Cholecystokinin (CCK) and its receptors (CCK1R and CCK2R) in chickens: functional analysis and tissue expression

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ABSTRACT Cholecystokinin (**CCK**) is widely distributed in the gastrointestinal tract and central nervous system, regulating a range of physiological functions by activating its receptors (CCK1R and CCK2R). Compared to those in mammals, the *CCK* gene and its receptors have already been cloned in various birds, such as chickens. However, knowledge regarding their functionality and tissue expression is limited. In this study, we examined the expression of *CCK* and its 2 receptors in chicken tissues. In addition, the functionality of the 2 receptors was investigated. Using 3 cell-based luciferase reporter systems and western blots, we demonstrated that chicken (c-) CCK1R could be potently activated by cCCK-8S but not cCCK-4, whereas cCCK2R could be activated by cCCK-8S and cCCK-4 with similar efficiency. Using RNA-sequencing, we revealed that *cCCK* is abundantly expressed in the testis, ileum, and several brain regions (cerebrum, midbrain, cerebellum, hindbrain, and hypothalamus). The abundant expression of CCK in the hypothalamus was further supported by immunofluorescence. In addition, *cCCK1R* is highly expressed in the pancreas and moderately expressed in various intestinal regions (ileum, cecum, and rectum) and the pituitary gland, whereas *cCCK2R* expression is primarily restricted to the brain. Our data reveal the differential specificities of CCK receptors for various CCK peptides. In combination with the differential tissue distribution of *CCK* and its receptors, the present study helps to understanding the physiological functions of CCK/CCKRs in birds.

Key words: chicken, cholecystokinin, CCK1R, CCK2R

2023 Poultry Science 102:102273
https://doi.org/10.1016/j.psj.2022.102273

INTRODUCTION

Cholecystokinin (**CCK**), a gastrointestinal hormone, is predominantly synthesized and secreted from endocrine I cells in the small intestine and regulates multiple digestive functions, including gallbladder contraction (Liddle et al., 1985), pancreatic enzyme secretion (Li and Owyang, 1993), intestinal motility (Grider, 1994), and food intake (Little et al., 2005; Cawthon and Claire, 2021). On the other hand, as a neurotransmitter, CCK is also abundantly expressed in the central nervous system (**CNS**) and peripheral neurons, and participates in anxiety and depression (Bowers et al., 2012; Vialou et al., 2014), panic disorder (Zwanzger et al., 2012), learning and memory processes (Yang et al., 2013).

To date, all bioactive CCK peptides of different lengths (e.g., CCK-58, CCK-33, CCK-22, CCK-8, and CCK-4) are processed from proCCK in a species-specific and tissue-specific manner (Beinfeld, 2003; Rehfeld, 2006). For instance, intestine enteroendocrine cells primarily express CCK-58 and CCK-33 (Rehfeld et al., 2001; Reeve et al., 2003), whereas brain neurons mostly express CCK-8 (Dockray et al., 1978; Beinfeld, 1981). Except for CCK-4, all CCK peptides include a conserved sulfation site at the seventh amino acid residue (tyrosine) from the C-terminus, which is essential for CCK function (Huang et al., 1989). Moreover, the sulfated CCK-8 peptide (CCK-8S) is the major form in vivo and exhibits all biological functions of CCK (Rehfeld, 2017).

In mammals, the biological activities of CCK are mediated by 2 G protein-coupled receptors, CCK1R (previously known as CCKAR) and CCK2R (previously known as CCKBR) (Noble et al., 1999; Dufresne et al., 2006). In response to ligand activation, both receptors can activate multiple signaling pathways, resulting in calcium mobilization and the MAPK/ERK signaling cascade (Zeng et al., 2020). Interestingly, CCK1R is more efficiently activated by sulfated CCK peptides than non-sulfated peptides, whereas CCK2R is activated by both sulfated and non-sulfated CCK peptides with similar affinity (Miller and Gao, 2008). In addition, the expression patterns of *CCK1R* and *CCK2R* are

Received April 26, 2022.
Accepted October 15, 2022.

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different, suggesting that CCK has distinct activities in target tissues (Miyasaka and Funakoshi, 2003). For instance, *CCK1R* is predominantly expressed in the gastrointestinal tract and brain subregions and is hence implicated in the control of digestive function and satiety (Weatherford et al., 1992; Wang et al., 2004). Unlike *CCK1R, CCK2R* is abundantly and widely expressed in the CNS, where it regulates anxiety, learning and memory (Noble and Roques, 1999; Sebret et al., 1999; Wang et al., 2005).

In addition to mammals, *CCK* has been identified in non-mammalian vertebrates, including birds (Jønson et al., 2000; Xie et al., 2016). In chickens, *CCK* and its receptors (*CCK1R* and *CCK2R*) have been cloned (Jønson et al., 2000; Nilsson et al., 2003; Ohkubo et al., 2007). As in mammals, CCK has been linked to food intake and energy balance (Furuse et al., 2000; Rodriguez-Sinovas et al., 1997; Tachibana et al., 2012). In addition, *CCK* is also expressed in the cerebrum and midbrain, and participates in imprinting behavior and crowing in chickens (Maekawa et al., 2007; Shimmura et al., 2019). However, how CCKs play their role through their receptors remains largely unknown. Therefore, using chicken as an animal model, 3 luciferase receptor systems were employed to examine how the 2 CCK peptides (cCCK-8S and cCCK-4) activate the 2 CCK receptors (cCCK1R and cCCK2R). RNA-seq analysis was used to determine the tissue expression of *CCK* and its receptors in multiple chicken tissues. Undoubtedly, our results will help shed light on the signaling pathway of chicken CCK receptors, providing novel information for CCK peptides in birds.

MATERIALS AND METHODS

Chemicals, Primers, Peptides, and Antibodies

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and restriction enzymes were obtained from TaKaRa (Dalian, China). All primers used in this study were synthesized by Youkang Biotechnology (Chengdu, China) and are listed in Supplementary Table 1. Chicken CCK-4 was synthesized using solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). The purity of the synthesized peptide was greater than 95% (analyzed by HPLC), and its structure was verified by mass spectrometry. (Tyr (SO_3H)^{27}) Cholecystokinin fragment 26-33 amide (CCK-8S, C2175-250UG) was purchased from Sigma-Aldrich. The antibodies used for western blotting in this study included phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) rabbit mAb (#9101), phospho-CREB (Ser133) (87G3) rabbit mAb (#9198), β-actin (13E5) rabbit mAb (#4970) and horseradish peroxidase (**HRP**)-conjugated goat anti-rabbit IgG antibody (#7074), all of which were purchased from Cell Signaling Technology (Danvers, MA). In addition, the rabbit polyclonal antibody to CCK (ab27441) was purchased from Abcam (Cambridge, UK). Alexa Fluor-488 goat anti-rabbit IgG antibody (A11008) and DAPI (D1306) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA).

Sequence Alignment

We searched protein sequences of CCK in several vertebrates, including chicken (*Gallus gallus*, NP_001001741), pigeon (*Columba livia*, NP_001290363), zebra finch (*Taeniopygia guttata*, NP_001232081), human (*Homo sapiens*, NP_001167609), mouse (*Mus musculus*, NP_112438), and pig (*Sus scrofa*, NP_999402). Amino acid sequences were aligned using the ClustalW program (BioEdit) (Hall et al., 2011). The signal peptide was labeled according to previous studies (Jønson et al., 2000; Xie et al., 2016).

Functional Characterization of Chicken CCK1R and CCK2R

According to the cDNA sequences of chicken *CCK1R* (NM_001081501) and *CCK2R* (NM_001001742) in previous reports (Nilsson et al., 2003; Ohkubo et al., 2007), gene-specific primers were designed to amplify the complete open reading frame (**ORF**) of *CCK1R* and *CCK2R* from the chicken brain. The amplified PCR products of *CCK1R* and *CCK2R* were cloned into the pcDNA3.1 (+) eukaryotic expression vector (Invitrogen, Carlsbad, CA) and sequenced (Youkang).

Chinese hamster ovary (**CHO**) cells were cultured in Dulbecco's Modified Eagle's Medium (**DMEM**) supplemented with 10% (vol/vol) fetal bovine serum (HyClone, Logan, UT), 100 U/mL penicillin G, and 100 g/mL streptomycin in 90-mm culture dishes (NUNC, Rochester, NY) and incubated at 37°C with 5% CO_2.

According to the methods described in our previous studies (Fang et al., 2021; Wan et al., 2022), the functionality and signaling property of chicken CCK1R and CCK2R were examined in CHO cells using 3 cell-based luciferase reporter systems (pGL3-CRE-Luciferase, pGL3-NFAT-RE-Luciferase, and pGL4-SRE-Luciferase reporter systems), which can monitor cAMP/PKA signaling pathway, calcium mobilization, and MAPK/ERK signaling pathway, respectively. Briefly, CHO cells were seeded one day prior to transfection in 6-well plates. The cells were then transfected with a mixture containing 250 ng of receptor expression plasmid (cCCK1R, cCCK2R, or empty pcDNA3.1 vector), 750 ng of luciferase reporter construct (pGL3-CRE-Luciferase, pGL3-NFAT-RE-Luciferase or pGL4-SRE-Luciferase), 2 μL of jetPRIME (Polyplus transfection, Illkirch, France) and 200 μL of jetPRIME transfection buffer. After 24 h, the cells were sub-cultured in 96-well plates and grown for 24 h prior to peptide treatment. After removal of medium from 96-well plates, the cells were treated with 100 μL DMEM containing the desired dosages of peptides (cCCK-8S/cCCK-4: 10^{-12} M - 10^{-6} M) for 6 h. Finally, CHO cells were lysed with 1 × cell culture lysis buffer for luciferase assay (Promega,

Madison, WI). The luciferase activity of the cell lysate was measured by a Multimode microplate Reader (TriStar LB941, Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's instruction.

Western Blot

To investigate whether the activation of cCCK1R or cCCK2R can enhance ERK1/2 (44/42 kDa) and CREB (43 kDa) phosphorylation, CHO cells were transfected with 1000 ng cCCK1R (or cCCK2R) expression plasmid and grown in 24-well plates at 37 °C. After 24 h of transfection, cells were treated with cCCK-8S (10 nM) or cCCK-4 (10 nM) for 10 min, respectively. After removal of the medium, cells were lysed and used for western blot detection of phosphorylated ERK1/2 (pERK1/2) and CREB (pCREB), as described in our previous studies (Wu et al., 2019; Jiang et al., 2022). Additionally, the levels of β-actin protein were also examined and used as internal controls in each experiment.

Tissue Expression Analysis of Chicken CCK, CCK1R, and CCK2R

Adult chickens (Lohmann layer, 1-yr-old) employed in this study were purchased from local commercial companies in Chengdu. All animal experimental protocols performed in this study were approved by the Animal Ethics Committee of the College of Life Sciences, Sichuan University, China, and the assurance number is 20210308008 (8 March 2021).

Six adult chickens (3 males and 3 females) were sacrificed, and various tissues including the cerebrum, midbrain, cerebellum, hindbrain, hypothalamus, spinal cord, retina, pineal body, pituitary gland, heart, liver, spleen, lung, kidney, bursa of Fabritius, skin, muscle, visceral fat, abdominal fat, thymus gland, thyroid gland, parathyroid gland, pancreas, adrenal gland, tongue, proventriculus, gizzard, crop, duodenum, jejunum, ileum, cecum, rectum, testis, ovary, uterus, infundibulum, and magnum were collected. Total RNA was extracted from chicken tissues using RNAzol reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and dissolved in diethylpyrocarbonate (**DEPC**)-treated H_2O. RNA samples were delivered to BGI (Wuhan, China) for RNA-sequencing (**RNA-seq**).

The RNA-seq data used in present study has been deposited in the CNGB Sequence Archive (**CNSA**) (Guo et al., 2020) of China National GeneBank DataBase (**CNGBdb**) (Chen et al., 2020) with accession number CNP0003404. In addition, the RNA-seq data have been published in a preprint (Zhang et al., 2022). An open-access chicken tissue gene expression atlas (**TGEA**) was presented based on the RNA-seq data. Expression estimates from the chicken TGEA are available via the Shiny Server (https://chickenatlas.avianscu.com/), which enables the searching for genes and downloading of high-solution figures, allows access to raw data, and provides links to external resources. In the present study, the relative abundance of chicken *CCK, CCK1R*, and *CCK2R* transcripts was expressed as transcripts per million (**TPM**).

Immunofluorescence Staining

To examine the distribution of CCK in chicken hypothalamus, immunofluorescence (**IF**) was used in our experiments, as described in our previous study (Liu et al., 2022). Whole brains collected from adult female chickens were fixed in 4% paraformaldehyde and embedded in paraffin for IF. Then, chicken hypothalamus sections of 5-μm thickness were obtained using a microtome. A rabbit polyclonal antibody against CCK (1:200) was used to probe the spatial distribution of CCK protein in the hypothalamus. Sections incubated with rabbit serum were used as negative controls. Next, sections were incubated with Alexa Fluor-488 secondary antibody (1:1,000), followed by counterstaining with DAPI (1:1,000).

Data Analysis

The protein bands of western blot were quantitated by densitometric analyses (ImageJ 1.52a, National Institutes of Health, Bethesda, MD). Relative pERK1/2 or pCREB levels were normalized by that of intracellular β-actin, and then expressed as the relative fold increase compared to respective control (without peptide treatment). The data were analyzed by Student's *t* text (for 2 groups), or by one-way ANOVA followed by Dunnett's test using GraphPad Prism 8 (GraphPad Software, San Diego, CA). All experiments were repeated at least 3 times to validate our results.

RESULTS

Functional Analyses of Chicken CCK1R and CCK2R

Chicken *CCK* has been cloned in a previous study (Jønson et al., 2000). As shown in Figure 1, the C-terminal octapeptide (CCK-8, DYMGWMDF), tetrapeptide (CCK-4, WMDF) and the sulfated tyrosine residue are conserved across the vertebrate species. As the major form with full biological activity in mammals (Rehfeld, 2006) and chickens (Fan et al., 1987; Jønson et al., 2000), CCK-8S peptide contains the sulfated tyrosine which is required for the activation of CCK1R. In contrast, the CCK-4 peptide is necessary for the activation of CCK2R (Miller and Gao, 2008). To determine whether chicken CCK-8S and CCK-4 can activate the 2 CCK receptors (CCK1R and CCK2R), each receptor expressed in CHO cells was treated by synthetic cCCK-8S and cCCK-4. Receptor-activated signaling pathways were then monitored by 3 cell-based luciferase reporter systems established in our laboratory (pGL3-NFAT-

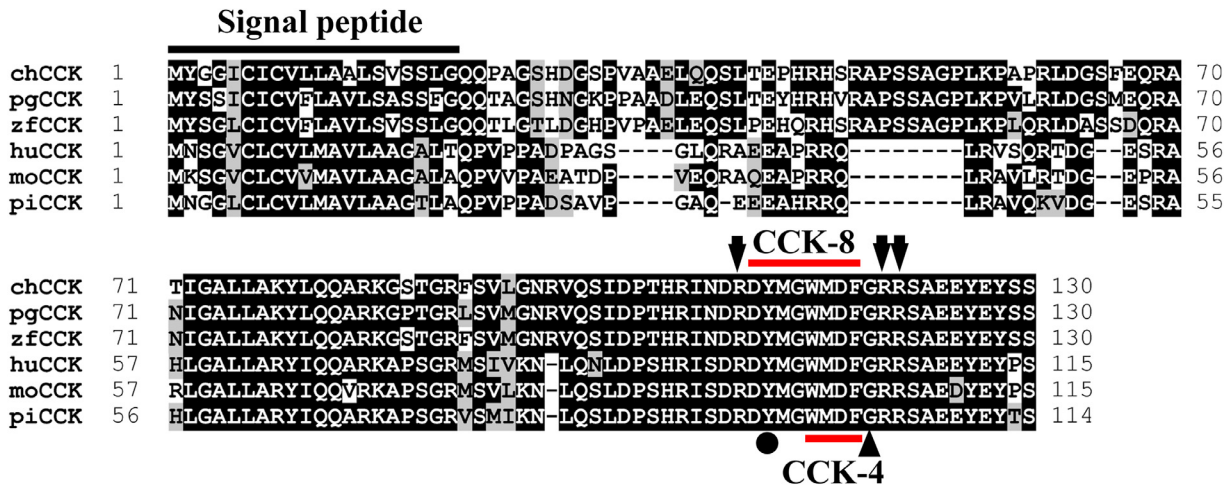

Figure 1. Alignment of chicken CCK precursor (chCCK, NP_001001741) with that of pigeon (pgCCK, NP_001290363), zebra finch (zfCCK, NP_001232081), human (huCCK, NP_001167609), mouse (moCCK, NP_112438), and pig (piCCK, NP_999402). The black horizontal line indicates the N-terminal signal peptide. The two red horizontal lines indicate the C-terminal octapeptide (CCK-8) and tetrapeptide (CCK-4), respectively. The dot indicates the sulfated tyrosine (Y) site. The putative cleavage sites are marked by arrows. An amidation donor site (G) is marked by triangle.

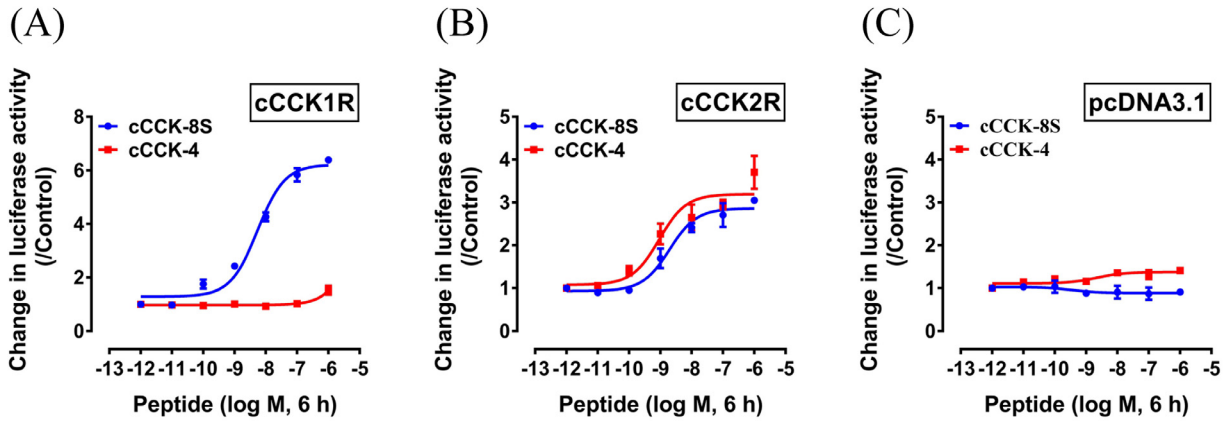

Figure 2. (A, B) Effects of chicken (c-) CCK-8S and CCK-4 on the activation of cCCK1R (A) or cCCK2R (B) expressed in CHO cells, as monitored by the pGL3-NFAT-RE-luciferase reporter system. (C) As a control, CHO cells co-transfected with an empty pcDNA3.1(+) vector (pcDNA3.1) and a pGL3-NFAT-RE-luciferase reporter construct showed no response to peptide treatment, confirming the specific effect of peptides on receptor activation. Each data point represents the mean ± SEM of triplicates ($N = 3$).

RE-luciferase, pGL3-CRE-luciferase, and pGL4-SRE-luciferase reporter systems).

Using the pGL3-NFAT-RE-luciferase reporter system, receptor-mediated activation of intracellular calcium mobilization was monitored according to our previous studies (Wan et al., 2018; Zhang et al., 2018). As shown in Figure 2, cCCK1R could only be activated by cCCK-8S (EC_{50}: 5.41 nM), but not by cCCK-4 (EC_{50}: > 100 nM). Unlike cCCK1R, cCCK2R could potently activated by cCCK-8S (EC_{50}: 1.93 nM) and cCCK-4 (EC_{50}: 0.91 nM) with similar potencies. As negative controls, cCCK-8S or cCCK-4 has no effect on pGL3-NFAT-RE-luciferase activity in CHO cells transfected with the empty pcDNA3.1(+) vector. The values of cCCK-8S and cCCK-4 in activating cCCK1R and cCCK2R are listed in the Table 1.

Using the pGL4-SRE-luciferase reporter system, receptor-mediated activation of intracellular MAPK/ERK signaling pathway was monitored according to our previous studies (Cui et al., 2021; Sun et al., 2021). As shown in Figure 3, cCCK-8S activates cCCK1R (EC_{50}: 0.32 nM) and cCCK2R (EC_{50}: 1.16 nM) in a dose-dependent manner, but cCCK-4 only efficiently activates cCCK2R (EC_{50}: 23.88 nM). As negative controls, cCCK-8S or cCCK-4 has no effect on pGL4-SRE-luciferase activity in CHO cells transfected with the

Table 1. EC_{50} values of chicken (c-) CCK-8S and CCK-4 in activating different signaling pathways of CHO cells expressing cCCK1R and cCCK2R.

EC_{50} (nM)		
Calcium mobilization		
	cCCK-8S	cCCK-4
cCCK1R	5.41 ± 1.15	> 100
cCCK2R	1.93 ± 0.70	0.91 ± 0.49
MAPK/ERK signaling pathway		
	cCCK-8S	cCCK-4
cCCK1R	0.32 ± 0.26	> 100
cCCK2R	1.16 ± 0.61	23.88 ± 6.32
cAMP/PKA signaling pathway		
	cCCK-8S	cCCK-4
cCCK1R	5.76 ± 1.55	> 100
cCCK2R	29.56 ± 7.68	89.66 ± 21.34

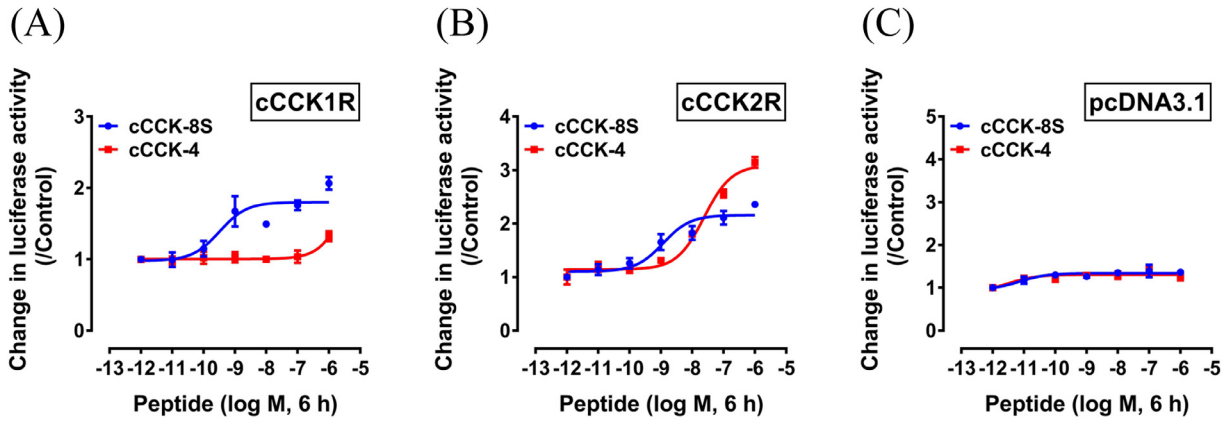

Figure 3. (A, B) Effects of chicken (c-) CCK-8S and CCK-4 on the activation of cCCK1R (A) or cCCK2R (B) expressed in CHO cells, as monitored by the pGL4-SRE-luciferase reporter system. (C) As a control, CHO cells co-transfected with an empty pcDNA3.1(+) vector (pcDNA3.1) and a pGL4-SRE-luciferase reporter construct showed no response to peptide treatment, confirming the specific effect of peptides on receptor activation. Each data point represents the mean ± SEM of triplicates ($N = 3$).

empty pcDNA3.1(+) vector. These findings suggest that activation of cCCK1R and cCCK2R can also stimulate the intracellular MAPK/ERK signaling cascade. This finding was further demonstrated by western blot results showing that cCCK-8S treatment (10 nM, 10 min) could strongly enhance ERK1/2 phosphorylation in CHO cells expressing cCCK1R (Figure 5A) and cCCK2R (Figure 5C). Moreover, cCCK-4 treatment (10 nM, 10 min) could potently enhance ERK1/2 phosphorylation in CHO cells expressing cCCK2R (Figure 5D) but not cCCK1R (Figure 5B).

Using the pGL3-CRE-luciferase reporter system, receptor-mediated activation of intracellular cAMP/PKA signaling pathway was monitored according to our previous studies (Wu et al., 2019; Sun et al., 2021). As shown in Figure 4, we also demonstrated that cCCK1R could only efficiently activated by cCCK-8S (EC_{50}: 5.76 nM), while cCCK2R could activate by both cCCK-8S (EC_{50}: 29.56 nM) and cCCK-4 (EC_{50}: 89.66 nM) with similar potencies. As negative controls, cCCK-8S or cCCK-4 has no effect on pGL3-CRE-luciferase activity in CHO cells transfected with the empty pcDNA3.1(+) vector. These findings suggest that activation of cCCK1R and cCCK2R can also stimulate the cAMP/PKA signaling pathway. In support of this finding, using western blotting, cCCK-8S treatment (10 nM, 10 min) could potently enhance CREB phosphorylation in CHO cells expressing cCCK1R (Figure 5A) and cCCK2R (Figure 5C). Additionally, cCCK-4 treatment (10 nM, 10 min) could significantly enhance CREB phosphorylation in CHO cells expressing cCCK2R (Figure 5D) but not cCCK1R (Figure 5B).

Tissue Distribution of CCK, CCK1R, and CCK2R in Chickens

In the present study, we analyzed the mRNA expression of *CCK, CCK1R,* and *CCK2R* in adult chicken tissues using RNA-seq data. As shown in Figure 6A, *cCCK* is abundantly expressed in the cerebrum, hypothalamus, ileum, and testis; and moderately expressed in the midbrain, cerebellum, hindbrain, thymus gland, and thyroid gland; and weakly expressed in the spinal cord, retina,

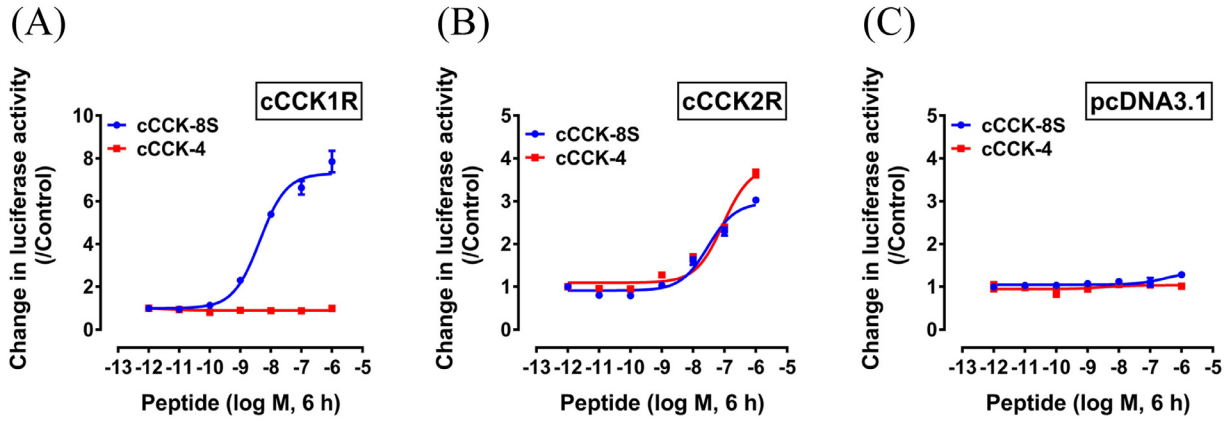

Figure 4. (A, B) Effects of chicken (c-) CCK-8S and CCK-4 on the activation of cCCK1R (A) or cCCK2R (B) expressed in CHO cells, as monitored by the pGL3-CRE-luciferase reporter system. (C) As a control, CHO cells co-transfected with an empty pcDNA3.1(+) vector (pcDNA3.1) and a pGL3-CRE-luciferase reporter construct showed no response to peptide treatment, confirming the specific effect of peptides on receptor activation. Each data point represents the mean ± SEM of triplicates ($N = 3$).

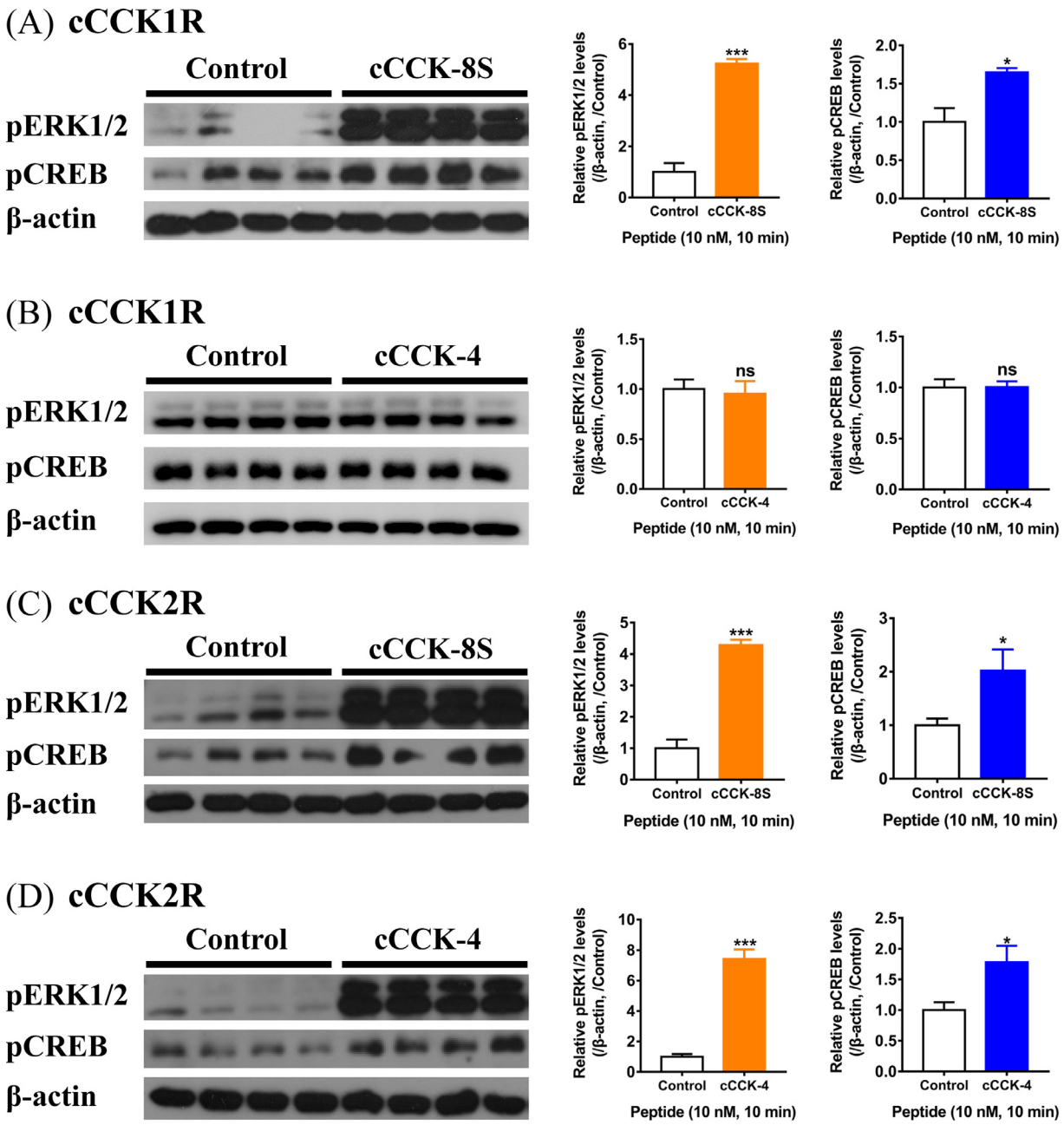

Figure 5. (A, B) Western blot detection of the phosphorylated ERK1/2 (pERK1/2) and CREB (pCREB) levels in CHO cells expressing cCCK1R treated by cCCK-8S (A) or cCCK-4 (B) for 10 min. (C, D) Western blot detection of the pERK1/2 and pCREB levels in CHO cells expressing cCCK2R treated by cCCK-8S (C) or cCCK-4 (D) for 10 min. The pERK1/2 and pCREB levels were quantified by densitometric analysis, normalized by that of cellular β-actin, and expressed as fold difference compared to the control. Each data point represents the mean ± SEM of four replicates ($N = 4$). The representative set of Western blotting is shown at the left of each graph. *, $P < 0.05$, ***, $P < 0.001$, ns, no statistical difference vs. respective control (without peptide treatment).

pineal body, parathyroid gland, duodenum, jejunum, and ovary. Moreover, IF staining confirmed that cCCK-immunoreactive (cCCK-ir) cells are distributed in different hypothalamus regions, including the nucleus dorsomedialis hypothalami (**DMN**), hypothalamic limbus, and stratum opticum (**SO**) (Figure 7).

In the present study, *cCCK1R* is abundantly expressed in the pancreas, and moderately expressed in the pituitary gland and various intestinal regions (ileum, cecum, rectum), and weakly expressed in the other tissues examined (Figure 6B). Unlike *cCCK1R, cCCK2R* is abundantly expressed in various parts of the CNS, including the cerebrum, midbrain, hindbrain, and hypothalamus (Figure 6C). In addition, it is moderately expressed in the pineal body and proventriculus, and weakly expressed in the ovary, retina, cerebellum, and pituitary.

DISCUSSION

In the present study, three cell-based luciferase reporter assays and western blots proved that cCCK-8S, but not cCCK-4, can potently activate cCCK1R, while both cCCK-8S and cCCK-4 can potently activate cCCK2R. Moreover, RNA-seq data revealed the

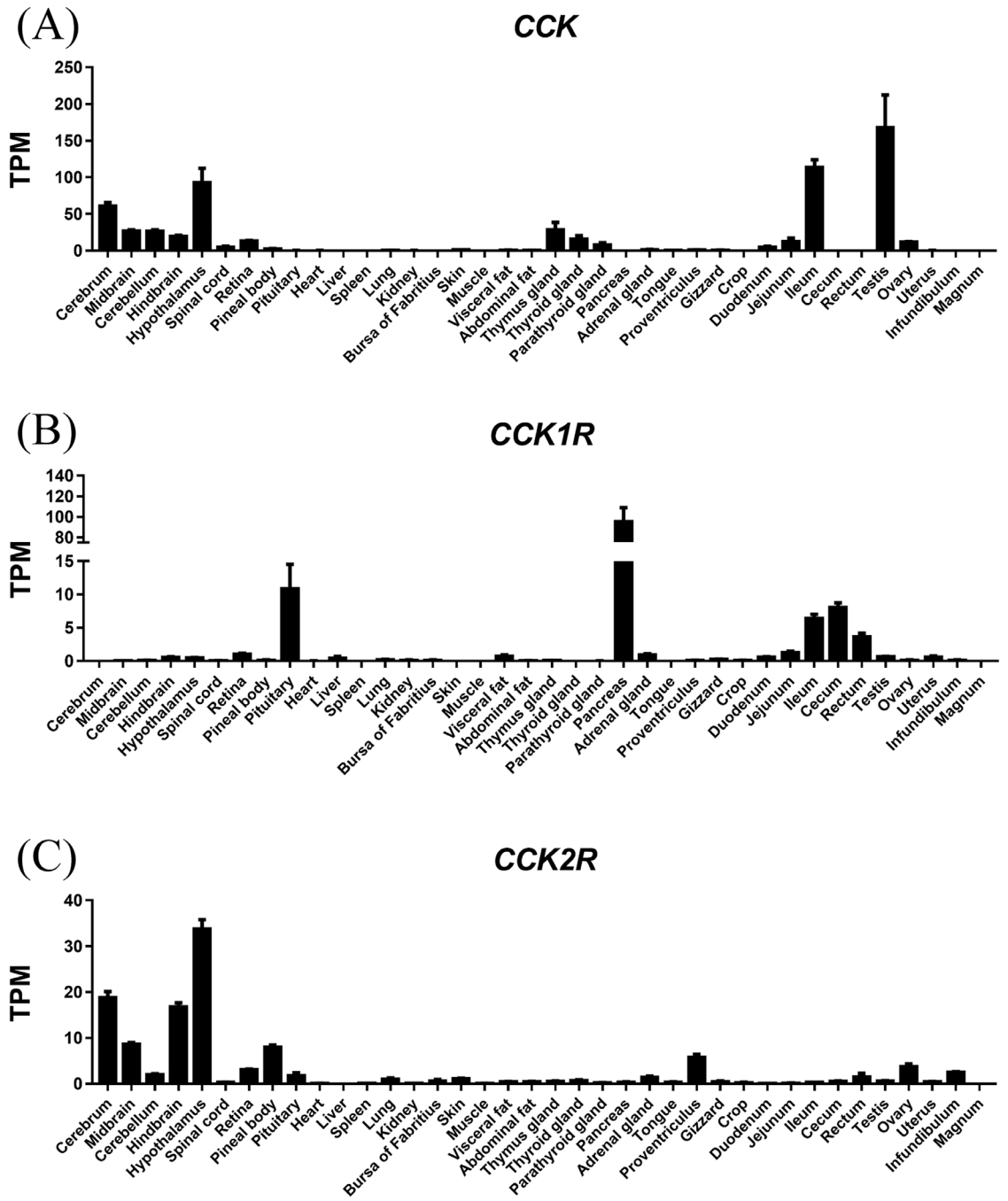

Figure 6. RNA-seq data showing the expression of *CCK* (A), *CCK1R* (B), and *CCK2R* (C) in adult chicken tissues. The transcripts per million (TPM) values were used to express the relative abundance of *CCK, CCK1R* and *CCK2R* transcripts. Each data point represents the mean ± SEM of 6 adult chickens (3 males and 3 females) ($N = 6$). However, uterus, infundibulum, magnum and ovary represent the mean ± SEM of three adult female chickens ($N = 3$). Similarly, testis represents the mean ± SEM of three male chickens ($N = 3$).

differential expression of *cCCK, cCCK1R,* and *cCCK1R* in adult chickens. Our study aids in understanding the signaling pathways of chicken CCK receptors, providing novel information for CCK peptides in birds.

Functional Analyses of cCCK-8S, cCCK-4, cCCK1R, and cCCK2R

Although chicken *CCK1R* and *CCK2R* have been cloned (Nilsson et al., 2003; Ohkubo et al., 2007), their downstream signaling pathways and relative potencies activated by cCCK-8S and cCCK-4 remain to be identified. In this study, we found that cCCK-8S is more effective than cCCK-4 in activating cCCK1R in CHO cells, while cCCK-8S and cCCK-4 are similar in activating cCCK2R, as monitored by the three reporter systems. This finding is in partial accordance with an earlier study, in which CCK2R had a high affinity for sulfated CCK-8 (IC50: 0.31 nM) and CCK-4 (IC50: 1.9 nM) (Nilsson et al., 2003). In chickens, the radioligand binding experiments showed that CCK-4 had a high affinity for brain and hypothalamus CCK receptors but a very low affinity for peripheral CCK receptors (Rodriguez-

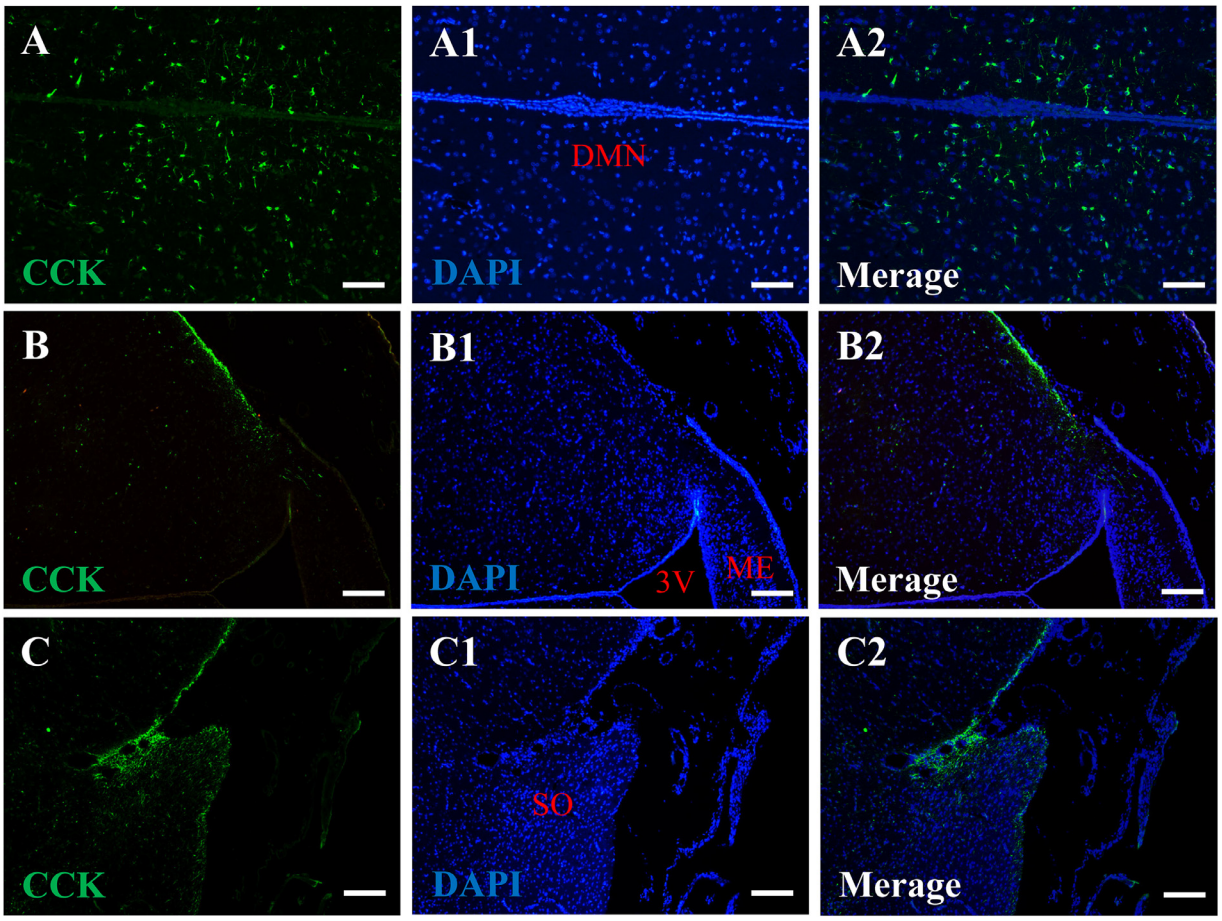

Figure 7. Immunofluorescence staining of CCK protein (green) in adult chicken hypothalamus, including nucleus dorsomedialis hypothalami (DMN, A), hypothalamic limbus (B) and stratum opticum (SO, C). (A1−C1) were nuclei staining images of (A−C). The nuclei were stained with DAPI (blue). (A2-C2) were merged figures of CCK protein and nucleus. Scale bar = 100 μm. Abbreviations: ME, median eminence; 3V, third ventricle.

Sinovas et al., 1995). In addition, our findings are consistent with those in mammals, in which CCK1R can be preferentially activated by sulfated CCK peptides, and CCK2R can be activated by both sulfated and non-sulfated CCK peptides (MMiyasaka and Funakoshi, 2003; iller and Gao, 2008; Staljanssens et al., 2011). According to reports in mammals, the binding affinity of CCK-8S to CCK1R is 500-fold higher than those of CCK-8NS (non-sulfated octapeptide of CCK) and 1,000 to 10,000-fold higher than those of CCK-4 (Noble and Roques, 2002; Miller and Gao, 2008). Unlike CCK1R, the binding affinities of CCK-8S and CCK-8NS to CCK2R are similar, which is 10-fold higher than that of CCK-4 (Noble and Roques, 2002; Miller and Gao, 2008). Moreover, cryo-electron microscopy (**EM**) experiments indicated that the residue $R197^{ECL2}$ of CCK1R is responsible for the high selective affinity of CCK-8S with CCK1R (Cheng and Shao, 2021; Liu et al., 2021).

In the present study, we demonstrated that both cCCK1R and cCCK2R couple with Gq protein to cause calcium mobilization and activation of the MAPK/ERK signaling pathway, as revealed by pGL3-NAFT-RE-luciferase reporter assay, pGL4-SRE-luciferase reporter assay, and western blotting. Our data are consistent with the findings in mammals. For instance, in pancreatic acinar cells, activation of CCK1R recruits Gq protein and activates intracellular PLC-IP_3-Ca^{2+} and PLC-DAG-PKC pathways, primarily stimulating the secretion of digestive enzymes (Paulssen et al., 2000; Murphy et al., 2008; Williams, 2011). Moreover, in COS cells, activation of CCK2R stimulates intracellular Ca^{2+} mobilization and PKC-dependent MAPK pathway (Silvente-Poirot et al., 1999; Galés et al., 2000). In addition, we also demonstrated that both cCCK1R and cCCK2R couple with Gs protein to stimulate cAMP/PKA signaling pathway, as revealed by pGL3-CRE-luciferase reporter assay and western blotting. Our result is consistent with the finding in mammals, in which activation of CCK1R leads to an increase in adenylyl cyclase activity and intracellular cAMP content in pancreatic cell lines and HEK293 cells (Marino et al., 1993; Wu et al., 1997). However, our result is distinct from the report by Wu et al. in that activation of CCK2R failed to stimulate the cAMP/PKA signaling pathway in HEK293 cells (Wu et al., 1997). The similarity and difference in G protein coupling between avian and mammalian CCK receptors may hint at their conserved and differential function during vertebrate evolution.

Tissue Expression of CCK1R, CCK2R, and CCK in Chickens

In this study, we found that *CCK1R, CCK2R,* and *CCK* are differentially expressed in adult chicken tissues, supporting their differential roles in birds. *cCCK1R* is primarily expressed in chicken peripheral tissues, with high expression levels in the pancreas and moderate expression levels in the ileum, cecum and rectum. This finding is partially in accordance with an earlier study, in which chicken *CCK1R* is widely expressed in peripheral tissues (pancreas, small intestine, pituitary, and gallbladder; Ohkubo et al., 2007). Our findings also coincide with reports in mammals (Kageyama et al., 2005; Konno et al., 2015), in which *CCK2R* is widely expressed in the peripheral tissues and is involved in the regulation of pancreatic enzyme secretion (Suzuki et al., 2001), intestinal mobility (Jiao et al., 2022), and satiety (Weatherford et al., 1992; Dunn et al., 2013). Interestingly, our results showed that *cCCK1R* is expressed in the pituitary gland, which has not been reported in mammals thus far. In contrast to the abundant distribution of *CCK1R* in mammalian CNS (Mercer and Beart, 2004), *cCCK1R* mRNA expression in the chicken CNS is rather low.

Compared to c*CCK1R, cCCK2R* expression is primarily restricted to the CNS, with the highest expression levels noted in the hypothalamus. This finding is partially in accordance with an earlier study, in which *cCCK2R* was found to be highly expressed in the brain and proventriculus by RT-PCR (Ohkubo et al., 2007). These findings are also consistent with reports in mammals (Noble and Roques, 1999; Mercer et al., 2000), in which *CCK2R* is widely and abundantly expressed in the CNS and is involved in the regulation of various physiological functions, including food intake (Clerc et al., 2007), anxiety (Wang et al., 2005), and memory processes (Sebret et al., 1999).

In the present study, *cCCK* is abundantly expressed in the ileum and widely expressed in different brain regions, including the cerebrum, midbrain, cerebellum, hindbrain, and hypothalamus. This observation is consistent with previous findings in mammals, in which CCK is widely expressed in the GIT and CNS, participating in the regulation of peripheral and central physiological functions, such as gallbladder contraction, pancreatic exocrine secretion, anxiety, and food intake (Crawley and Corwin, 1994; Rehfeld et al., 2007). In birds, *CCK* is reported to be highly expressed in the proximal ileum, cerebrum, and midbrain, indicating that it is involved in the regulation of feeding (Martinez et al., 1993), imprinting behavior (Maekawa et al., 2007) and crowing (Reid and Dunn, 2018). Our results demonstrated for the first time that cCCK is expressed in various hypothalamic regions, especially the DMN. The DMN is involved in the regulation of food intake and satiety (Bellinger and Bernardis, 2002). In rats, CCK in the DMN is reported to play an important role in the control of food intake (Chen et al., 2008; Kobelt et al., 2006). Given that *CCK* and *CCK2R* are highly expressed in the chicken hypothalamus, further studies are needed to elucidate the critical role of the

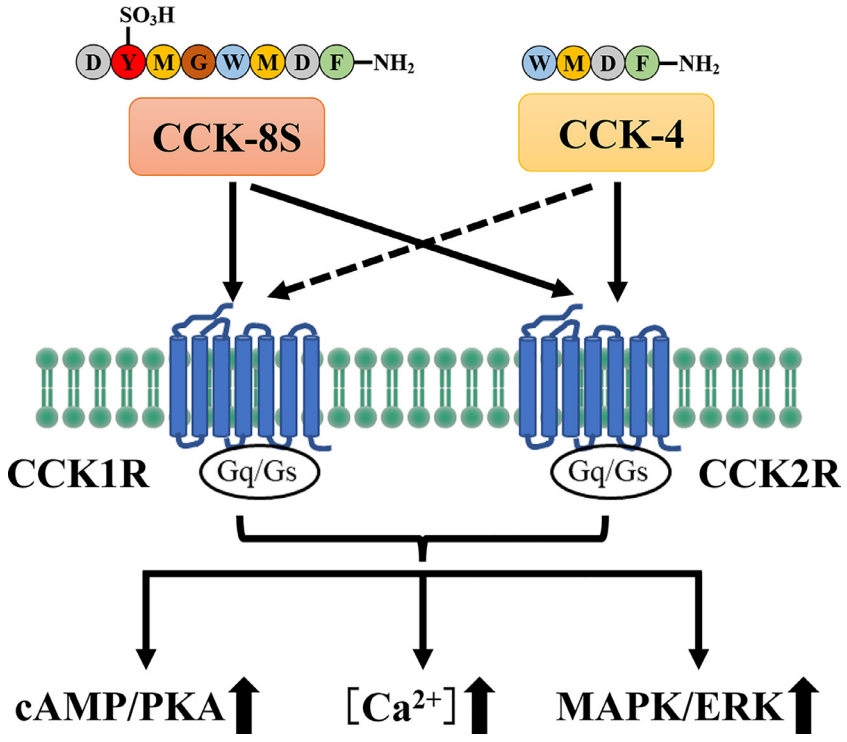

Figure 8. Schematic diagram shows the ligand-receptor interaction of CCK and its receptors (CCK1R, CCK2R) in chickens. CCK1R can only be activated by CCK-8S, suggesting that CCK1R is highly selective for sulfated CCK analogues. In contrast, CCK2R can be activated by CCK-8S and CCK-4 potently, indicating that CCK2R has high affinity for both sulfated and non-sulfated CCK analogues. In chickens, CCK1R and CCK2R are likely coupled to Gq (and Gs) protein, and their activation stimulates calcium mobilization, MAPK/ERK and cAMP/PKA signaling pathways.

CCK-CCK2R axis in regulating food intake and energy balance in chicken brain.

In addition to the CNS and GIT, *cCCK* is highly expressed in the testis, which has not been studied in chickens thus far. However, in mammals, studies showed that *CCK* is expressed in the peripheral parts of the seminiferous tubules, sperm cells, and acrosomal granules, suggesting that *CCK* may be involved in regulating the process of fertilization (Persson et al., 1988; Pelto-Huikko et al., 1989; Persson et al., 1989). Moreover, a study in 2015 demonstrated that expression of *CCK* and its receptors in mature sperm constituted the intrinsic mechanism of CCK and its receptors regulating sperm capacitation and fertilization (Zhou et al., 2015). Then, further investigation will be required to specify the CCK function in chicken testis.

In summary, cCCK1R and cCCK2R show differential binding affinities for CCK peptides. The cCCK1R is activated preferentially by cCCK-8S while cCCK2R is activated by cCCK-8S and cCCK-4 with similar potencies. In response to ligand activation, cCCK1R and cCCK2R couple with Gq (and Gs) proteins, hence stimulating calcium mobilization and the MAPK/ERK and cAMP/PKA signaling pathways (Figure 8). In combination with RNA-seq analyses revealing that *CCK, CCK1R* and *CCK2R* are differentially expressed in multiple chicken tissues, the present study demonstrates that CCK peptides act in a tissue-specific manner through their receptors. The present study helps to reveal functional conservation and changes in the CCK-CCKRs axis across vertebrates.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (32072706, U1901206, 31802056), the Sichuan University Postdoctoral Interdisciplinary Innovation Start-up Fund (0020404153033), and the Sichuan University Postdoctoral Research and Development Fund (2021SCU12101).

DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102273.

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