Cholecystokinin is the follicle-stimulating hormone (FSH)-releasing hormone

Shun Kenny Uehara[1]†, Yuji Nishiike[2]†, Kazuki Maeda[1], Tomomi Karigo[3], Shigehiro Kuraku[4,5], Kataaki Okubo[2], Shinji Kanda[1*]

*Corresponding author (e-mail address: shinji@aori.u-tokyo.ac.jp)

Affiliations:

1 Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwa, Chiba, 277-8564, Japan
2 Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, 113-8657, Japan
3 Kennedy Krieger Institute, Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205
4 Laboratory for Phyloinformatics, RIKEN Center for Biosystems Dynamics Research, Kobe 650-0047, Japan
5 Department of Genomics and Evolutionary Biology, Molecular Life History Laboratory, National Institute of Genetics, Mishima 411-8540, Japan
† These authors contributed equally to this work.

Abstract:

Follicle-stimulating hormone (FSH) is a gonadotropin released from the pituitary essential for the regulation of folliculogenesis in vertebrates. Unlike mammals, the regulatory mechanism of FSH is unknown in teleosts because gonadotropin-releasing hormone (GnRH) was proven to be dispensable for FSH function. Here, we aimed to identify the FSH-releasing hormone (FSH-RH) by using a model teleost, medaka. We found that knockout of either cholecystokinin receptor, *cckbr1,* which is highly expressed in the FSH cells, or its ligand cholecystokinin (*ccka*/*cckb)*, which is expressed in the pituitary projecting neurons, drastically reduced the *fshb* mRNA expression in the pituitary, resulting in infertility. Also, *in vitro* experiments clearly demonstrated that cholecystokinin peptide increases $[Ca^{2+}]_i$ of FSH cells and *fshb* expression. We hypothesize that this system constitutes the long-missing FSH-RH in teleosts.

One-Sentence Summary:

Cholecystokinin is proven to be essential for FSH secretion in a non-mammalian vertebrate.

Main Text:

The pituitary gland secretes two essential hormones for reproduction, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Given the hypothesis of Geoffrey Harris, who is also known as the father of neuroendocrinology, the secretion of anterior pituitary hormones is mostly regulated by the hypothalamic neuropeptides (*1*). In lines with this hypothesis, it is believed that gonadotropins, including FSH and LH, are also regulated by hypothalamic neuropeptides. In 1971, the discovery of LH-releasing hormone (LH-RH) as the key regulator of LH release marked a significant breakthrough (*1-3*). Although LH-RH not only stimulates LH secretion but also has some effect on FSH release, the impact of LH-RH on FSH is relatively weaker compared to its effect on LH. Thus, the existence of another factor, FSH-releasing hormone (FSH-RH), was proposed to explain the stimulation of FSH secretion. Despite numerous research efforts, a distinct factor that strongly stimulates FSH release has yet to be identified (*4*). Consequently, there is a growing recognition that LH-RH may be the sole gonadotropin-releasing hormone (GnRH) in vertebrates, responsible for regulating both LH and FSH secretion(*5*).

However, recent knockout (KO) studies using non-mammalian vertebrates suggested that GnRH may not be the primary regulator of FSH release, because GnRH KO did not affect its FSH function in teleosts (*1, 6, 7*). Furthermore, there is evidence that GnRH exclusively acts on LH secretion, but only mildly acts upon FSH secretion (*1, 8-10*). Thus, the regulator of FSH other than GnRH should exist somewhere in the body in non-mammalian vertebrates. Given that gonadal substance should not be such a regulator, because gonadectomy does not suppress FSH expression (*1, 11, 12*), we speculated that the regulator of FSH, FSH-RH, should exist in the hypothalamus.

Here, we aimed to identify the FSH-RH in the hypothalamus. Among the non-mammalian vertebrates, we used medaka because of their amenability to genetic modification and plenty of knowledge in reproductive biology. Because we considered the identification of the receptor expressed in the FSH cells important, we first performed FSH-cell specific RNA-seq by collecting GFP-positive cells in the pituitary of FSH-GFP medaka. Among the metabolic receptors expressed, we identified cholecystokinin receptor B1 (Cckbr1) had the highest expression (Table S1). Therefore, we conducted the double *in situ* hybridization of *fshb* and *cckbr1* to examine the possible expression in the pituitary. From the results of double *in situ* hybridization, we proved that Cckbr1 is expressed in the FSH cells. (Figure 1A). To determine the functional essentiality of the identified receptor, we *cckbr1* KO and analyzed both the phenotype and the gonadosomatic index (GSI) of both ovaries and testes. The *cckbr1* KO showed a drastic decrease in the ovary as well as the testis. Especially in ovaries, a severe reduction in the gonadal weight was observed (Figure 1B). There was no apparent change in their growth except for the gonadal development (Figure S1). Additionally, histological analysis revealed that the ovary of the *cckbr1* KO includes only the previtellogenic oocyte. On the other hand, in males, although the testicular size is smaller, they showed normal spermatogenesis (Figure 1C). Estradiol (E2) content of the whole body was analyzed in *cckbr1* homo KO ($cckbr1^{+/+}$) and *cckbr1* homo KO ($cckbr1^{-/-}$) and indicated that the E2 concentration was significantly lower in $cckbr1^{-/-}$ compared to $cckbr1^{+/+}$ (Figure 1E). Finally, the pituitary of $cckbr1^{+/+}$ and $cckbr1^{-/-}$ was excised, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to examine the effects of knockouts on gonadotropin gene expression in females and males. In both sexes, it was revealed that *fshb* expression was by far lower, and *lhb* expression was lower in $cckbr1^{-/-}$ compared to $cckbr1^{+/+}$ while *tshb* expression did not change, suggesting that Cckbr1 is the strongest candidate for the receptor of FSH cells in the

pituitary (Figure 1F-K). Because FSH has indispensable roles in folliculogenesis in females whereas FSH KO males show spermatogenesis (*6*), the phenotype that *cckbr1* KO males showed fertility is reasonable. However, the drastic reduction of *fshb* mRNA in the pituitary in both sexes indicates that this mechanism is common in females and males. Thus, it strongly suggests that Cckbr1, which is expressed in FSH cells, is essential for the normal function of *fshb*. Note that *lhb* expression should have been secondarily reduced due to the reduction of estradiol, which strongly enhances the expression of *lhb* (*1, 11*).

After finding Cckbr1 as the prime candidate for the FSH-RH receptor, the ligands for Cckbr1 were explored. The ligands that bind to Cckbr1 are the *gastrin/cholecystokinin* family. In medaka, the gene family consists of cholecystokinin (CCK) a (*ccka*) and b (*cckb*), and gastrin (*13*). Because the highly conserved 8 amino acid peptide of CCK and gastrin are reported to show high biological activity, we used CCK8 (DY(SO_3H)LGWMDF-$_{NH2}$) and Gastrin8 (DY(SO_3H)RGWLDF-$_{NH2}$) for luciferase reporter assay. Note that both *ccka* and *cckb* genes result in the identical deduced 8 amino acid residue peptide, CCK8. In the luciferase assay, for all cyclic adenosine monophosphate (cAMP), Ca^{2+}, and Mitogen-activated Protein Kinase (MAPK) reporter systems, Cckbr1 was activated by both CCK8 and Gastrin8 in dose-dependent manners (Figure 2A). EC50 of these results is summarized in Table S2. Thus, CCK and gastrin can activate the receptor, Cckbr1 in a physiological concentration.

Next, *in situ* hybridization was performed using DIG-labelled *ccka*, *cckb*, and *gastrin* probes to analyze their possible expression in the brain. Here, *ccka* and *cckb* were expressed in various regions of the brain including the nuclei that contain the hypophysiotropic (pituitary-projecting) neurons, the pre-optic area (POA), and the nucleus ventralis tuberis (NVT) in the hypothalamus (Figure 2B). On the other hand, *gastrin* mRNA was not detected in any of the regions, which lead us to exclude gastrin from the candidate of FSH-RH. Since the expression of *ccka* and *cckb* in the NVT apparently overlapped, we examined the possible co-expression of *ccka* and *cckb* by double *in situ* hybridization. Here we found out that the vast majority of CCK neurons in NVT co-express *ccka* and *cckb* mRNA (Figure 2C). To observe their projection, we used a commercial cholecystokinin antibody. As expected from the classical study of comparative anatomy (*14, 15*), cell bodies (Figure 2D), as well as dense axonal fibers in the pituitary (Figure 2E), were labeled. Note that all the cell bodies and fibers were diminished in the double knockout of *ccka* and *cckb*; the cell bodies and the axonal projection in the pituitary are strongly suggested to be *ccka* and/or *cckb* expressing neurons (Figure S2). Next, to examine which population of these neurons project to the pituitary, double labeling of *ccka* in situ hybridization and retrograde tracer labeling from the pituitary was performed. From the sample that underwent biocytin tracer in the area where FSH cells are localized in the pituitary, we observed co-labeling of retrogradely labeled biocytin and *ccka* mRNA in the neurons in NVT. These results strongly suggest that the CCK neurons in the NVT are hypophysiotropic, projecting their axons to the pituitary FSH cells (Figure 2F).

To determine if FSH cells respond to CCK, Ca^{2+} imaging was performed. The pituitaries of females were isolated from the *fsh*: Inverse Pericam (*fsh*:IP) transgenic medaka (*16*), whose FSH cells express a genetically encoded Ca^{2+} indicator, Inverse Pericam. In FSH cells, 1 μM of CCK8 induced a rapid and strong Ca^{2+} rise, which suggests hormonal release from the FSH cells (Figure 3A). This effect was similarly observed in males as well (Figure S3). Furthermore, perfusion of various concentrations of CCK8 demonstrated that this action on FSH cells is in a dose-dependent manner (EC50=19nM, Figure 3B). Unlike FSH cells, Ca^{2+} imaging experiment in LH cells using *lh*:IP transgenic medaka did not show CCK8-induced Ca^{2+} response (Figure

3C, D). It was concluded that CCK acts upon FSH cells to induce hormonal release, but not on LH cells (Figure 3D).

We next examined if CCK stimulates the FSH expression in addition to the response by using *in vitro* experiment of isolated pituitary. The pituitaries of the females were isolated and incubated for 48 hours in a culture medium containing 0 nM, 10 nM, and 100 nM CCK8. After the incubation, the expression of *fshb*, *lhb*, and *tshb* mRNA was analyzed by qRT-PCR. It was revealed that the *fshb* expression dose-dependently increased (Figure 3E-G). In the presence of 10nM or 100nM CCK8 during the incubation, *fshb* increases up to around 10 folds compared to 0nM control (Figure 3E). Expression of *lhb* mRNA showed a slight increase with no statistical difference (Figure 3F). Considering the results of Ca^{2+} imaging and double in situ hybridization, LH cells do not likely to possess CCK receptors. Thus, this effect may be some indirect effect. Also, CCK8 did not affect the *tshb* expression (Figure 3G). A similar procedure was conducted by using the pituitary of *cckbr1*$^{+/-}$ and *cckbr1*$^{-/-}$. As expected, CCK8 increased *fshb* expression in the *cckbr1*$^{+/-}$ pituitary but not in the *cckbr1*$^{-/-}$ pituitary (Figure 3H). These results support that the action of CCK on FSH cell is mediated by Cckbr1. Similar to the results in the wild type, CCK8 did not significantly increase the expression of *lhb* or *tshb* in both genotypes (Figure 3I, 3J).

From the results above, CCK was proven to strongly stimulate FSH secretion, both in hormone synthesis and release. To examine the essentiality of intrinsic CCK, we generated knockouts of the two genes for CCK, *ccka* and *cckb* by CRISPR. Interestingly, although either single knockout of *ccka* or *cckb* showed normal phenotype, the double knockout showed severe change in phenotype (Figure 4, S4). Overall phenotype of the double knockout was similar to that of *cckbr1* knockout. The double knockout showed significantly smaller ovarian size (Figure 4A). As explained in the receptor knockout results, since FSH is not essential for male reproduction, the effects on testes were milder (Figure 4B). As expected, the pituitary mRNA expression of *fshb* in *ccka/cckb* double knockout was far smaller than those of the other genotypes (Figure 4C). Also, *lhb* expression also decreased in the double knockout, whereas *tshb* did not show difference among all genotypes (Figure 4E, F). The fact that only double knockout showed severe phenotype suggests that *ccka* and *cckb* have redundancy in their function of FSH regulation, which is also consistent with the fact that *ccka* and *cckb* are co-localize in the NVT hypophysiotropic neurons (Figure 2).

In the present study, we identified hypothalamic CCK as the most potent regulator of FSH release. CCK and its receptor is widely conserved in vertebrates (*13*). CCK was originally found as an intestinal peptide, and its function has been extensively studied in mammalian digestive system both in basic science and medical area. Since the knockout medaka of *ccka/cckb* did not show deficiency in their growth (Figure S5), the most prominent function of CCK system is not the one related to digestive system at least in medaka. Instead, all the evidence demonstrated in the present study suggest that CCK in medaka falls into criteria of imaginary FSH-RH.

Although the possibility of CCK being the FSH-RH is demonstrated in medaka, there are some lines of evidence to suggest this mechanism can be applied to teleosts in general. In teleosts, there have been some immunohistochemical studies suggesting the existence of CCK immunoreactive neurons that project to the pituitary in hypothalamus (*14, 15*). Also, recent single cell RNA-seq study indicated that FSH expressing cells showed high expression of CCK receptor in tilapia (*1, 17*). Additionally, the fact shown in the present study that hypophysiotropic CCK neurons co-expressed the paralogous *ccka* and *cckb* in medaka strongly suggests that the enhancer that enabled the expression of CCK in the hypophysiotropic neurons had been acquired before the divergence of *ccka* and *cckb* genes, which occurred in the teleost specific whole genome duplication (*18*). This can be also interpreted that ancestral vertebrates before emergence

of teleosts may also possessed hypophysiotropic CCK neurons in their hypothalamus. These lines of evidence suggests that the mechanism proved in this study is widely conserved at least among teleosts. On the other hand, in mammals, the fact that CCK agonist/antagonist are widely used in medical use (*19*).without showing strong side effects on reproduction, it is unlikely that this mechanism is not prominent in mammals, if exists. Interestingly, teleosts have separate FSH and LH cells in the pituitary, whereas mammals possess one gonadotroph that secrete both FSH and LH (*11, 20-23*). Thus, it should be an interesting theme to identify when this system was acquired or lost in the ancestors of teleosts/mammals.

The physiological, morphological, and knockout results in the present study led us to hypothesize that CCK is the FSH-RH in teleosts. We summarized the results and the hypothesis of the dual regulatory systems of gonadotropins, FSH-RH and LH-RH in Figure 4G. The long-lasting solid hypothesis that GnRH is the exclusive regulator of gonadotropin release in vertebrates is now updated by the finding in a teleost.

References and Notes

1. G. Raisman, AN URGE TO EXPLAIN THE INCOMPREHENSIBLE: Geoffrey Harris and the Discovery of the Neural Control of the Pituitary Gland. *Annu Rev Neurosci* **20**, 533-566 (1997).
2. M. Amoss *et al.*, Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. *Biochem Biophys Res Commun* **44**, 205-210 (1971).
3. H. Matsuo, Y. Baba, R. M. Nair, A. Arimura, A. V. Schally, Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem Biophys Res Commun* **43**, 1334-1339 (1971).
4. M. IGARASHI, S. M. MCCANN, Endocrinology: a hypothalamic follicle stimulating hormone-releasing factor. *Obstetrical & Gynecological Survey* **19**, 838-839 (1964).
5. A. E. Herbison, in *Knobil and Neill's Physiology of Reproduction, 4th Editon,* T. M. Plant, A. J. Zeleznik, Eds. (Academic Press, 2014), pp. 399-467.
6. A. Takahashi, S. Kanda, T. Abe, Y. Oka, Evolution of the hypothalamic-pituitary-gonadal axis regulation in vertebrates revealed by knockout medaka. *Endocrinology* **157**, 3994-4002 (2016).
7. Z. Zhang, B. Zhu, W. Ge, Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. *Mol Endocrinol* **29**, 76-98 (2015).
8. M. Nyuji, I. Yamamoto, K. Hamada, Y. Kazeto, K. Okuzawa, Effect of GnRHa on plasma levels of Fsh and Lh in the female greater amberjack *Seriola dumerili*. *J Fish Biol* **95**, 1350-1354 (2019).
9. K. Okuzawa *et al.*, Development of a homologous radioimmunoassay for red seabream follicle stimulating hormone and regulation of gonadotropins by GnRH in red seabream, Pagrus major. *Gen Comp Endocrinol* **239**, 4-12 (2016).
10. R. Fontaine, M. R. Royan, K. von Krogh, F. A. Weltzien, D. M. Baker, Direct and indirect effects of sex steroids on gonadotrope cell plasticity in the teleost fish pituitary. *Front Endocrinol (Lausanne)* **11**, 605068 (2020).
11. S. Kanda, K. Okubo, Y. Oka, Differential regulation of the luteinizing hormone genes in teleosts and tetrapods due to their distinct genomic environments - Insights into gonadotropin beta subunit

evolution. *Gen Comp Endocrinol* **173**, 253-258 (2011).
12. D. Kayo, B. Zempo, S. Tomihara, Y. Oka, S. Kanda, Gene knockout analysis reveals essentiality of estrogen receptor beta1 (Esr2a) for female reproduction in medaka. *Sci Rep* **9**, 8868 (2019).
13. T. Sekiguchi, *Cholecystokinin*. Y. Takei, H. Ando, K. Tsutsui, Eds., Handbook of hormones: comparative endocrinology for basic and clinical research (2016), pp. 177-178.
14. T. F. Batten, M. L. Cambre, L. Moons, F. Vandesande, Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, *Poecilia latipinna*. *J Comp Neurol* **302**, 893-919 (1990).
15. B. A. Himick, R. E. Peter, CCK/gastrin-like immunoreactivity in brain and gut, and CCK suppression of feeding in goldfish. *American Journal of Physiology-Regulatory, Integrative and Comp Physiol* **267**, 841-851 (1994).
16. T. Karigo *et al.*, Whole brain-pituitary in vitro preparation of the transgenic medaka (Oryzias latipes) as a tool for analyzing the differential regulatory mechanisms of LH and FSH release. *Endocrinology* **155**, 536-547 (2014).
17. L. Hollander-Cohen, M. Golan, B. Levavi-Sivan, Differential Regulation of Gonadotropins as Revealed by Transcriptomes of Distinct LH and FSH Cells of Fish Pituitary. *Int J Mol Sci* **22**, 6478 (2021).
18. D. Dupré, Evolution of the gastrin–cholecystokinin gene family revealed by synteny analysis. *Gen Comp Endocrinol* **195**, 164-173 (2014).
19. J. C. Branum, R. S. Kline, C. S. Whisnant, The effects of neurotransmitter antagonists and glucose on luteinizing hormone secretion in growth-restricted wethers. *Animal Reproduction Science* **45**, 263-272 (1997).
20. L. A. Miranda, C. A. Strussmann, G. M. Somoza, Immunocytochemical identification of GtH1 and GtH2 cells during the temperature-sensitive period for sex determination in pejerrey, *Odontesthes bonariensis*. *Gen Comp Endocrinol* **124**, 45-52 (2001).
21. M. Nozaki *et al.*, Salmonid pituitary gonadotrophs. I. Distinct cellular distributions of two gonadotropins, GTH I and GTH II. *Gen Comp Endocrinol* **77**, 348-357 (1990).
22. Z. Yaron *et al.*, Regulation of fish gonadotropins. *Int Rev Cytol* **225**, 131-185 (2003).
23. S. Kanda, Evolution of the regulatory mechanisms for the hypothalamic-pituitary-gonadal axis in vertebrates-hypothesis from a comparative view. *Gen Comp Endocrinol* **284**, 113075 (2019).

Acknowledgments: We thank Dr. Shin-ichi Higashijima (National Institute for Basic Biology, Japan) for providing plasmid containing medaka heat shock promoter sequence. We are also grateful to Drs. Daichi Kayo (Tohoku University, Japan) for helpful discussion and comments. We also thank Dr. Soma Tomihara (Nagahama Institute of Bio-Science and Technology, Japan) for help in construction of plasmid DNA.

Funding:
Japan Society for the Promotion of Science 23H02306 (SKa, KO)
Japan Society for the Promotion of Science 18K19323 (SKa)
Japan Society for the Promotion of Science 18H04881 (SKa)

Research grants in the Natural Sciences of Mitsubishi Foundation (SKa)
Grant for Basic Science Research Projects of Sumitomo Foundation (SKa)

Author contributions:

Conceptualization: SKU, YN, KO, SKa

Methodology: TK, SKu

Investigation: SKU, YN, KM, SKa

Visualization: SKU, YN, SKa

Funding acquisition: KO, SKa

Project administration: SKa

Supervision: KO, SKa

Writing – original draft: SKU, YN, SKa

Writing – review & editing: SKU, YN, TK, KO, SKa

Competing interests:

KO and SKa (The University of Tokyo) have filed a patent related to this study (Japanese Patent Application No. 2023-25897).

Data and materials availability:

All data are available in the main text or the supplementary materials.

Supplementary Materials

Materials and Methods

Figs. S1 to S6

Tables S1 to S2

Figure 1. CCKBR1 expressed in the pituitary FSH cells is essential for normal FSH expression. (**A**) Double *in situ* hybridization indicates that *fsh* (magenta) and *cckbr1* (green) are co-expressed in the pituitary of medaka. (**B –C**) Knockout of *cckbr1* results in abnormally smaller gonads in both male and female. Gross morphology (B) and histological section of (C) ovary and testis are shown in *cckbr1*$^{+/+}$ and *cckbr1*$^{-/-}$ medaka. (**D**) The ovarian size of *cckbr1* knockout is significantly smaller than that of wild type (**E**) Estradiol content of females is significantly lower than that of wildtype. (**F –K**) Expression of *fshb* (F, I), *lhb* (G, J), and *tshb* (H, K) in the pituitary was analyzed by quantitative RT-PCR. *fshb* mRNA is drastically reduced in *cckbr1*$^{-/-}$ in both females (F) and males (I). *lhb* mRNA is also reduced in *cckbr1*$^{-/-}$, which may be due to the reduction of serum estradiol in females (G) whereas it was not affect by knockout in males (J). There is no significant change in *tshb* mRNA between *cckbr1*$^{+/-}$ and *cckbr1*$^{-/-}$ in both females (H) and males (K). ***P<0.001, N.S. not significant.

Figure 2. Hypophysiotropic Neurons Expressing *ccka* and *cckb* exist in the Hypothalamus. (**A**) Luciferase assay revealed that cholecystokinin family peptides, CCK8 and Gastrin8, dose-

dependently increase cAMP, Ca^{2+}, and MAPK pathways in Hela cells expressing Cckbr1. (**B**) *in situ* hybridization indicates that *ccka* and *cckb* mRNA are expressed in various brain regions including the nuclei known to be containing hypophysiotropic neurons, pre-optic area (POA), and nucleus ventralis tuberis (NVT) of the hypothalamus. On the other hand, *gastrin* mRNA is not expressed in any brain regions. (**C**) Double *in situ* hybridization reveals co-expression of *ccka* (magenta) *cckb* (green) mRNA expression in the NVT. (**D –E**) Immunohistochemistry (IHC) using CCK-specific antibody labeled CCK neurons in the NVT (D) and the axonal fibers in the pituitary (E). (**F**) Dual labeling of retrogradely induced biocytin (green) from pituitary and *ccka* mRNA (magenta) show co-expression in the NVT, suggesting that CCK neurons in the NVT population are the ones that project their axons to the pituitary.

Figure 3. CCK robustly increases both $[Ca^{2+}]_i$, which causes the hormonal release, and mRNA expression of *fshb*. (**A**) Ca^{2+} imaging of FSH cells indicates that the application of 1 μM of CCK8 increases $[Ca^{2+}]_i$ of FSH cells. (**B**) CCK8 dose-dependently increases $[Ca^{2+}]_i$ of FSH cells. (**C**) Ca^{2+} imaging of LH cells indicates that the application of 1 μm of CCK8 does not alter $[Ca^{2+}]_i$ of LH cells. (**D**) Repetitive trials also did not show the effect of CCK8 on $[Ca^{2+}]_i$ of LH cells. (**E –G**) *in vitro* incubation with CCK8 for 48 hours, *fshb* is robustly increased among the pituitary glycoprotein hormone genes. (E) CCK dose-dependently increases the *fshb* expression. (F) CCK8 slightly increases *lhb* expression with no statistical difference. (G) CCK8 does not change *tshb* expression. [***P<0.001, N.S. not significant, Dunnett's test.]. (**H –J**) This in vitro incubation is mediated by Cckbr1 receptor. The effects of the CCK8 clearly disappear in the experiments using $cckbr1^{-/-}$ pituitary whereas those of $cckbr1^{+/-}$ show the same tendency as those of wild type (H, *fshb*; I, *lhb*; J, *tshb*). [**P<0.01, N.S. not significant, Student's T test.]

Figure 4. CCK is essential for FSH function. (**A –B**) Gross morphology of the ovaries (A) and testes (B) of CCK KO medaka reveals a decrease in gonads only when *ccka* and *cckb* are both knocked out. (**C**)The gonadosomatic index (GSI) reveals a decrease in ovarian size in $ccka^{-/-}$;$cckb^{-/-}$. (**D**) qRT-PCR indicates that *fshb* expression also drastically decreases when *ccka* and *cckb* are both knocked out. (**E**)*lhb* expression also decreased in $ccka^{-/-}$;$cckb^{-/-}$ probably as a secondary effect from the reduction of estradiol from the ovary [**P<0.01, N.S. not significant, Dunnett's test]. (**F**) *tshb* expression did not change even when *ccka* and *cckb* were knocked out [**P<0.01, N.S. not significant, Dunnett's test]. (**G**) The schematic illustration of the working hypothesis. The present study identified that CCK is the hypothalamic FSH-RH in teleosts, which stimulates the expression and release of FSH to induce folliculogenesis. After folliculogenesis is completed, GnRH (LH-RH) induced LH induces final oocyte maturation followed by ovulation.

Figure 1

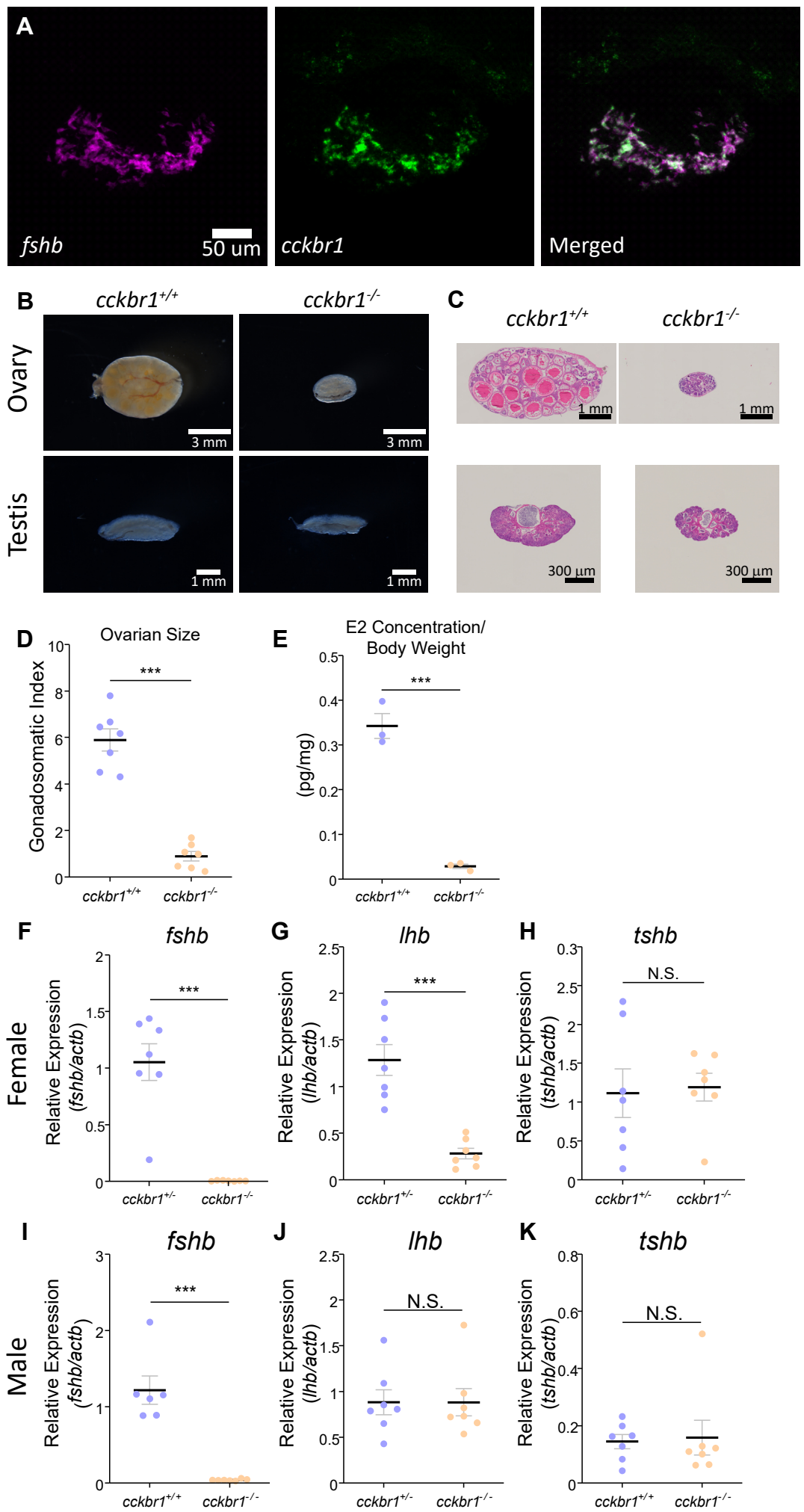

A
fshb
50 um
cckbr1
Merged
B
cckbr1+/+
cckbr1-/-
Ovary
Testis
3 mm
1 mm
C
cckbr1+/+
cckbr1-/-
1 mm
300 µm
D
Ovarian Size
Gonadosomatic Index
***
cckbr1+/+
cckbr1-/-
E
E2 Concentration/
Body Weight
(pg/mg)
***
F
fshb
Female
Relative Expression
(fshb/actb)
***
cckbr1+/-
cckbr1-/-
G
lhb
Relative Expression
(lhb/actb)
***
H
tshb
Relative Expression
(tshb/actb)
N.S.
I
fshb
Male
Relative Expression
(fshb/actb)
***
J
lhb
Relative Expression
(lhb/actb)
N.S.
K
tshb
Relative Expression
(tshb/actb)
N.S.

Figure2

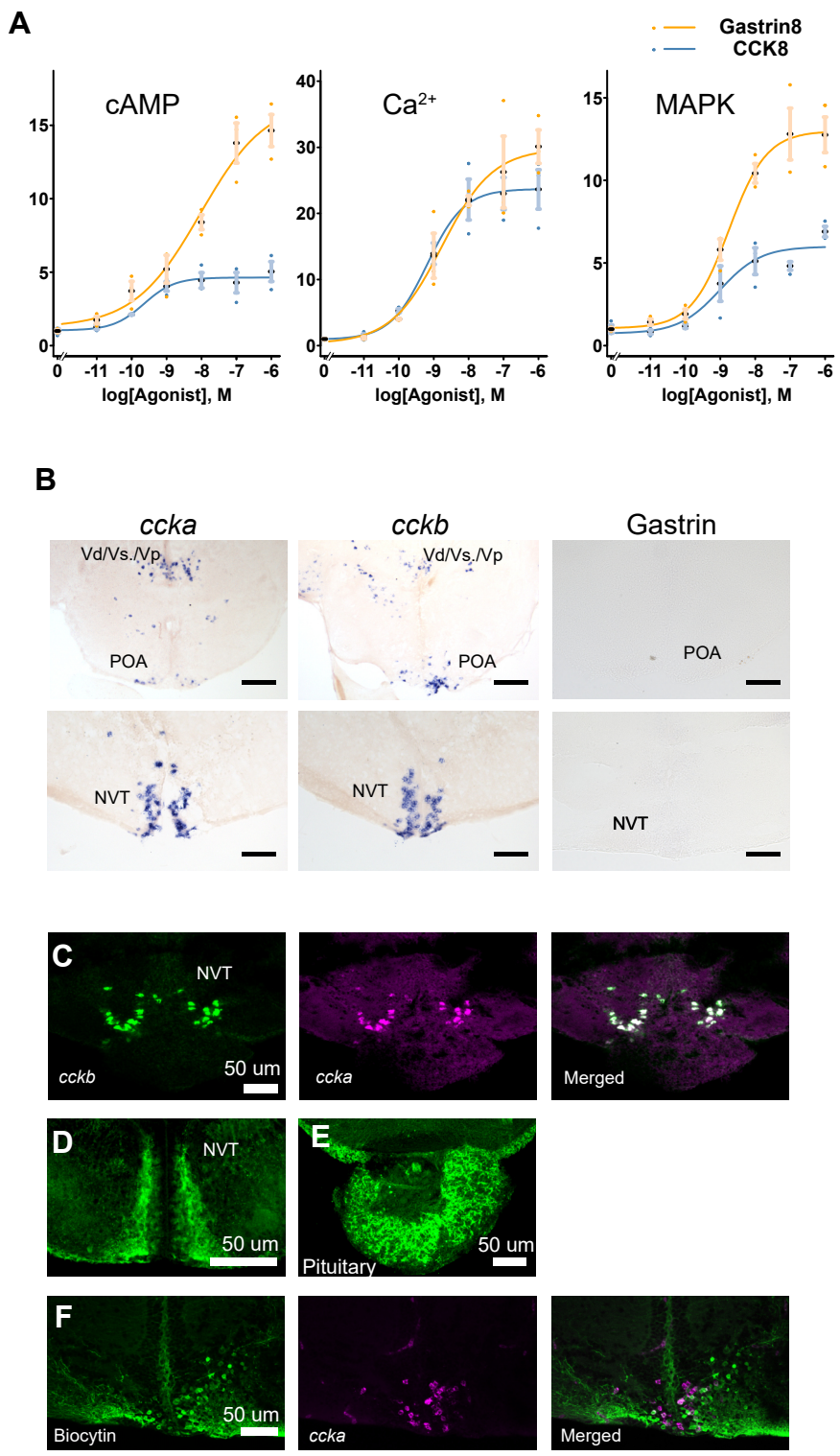
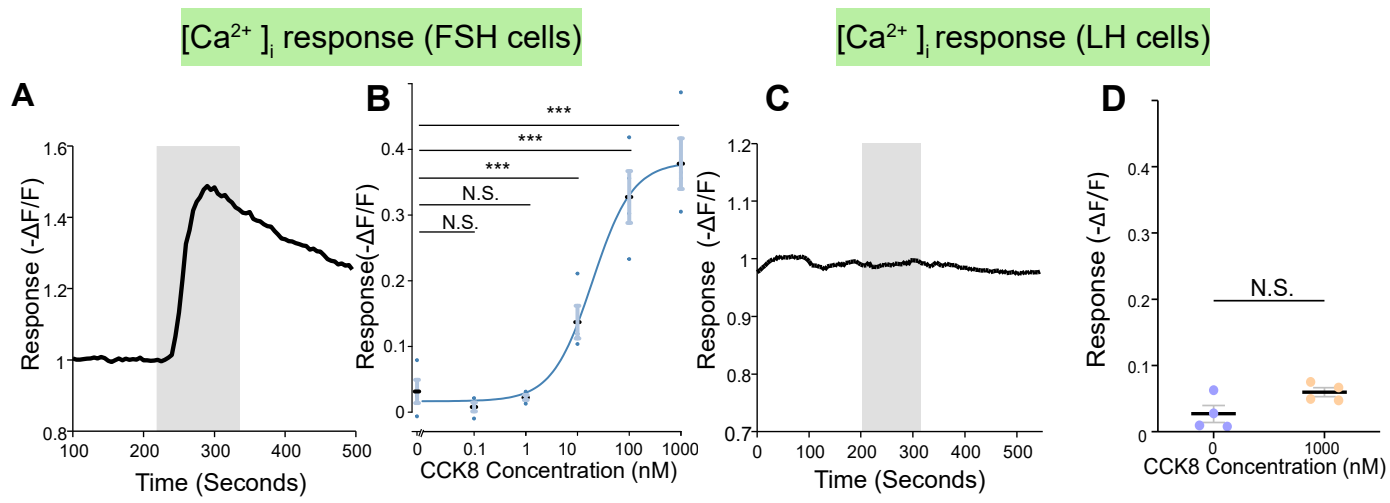

A
Gastrin8
CCK8
cAMP
Ca2+
MAPK
log[Agonist], M
B
cckb
Gastrin
Vd/Vs./Vp
POA
NVT
C
D
E
F
50 um
Pituitary
Biocytin
Merged

Figure 3



Expressoin of pituitary genes after 48 hours application of CCK8 *in vitro*

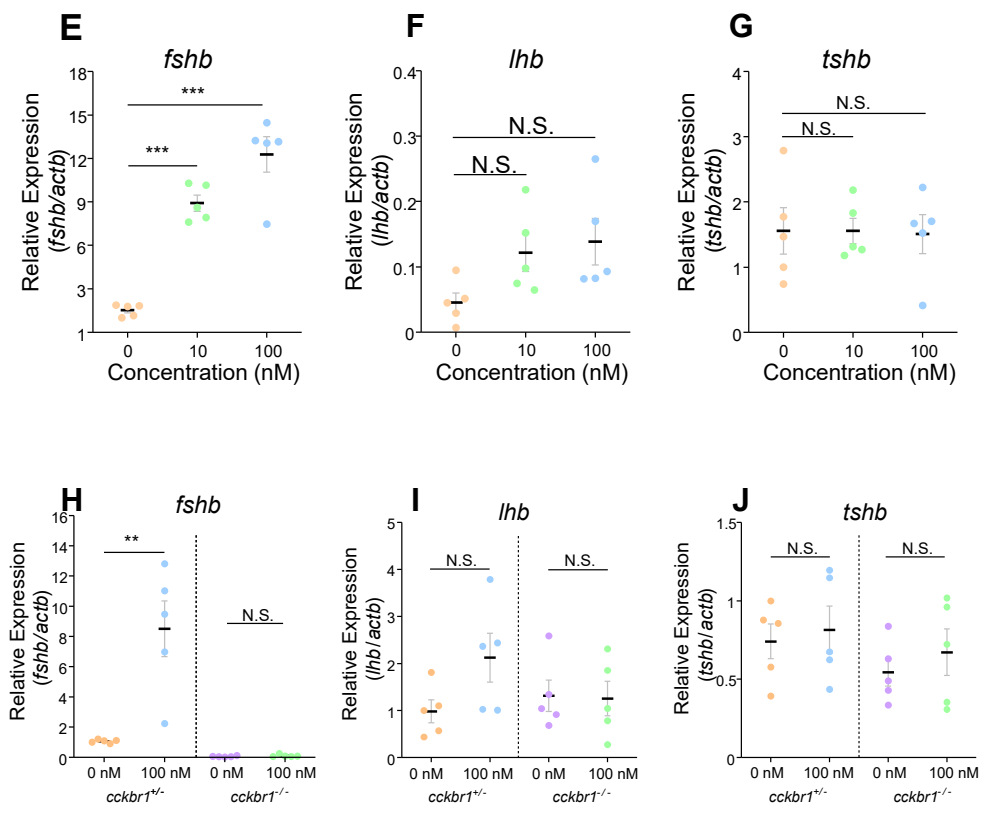
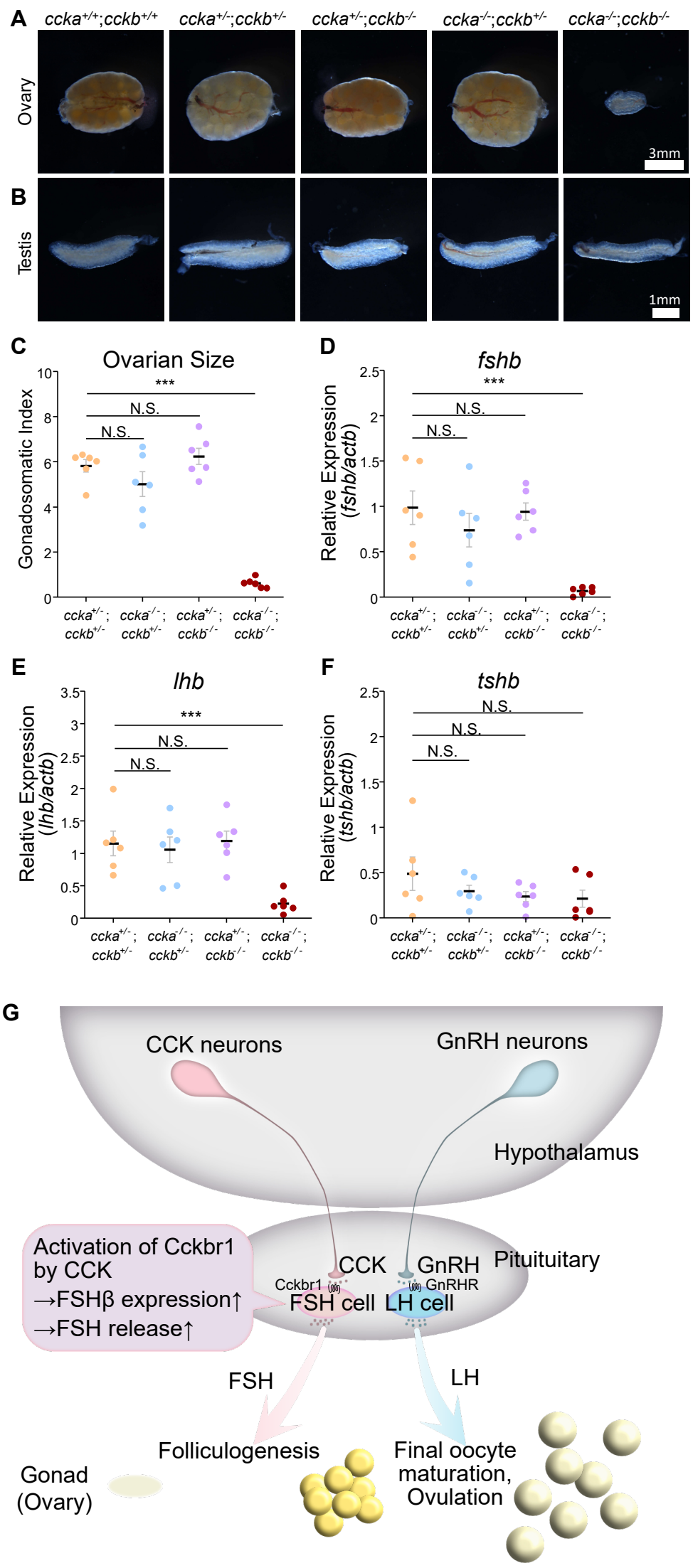


Figure 4


A
ccka+/+;cckb+/+
ccka+/-;cckb+/-
ccka+/-;cckb-/-
ccka-/-;cckb+/-
ccka-/-;cckb-/-
Ovary
3mm
B
Testis
1mm
C
Ovarian Size
Gonadosomatic Index
***
N.S.
N.S.
D
fshb
Relative Expression (fshb/actb)
E
lhb
Relative Expression (lhb/actb)
F
tshb
Relative Expression (tshb/actb)
G
CCK neurons
GnRH neurons
Hypothalamus
Pituitary
CCK
GnRH
Cckbr1
GnRHR
FSH cell
LH cell
Activation of Cckbr1 by CCK
→FSHβ expression↑
→FSH release↑
FSH
LH
Gonad (Ovary)
Folliculogenesis
Final oocyte maturation, Ovulation