analysis in Condition 1 and 2, the timing of clasping/spawning was observed by visual inspection of the recordings. As for the detailed behavior analysis recorded by ZebraCube, the timing of male courtship to female, the timing/duration of following and clasping, and the timing of spawning were annotated using the Excel macro for behavior analysis that we previously reported.[34] In each record, 0-30 min periods after the separation was removed were used for the analysis. Time to spawning was defined as the time spent until the pair finished spawning within 30 min. All the movie files were played at double speed by VLC media player (VideoLAN, Version 2.2.6; https://www.videolan.org). For statistical analysis, Mann-Whitney *U* test or Wilcoxon Signed-rank test for comparison of two groups and Steel test for that of multiple groups were used. We used log-rank test and Kruskal-Wallis test for the comparisons of survival curves showing time to spawning. Chi-square test with Bonferroni's correction was used for the data on the number of pairs with attack-like behavior. Significance values are reported as follows: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. All statistical analyses were performed by using Kyplot 6.0 software (Kyence, Tokyo, Japan) or GraphPad Prism 9.5.1 (GraphPad Software, Boston, MA).