Neuropeptide S (NPS) and its receptor (NPSR1) in chickens: cloning, tissue expression, and functional analysis

Chao Fang ,*,†,[1] Jiannan Zhang,*,[1] Yiping Wan,* Zejiao Li,* Feiyang Qi,* Yuanhao Dang,* Juan Li,* and Yajun Wang*,[2]

*Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province, College of Life Sciences, Sichuan University, Chengdu 610064, China; and †The Brain Cognition & Brain Disease Institute, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China

ABSTRACT Neuropeptide S (**NPS**) and its receptor neuropeptide S receptor 1 (**NPSR1**) have been suggested to regulate many physiological processes in the central nervous system (**CNS**), such as arousal, anxiety, and food intake in mammals and birds, however, the functionality and tissue expression of this NPS-NPSR1 system remain unknown in birds. Here, we cloned *NPS* and *NPSR1* cDNAs from the chicken brain and reported their functionality and tissue expression. The cloned chicken *NPS* is predicted to encode a mature NPS peptide of 20 amino acids, which shows a remarkable sequence identity (∼94%) among tetrapod species examined, while *NPSR1* encodes a receptor of 373 amino acids conserved across vertebrates. Using cell-based luciferase reporter systems, we demonstrated that chicken NPS could potently activate NPSR1 expressed in vitro and thus stimulates multiple signaling pathways, including calcium mobilization, cyclic adenosine monophosphate/protein kinase A (**cAMP**/**PKA**), and mitogen-activated protein kinase/extracellular signal-regulated kinase (**MAPK**/**ERK**) signaling pathways, indicating that NPS actions could be mediated by NPSR1 in birds. Quantitative real-time PCR revealed that *NPS* and *NPSR1* are widely expressed in chicken tissues, including the hypothalamus, and *NPSR1* expression is likely controlled by a promoter upstream exon 1, which shows strong promoter activities in cultured DF-1 cells. Taken together, our data provide the first proof that the avian NPS-NPSR1 system is functional and helps to explore the conserved role of NPS and NPSR1 signaling in tetrapods.

Key words: chicken, NPS, NPSR1, tissue distribution, functional analysis

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INTRODUCTION

In mammals, neuropeptide S (**NPS**) is a neuropeptide of 20 amino acids, which was identified by reverse pharmacology, and proved to be the endogenous ligand of the neuropeptide S receptor 1 (**NPSR1**, also called VRR1/GPRA/GPR154). Since its discovery, the NPS-NPSR1 system has been reported to have multiple effects in the central nervous system (**CNS**). In rodents, central administration of NPS caused anxiolytic effects (Xu et al., 2004), increased locomotor activity (Xu et al., 2004; Ahnaou and Drinkenburg, 2012), enhanced memory (Okamura et al., 2011), and decreased the length of ketamine-induced anesthesia (Kushikata et al., 2011). Moreover, NPS can stimulate hypothalamic-pituitary-adrenal axis activity including stimulation of adrenocorticotropic hormone (**ACTH**) and corticosterone secretion in rats (Smith et al., 2006). In humans, genetic variation at the *NPSR1* locus is involved in many pathological processes, including intermediate phenotypes of functional gastrointestinal disorders (Camilleri et al., 2010) and inflammatory bowel disease (D'Amato et al., 2007), panic disorder (Domschke et al., 2011), rheumatoid arthritis (D'Amato et al., 2010), and asthma (Allen et al., 2006).

NPS exerts its actions through NPSR1, which is coupled to multiple signaling pathways (Reinscheid and Xu, 2005). In humans and mice, NPSR1 has been reported to be capable of coupling to Gq and Gs proteins, and its activation can trigger intracellular calcium mobilization (Guerrini et al., 2010), stimulate intracellular the cyclic adenosine monophosphate (**cAMP**) levels (Reinscheid et al., 2005), and enhance the extracellular

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[1]These authors contributed equally to the work.

[2]Corresponding author: cdwyjhk@gmail.com

signal-regulated kinase 1/2 (**ERK1/2**) phosphorylation (Liao et al., 2016). The tissue expression patterns of *NPS* and *NPSR1* are consistent with the biological functions of the NPS system reported in mammals. In rats, *NPS* mRNA is widely expressed in various tissues with the highest levels noted in the brain, salivary, thyroid, and mammary glands (Xu et al., 2004). Within the CNS, *NPS* is highly expressed in 3 nuclei of brainstem, principal sensory trigeminal nucleus (**PSTN**), the locus coeruleus (**LC**) area, and the lateral parabrachial nucleus (**LPBN**) (Xu et al., 2007). Similarly, *NPSR1* mRNA is widely expressed in rat tissues and mainly expressed in the brain regions including thalamus, hypothalamus, and amygdala associated with regulation of learning and memory, energy balance, and arousal (Leonard and Ring, 2011).

Interestingly, the NPS-NPSR1 system has been predicted to exist only in tetrapods including mammals, frogs, and birds, whereas it seems to be absent in teleosts (Reinscheid, 2007; Valsalan and Manoj, 2014). Although NPS and NPSR1 has been extensively studied in mammals, our knowledge regarding the functionality and tissue expression of the NPS-NPSR1 system remains extremely limited in non-mammalian vertebrates including birds. As in mammals, intracerebroventricular (**ICV**) injection of mammalian NPS has been reported to inhibit food intake in chicks (Cline et al., 2007, 2008) and Japanese quail (Webster et al., 2020), implying that the NPS-NPSR1 system may function in avian and possibly involved in the control of energy balance. Therefore, using chicken as an experimental model, our present study aims to: 1) clone the *NPS* and *NPSR1* and examine their tissue expression; 2) investigate whether the NPS-NPSR1 system is functional in vitro. The results from the present study provide a clear proof that an NPS-NPSR1 system exists and functions in birds, which will facilitate our understanding of physiological roles of NPS and NPSR1 in vertebrates.

MATERIALS AND METHODS

Peptides and Chemicals

Chicken 20-amino-acid peptide neuropeptide S (**cNPS$_{20}$**: Ser-Phe-Arg-Asn-Gly- Val-Gly-Ser-Gly-Ile-Lys-Lys-Thr-Ser-Phe-Arg-Arg-Ala-Lys-Ser) was synthesized using solid-phase Fmoc chemistry (GL Biochem, Shanghai, China) and dissolved in double-distilled deionized water. The purity of synthesized chicken peptide is greater than 95% (analyzed by HPLC), and its structure was verified by mass spectrometry (GL Biochem). Restriction enzymes and KOD DNA polymerase were purchased from Takara (Takara, Dalian, China). Antibodies used in this study include phospho-p44/42 MAPK (**ERK1/2**) (Thr202/Tyr204) rabbit mAb (1:1,000, #9101), β-Actin (13E5) rabbit mAb (1:2,000, #4970) and anti-rabbit IgG, HRP-linked antibody (1:5,000, #7074) were purchased from Cell Signaling Technology (CST, Danvers, MA). All the other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Total RNA Extraction, RT-PCR, and Quantitative Real-time PCR

Adult chickens (Lohmann layer, 1-year-old, 3 males and 3 females) from local suppliers were killed by decapitation, and tissues were collected immediately. Tissue samples including heart, kidneys, liver, lung, breast muscle, ovary, pancreas, anterior pituitary, duodenum, spleen, testis, and different brain regions, including telencephalon, cerebellum, hindbrain, midbrain, and hypothalamus were frozen in liquid nitrogen. All tissue samples were stored at −80°C before RNA extraction. All experimental protocols involving animals were approved by the Animal Ethics Committee of College of Life Sciences, Sichuan University (Chengdu, China).

Total RNA extracted from chicken tissues using RNAzol reagent (Molecular Research Center, Cincinnati, OH) was dissolved in diethylpyrocarbonate (**DEPC**)-treated H_2O and reversely transcribed using M-MLV (TaKaRa). In brief, oligodeoxythymide (0.5 μg) and total RNA (2 μg) were mixed in a total volume of 5 μL, incubated at 70°C for 10 min, and cooled at 4°C for 2 min. Then, the buffer containing 0.5 mM each of deoxynucleotide triphosphate and 100 U MMLV reverse transcriptase were added into the reaction mix, for a total volume of 10 μL. Reverse transcription (**RT**) was performed at 42°C for 90 min.

After RNA extraction and reverse transcription, the cDNA samples obtained were subjected to quantitative real-time PCR assay of chicken *NPS* and *NPSR1* mRNA levels in different chicken tissues. Quantitative real-time PCR (**qPCR**) was performed on the CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA), based on our previously established experimental protocol (Zhang et al., 2018; Wu et al., 2019). Briefly, the reaction system contained 1 μL of EvaGreen (Biotium Inc., Hayward, CA), 1 μL of cDNA, 1 × PCR buffer, 0.2 mM each dNTP, 0.2 mM each primer, 0.5 U Taq DNA polymerase (TaKaRa) and RNase-free H_2O to a final volume of 20 μL. The PCR profile consisted of 40 cycles of 94°C for 3 min, followed by 94°C for 15 s, 60°C for 15 s, and 72°C for 20 s. To assess the specificity of PCR amplification, melting curve analysis and agarose gel electrophoresis were performed at the end of the PCR reaction to confirm that a specific PCR band was produced. In addition, the identity of PCR products for all genes was confirmed by sequencing.

Cloning of cNPS and NPSR1 cDNAs and Construction of the Expression Plasmids

To isolate the cDNA of chicken *NPS* and *NPSR1*, gene-specific primers (Supplementary Table 1) based on the predicted cDNA sequences of chicken *NPSR1* deposited in GenBank (XM_426022) or Ensembl Database (www.ensembl.org/gallus_gallus) were designed to amplify the

complete open reading frame (**ORF**) of chicken *NPS* and *NPSR1* gene. The amplified PCR products were cloned into pTA2 vector (TOYOBO, Osaka, Japan) and sequenced by TSINGKE (Beijing, China).

To determine the complete gene structure of chicken *NPSR1*, gene-specific primers (Supplementary Table 1) were used to amplify the 5'-untranslated region (5'-**UTR**) and 3'-UTR of chicken *NPSR1* from the adult chicken brain using SMART-RACE cDNA amplification Kit (Clontech, Palo Alto, CA). The amplified PCR products were cloned into pTA2 vector (TOYOBO) and sequenced by TSINGKE (Beijing, China). The sequence of 5'-UTR and 3'-UTR of chicken *NPSR1* gene were compared to the chicken genome database (www.ensembl.org/gallus_gallus).

To construct expression plasmid of cNPSR1 for functional assay, we designed one pair of gene-specific primers flanking the start and stop codon of *NPSR1* to amplify complete ORF with high-fidelity KOD Taq DNA polymerase. The amplified PCR products (1190 bp) were cloned into pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA) and sequenced by TSINGKE company.

Sequence Alignment and Analysis

Amino acid sequences of cNPS and cNPSR1 of various species, as listed in Figure 1, were retrieved from the GenBank database (Supplementary Table 2). The deduced amino acid sequence of cNPSR1 was aligned with that of human, mouse, rat, and zebra finch with the use of the ClustalW program (Thompson et al., 1994). The putative transmembrane domains of cNPSR1 were predicted with the online protein topology prediction tool TMpred (http://embnet.vital-it.ch/software/TMPRED_form.html).

Functional Characterization of cNPS-cNPSR1

Based on our previous studies (Zhang et al., 2017, 2020), the functionality of cNPSR1 was examined in Chinese hamster ovary (**CHO**) cells by three luciferase reporter systems (pGL3-NFAT-RE-Luc, pGL3-CRE-Luc, and pGL4-SRE-Luc), which can monitor three different signaling pathways (the intracellular calcium signaling pathway, the cyclic adenosine monophosphate/protein kinase A (**cAMP**/**PKA**) signaling pathway, and the mitogen-activated protein kinase/extracellular signal-regulated kinase (**MAPK**/**ERK**) signaling pathway). Receptor-stimulated luciferase activity was detected with the Dual-Luciferase Reporter (**DLR**) assay kit (Promega, Madison, WI). In brief, CHO cells were cultured on a 6-well plate (Nunc, Roskilde, Denmark) and grown for 24 h before transfection. The cells were then transfected with a mixture containing 700 ng pGL3-NFAT-RE-Luciferase reporter construct (or pGL4-SRE-Luc or pGL3-CRE-Luc), 200 ng of receptor expression plasmid (or empty pcDNA3.1 vector as a negative control), 20 ng of pRL-TK construct (containing a *Renilla* luciferase gene, used as an internal control), and 2 μL jetPRIME transfection reagent (Polyplus Transfection, Illkirch, France) in 200 μL buffer. Twenty-four hour later, CHO cells were subcultured into a 96-well plate at 37°C for an additional 24 h before treatment. After removal of medium from the 96-well plate, the cells were treated with 100 μL $cNPS_{20}$-containing medium (or $cNPS_{20}$-free medium) for 6 h. Finally, CHO cells were lysed with 1 × passive lysis buffer for luciferase assay (Promega) and the luciferase activity of the cell lysate was measured by a Multimode microplate Reader (TriStar LB941, EG&G Berthold, Germany) according to the manufacturer's instruction.

Western Blotting

To investigate whether the activation of cNPSR1 can enhance ERK1/2 phosphorylation, 100 ng of cNPSR1 expression plasmid, or an empty pcDNA3.1 (+) vector, was transfected into CHO cells cultured in a 24-well plate (Nunc) using jetPRIME transfection reagent (Polyplus Transfection). After 24 h transfection, $cNPS_{20}$ (100 nM) was added to treat the cells for 0 to 30 min. Then the whole-cell lysates were used to examine the level of phosphorylated ERK1/2 using western blot analysis according to the manufacturer's instructions (Cell Signaling Technology). The levels of β-actin protein were also examined and used as internal controls in each experiment. The phosphorylated ERK1/2 (pERK1/2) levels were quantified by densitometric analysis, normalized by that of cellular β-actin, and expressed as fold difference compared to the control.

Identification of the Promoter Regions of cNPSR1

To identify the promoter region of chicken *NPSR1*, we cloned the 5'-flanking regions of *NPSR1* into a pGL3-Basic vector and tested their promoter activity in cultured chicken embryonic fibroblast cells DF-1 (CRL-12203, ATCC). In brief, several promoter-luciferase plasmids (−1,860/+334-Luc; −847/+334-Luc; −403/+334-Luc; −34/+334-Luc. Note: the first nucleotide on exon 1 of chicken *NPSR1* gene determined by 5'-RACE was termed as "+1", and the first one on exon 1 upstream was termed as '-1') of the cNPSR1 were transfected into cultured chicken DF-1 cells. The promoter activities were analyzed by the Dual-Luciferase Reporter assay (Promega), as described in our previous study (He et al., 2016; Gao et al., 2017). In brief, DF-1 cells were cultured in a 48-well plate at a density of 1×10^5 cells per well before transfection. After 24 h incubation, a mixture containing 100 ng of promoter construct, 5 ng of pRL-TK construct and 0.5 μL of JetPRIME (Polyplus-transfection, France) was prepared in 200 μL of buffer and transfected following the manufacturer's instructions when the cells reached 70% confluence. Twenty-four hours later, the medium was removed and

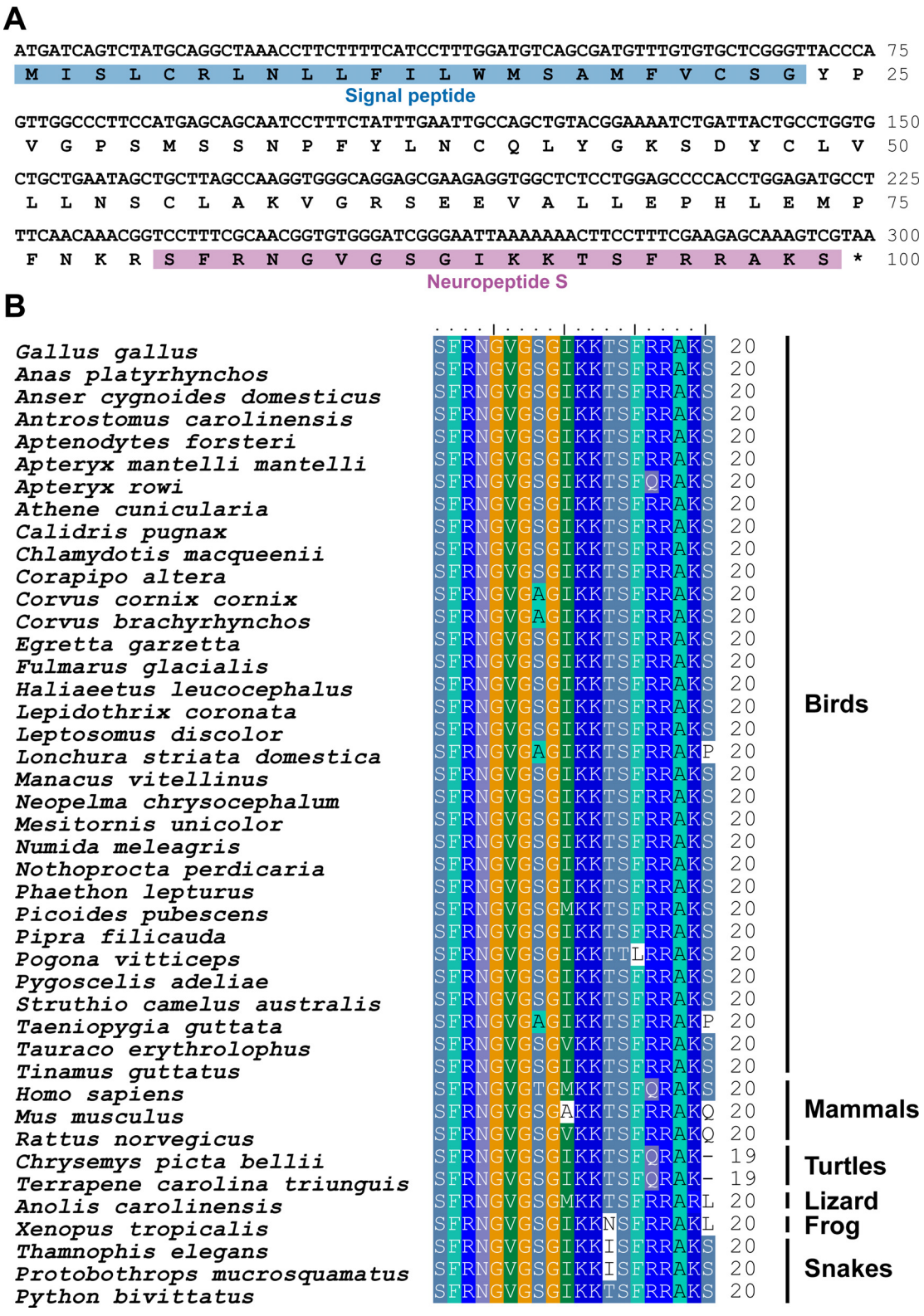

Figure 1. (A) The cDNA and deduced amino acid sequence of chicken neuropeptide S (*NPS*) (KX595274). The signal peptide (23 amino acids) and mature NPS (20 amino acids) are shaded. (B) Alignment of mature NPS amino acid sequences among 43 species. NCBI accession numbers of sequences are available in Table S2.

100 μL 1 × passive lysis buffer (Promega) was added to each well. Luciferase activities of 15 μL of cellular lysates were measured by using DLR assay kit (Promega). The cells transfected with the empty pGL3-Basic vector was used as an internal control group.

Data Analysis

The relative mRNA levels of *NPS* or *NPSR1* in chicken tissues were normalized by that of *GAPDH* and then expressed as fold difference compared with that of

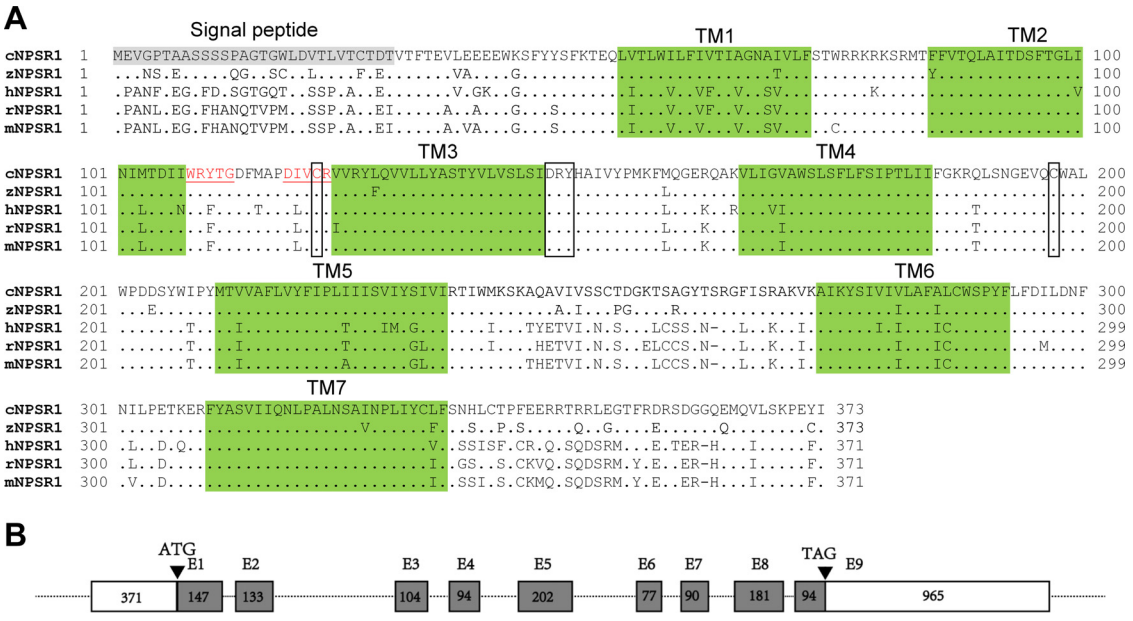

Figure 2. (A) Alignment of chicken neuropeptide S receptor (cNPSR1, KX595273) with that of humans (hNPSR1, NP_997055.1), rats (rNPSR1, NP_001100278.1), mice (mNPSR1, NP_783609.1), and zebra finch (zNPSR1, ENSTGUT00000005548.1). The predicted seven transmembrane domains (TM1-7) are shaded and labeled. The conserved two cysteine residues for disulfide bond formation are boxed. The DRY motif highly conserved in rhodopsin family of GPCRs is boxed. The 'DXXCR' (where X represents any amino acid residue except proline) and 'WRYTG' motifs in the first extracellular loop (ECL1) are underlined. Dots indicate amino acids identical to cNPSR1. (B) Exon (E)-intron organization of chicken NPSR1, the coding region was shaded. The numbers indicate the sizes of coding exons (or untranslated regions). Arrowheads indicate the locations of start codon and stop codon.

telencephalon. Luciferase activity of promoter-luciferase construct in CHO or DF-1 cells was normalized to *Renilla* luciferase activity derived from the pRL-TK vector (Promega). Then, the luciferase activities in each treatment group were expressed as fold change as compared with the control group (without peptide treatment or pGL3-Basic vector). The data were analyzed by one-way ANOVA followed by Dunnett's test using Graph Pad Prism 7 (GraphPad Software, San Diego, CA). To validate our results, all in-vitro experiments were repeated 3 times, and representative data are reported.

RESULTS

Cloning the cDNA of Chicken NPS and NPSR1

According to the predicted cDNA sequences of NPS and NPSR1 deposited in Ensembl database (NPS, ENSGALT00000105078.1) or GenBank (NPSR1, XM_426022), using RT-PCR or RACE-PCR, we amplified and cloned the cDNAs of NPS and NPSR1 from chicken brain tissue, respectively. The cloned *NPS* cDNA consists of 3 coding exons and encodes a precursor of 99 amino acids (accession no. KX595274). As in mammals, chicken NPS precursor could also produce a mature NPS peptide of 20 amino acids, which is highly conserved among vertebrate species. The cloned chicken full-length *NPSR1* is predicted to encode a receptor of 373 amino acids (Figure 1A). The chicken *NPSR1* gene contains 9 exons (accession no. KX595273), and its 5'-untranslated region (5'-UTR) and 3'-UTR is 371 bp and 935 bp in length, respectively (Figure 2B).

Amino acid sequence alignment of chicken NPS and NPSR1 with their corresponding orthologs in other vertebrate species was shown in Figure 1B and Figure 2A, respectively. The mature chicken NPS of 20 amino acids shows a remarkable degree of amino acid sequence identity with that of human, mouse, *Xenopus tropicalis*, anole lizards, and other avian species examined. Similarly, cNPSR1 displays a high degree of amino acid sequence identity to that of human (71%), rat (73%), mouse (73%), zebra finch (91%), *Xenopus tropicalis* (68%), and 43% identity to that of NPSR1-like sequence (XM_019758052) in lancelet. Like mammalian NPSR1, chicken NPSR1 contains seven hydrophobic transmembrane domains (**TM1-7**), a DRY motif of the class A G protein-coupled receptors in the intracellular loops 2 (**ICL2**) (Rovati et al., 2007). Moreover, a well-conserved motif (DXXCR), which has been reported to be linked to signal transduction (Clark et al., 2010), was also observed in the first extracellular loop (**ECL1**) of cNPSR1.

Synteny Analysis of NPS and NPSR1 in Vertebrates

To determine whether the cloned chicken *NPS* and *NPSR1* are orthologous to *NPS* and *NPSR1* identified in other vertebrate species, we examined the neighboring genes of *NPS* and *NPSR1* in genomic regions of chicken and other vertebrate species. As shown in Figure 3, *NPS* and *NPSR1* and their neighboring genes could be identified in human, mouse, turkeys, anole lizards, and *Xenopus tropicalis*, indicating that chicken *NPS* and *NPSR1* are orthologous to *NPS* and *NPSR1* identified in other tetrapods.

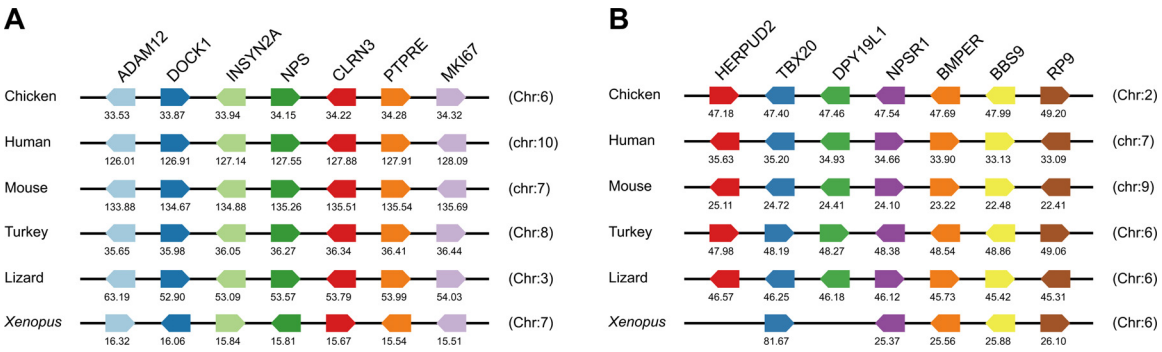

Figure 3. Syntenic analysis of the genomic region contained vertebrate NPS (A) and NPSR1 (B). The genomic locations of NPS and NPSR with their neighboring genes were compared with their orthologous genes in humans, mice, turkeys and lizards, *Xenopus tropicalis*. Orthologous genes are on the same color. For each species, the chromosome number is indicated on the right. The numbers below the genes indicate their locations (Megabyte, Mb) in each chromosome.

Functional Analysis of cNPS-cNPSR1 in Cultured CHO Cells

To investigate the functionality of cNPS-cNPSR1, 3 cell-based luciferase reporter systems including the pGL3-NFAT-RE-luciferase, pGL3-CRE-luciferase, and pGL4-SRE-luciferase reporter systems were used to test whether cNPSR1 could be activated by synthetic chicken NPS.

Using the pGL3-NFAT-RE-luciferase reporter assay, we first investigated whether like mammalian NPSR1 (McCoy et al., 2010), cNPSR1 activation could trigger calcium mobilization. As shown in Figure 4A, synthetic $cNPS_{20}$ could potently stimulate NFAT-RE-driven luciferase activities of CHO cells expressing cNPSR1 with an EC_{50} value of 0.23 nM, indicating that cNPSR1 is a functional receptor for NPS and its activation triggers Ca^{2+} mobilization from intracellular stores. This idea is also supported by the fact that 2-APB, a specific inhibitor of IP3-induced Ca^{2+} release from intracellular stores, could block $cNPS_{20}$-induced luciferase activity of CHO cells expressing cNPSR1 (Figure 4D).

Using the pGL3-CRE-luciferase reporter assay (Figure 4B), we found that $cNPS_{20}$ could also dose-dependently induce CRE-driven luciferase activities of CHO cells expressing cNPSR1 (EC_{50}: 0.90 nM), and the stimulatory effect of $cNPS_{20}$ (10 nM) could be significantly inhibited by H89 (10 μM), a specific inhibitor of protein kinase A (**PKA**) (Figure 4E). It indicates that like mammalian NPSR1, chicken NPSR1 is likely coupled to $G\alpha_s$-cAMP/PKA signaling pathway.

Using pGL4-SRE-luciferase reporter assay (Figure 4C), we also demonstrated that $cNPS_{20}$ could activate NPSR expressed in CHO cells and thus stimulate luciferase activity dose-dependently (EC_{50}: 5.86 nM). It suggests that cNPSR1 activation could also stimulate the MAPK/ERK signaling pathway. In agreement with this idea, $cNPS_{20}$ treatment (100 nM) could strongly enhance ERK1/2 phosphorylation of CHO cells expressing cNPSR1 in a time-dependent manner, with the maximal effect observed at 5 min treatment (Figure 4F).

In parallel with the above experiments, peptide treatment (10^{-12} to 10^{-6} M) did not alter luciferase activity of CHO cells transfected with empty pcDNA3.1 (+) vector, confirming specific action of the peptide on receptor activation (Figures 4A–4C).

Tissue Expression Patterns of NPS and NPSR1 in Chicken Tissues

In this experiment, quantitative real-time PCR was used to examine the tissue distribution of *NPS* and *NPSR1* in adult chicken tissues, including anterior pituitary, heart, duodenum, kidney, lung, breast muscle, ovary, testes, spleen, pancreas, and various brain regions including telencephalon, midbrain, cerebellum, hindbrain, and hypothalamus. As shown in Figure 5A, chicken *NPS* is highly expressed in the hindbrain, moderately expressed in the telencephalon and anterior pituitary, and weakly expressed in other tissues. chicken *NPSR1* is highly expressed in the midbrain and hypothalamus, and weakly expressed in other tissues (Figure 5B).

Identification of cNPSR1 Promoter

The identification of 5'-cDNA end of chicken *NPSR1* led us to further test whether a promoter region driving *cNPSR1* expression is located near exon 1, therefore, we constructed several promoter-luciferase constructs containing the 5' flanking region of *NPSR1* with different lengths and test their promoter activities in cultured DF-1 cells.

As shown in Figure 6A, the 5'-flanking regions (−1,860/+334, −870/+334, and −403/+334) of chicken *NPSR1* displayed strong promoter activities in cultured DF-1 cells, compared with control cells transfected with the promoter-less pGL3-Basic vector. Since the promoter construct containing −34/+334 still maintain a strong promoter activity, it indicates that the 5'-flanking region −34/+334 is likely the putative core promoter region of chicken *NPSR1* gene. Using the online software AnimalTFDB (v3.0) (Hu et al., 2019), the putative binding sites for many transcription factors, including POU3F3, HSF2, HNF4A, and CREBBP were predicted to exist within or near the core promoter region (Figure 6B).

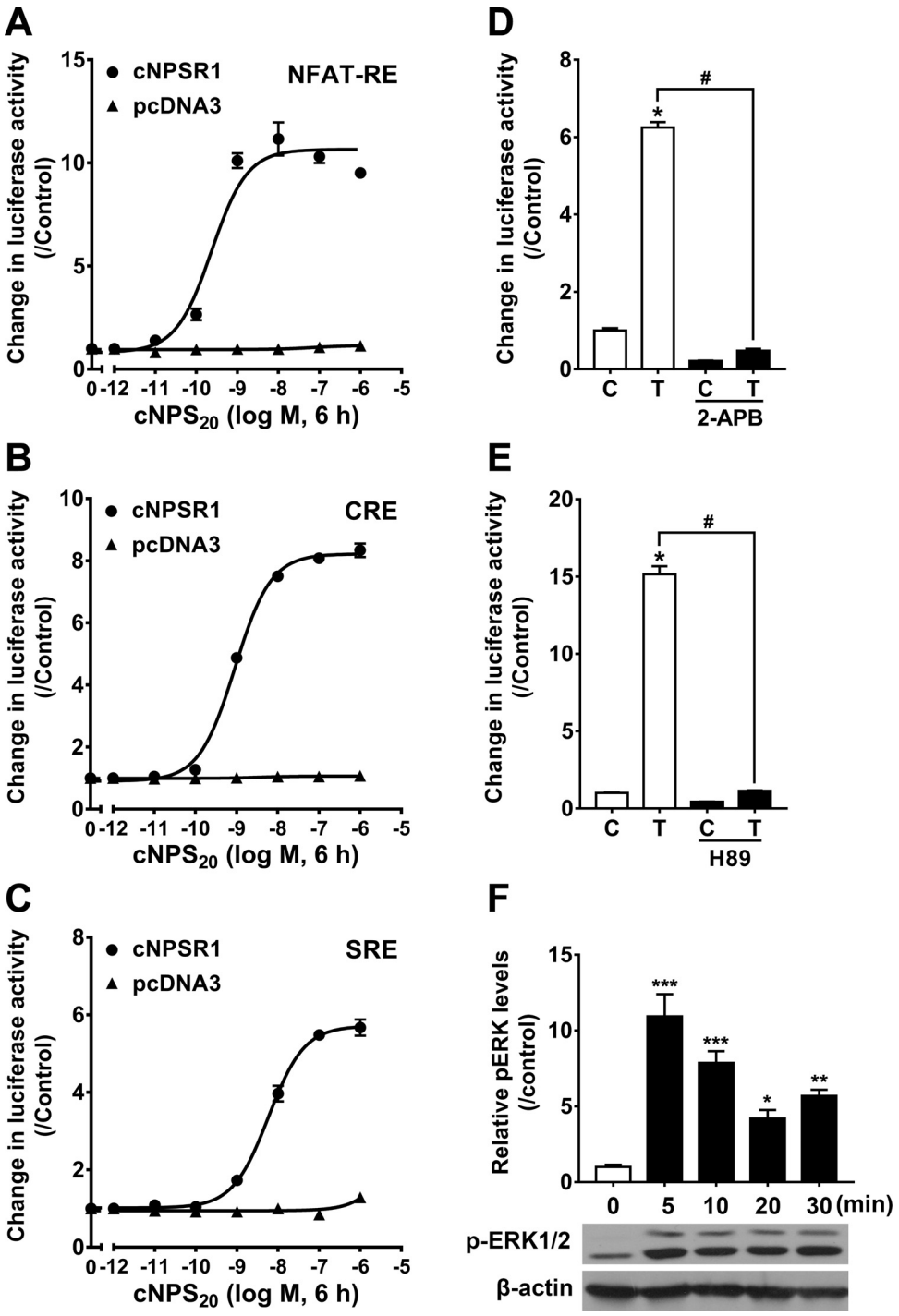

Figure 4. (A−C) Activation of cNPSR1 expressed in CHO cells upon chicken 20-amino-acid peptide neuropeptide S ($cNPS_{20}$) treatment, monitored by pGL3-NFAT-RE-luciferase (A), pGL3-CRE-luciferase (B), and pGL4-SRE-luciferase (C) reporter systems. The cells transfected by pcDNA3.1(+) empty vector were used as the negative control. Data are shown as the mean ± SEM of three replicates ($N = 3$) and are representative of three independent experiments. (D, E) Effects of 2-APB (100 μM) (D), or H89 (10 μM) (E) on $cNPS_{20}$ (10 nM, 6 h)-induced luciferase activities of CHO cells expressing cNPSR1, monitored by pGL3-NFAT-RE-luciferase (D) and pGL3-CRE-luciferase (E), respectively. 2-APB or H89 was added 0.5 h before treatment. In figure D and E, T represents peptide treatment, and C represents control without peptide treatment. Each figure shows one representative experiment repeated three times. *, P<0.01 vs. control (in the absence of drug); #, P<0.01 vs. peptide treatment (in the presence of drug). (F) cNPS treatment (100 nM, 0−30 min) could enhance ERK1/2 phosphorylation levels of CHO cells expressing cNPSR1. The phosphorylated ERK1/2 (p-ERK1/2) levels were quantified by densitometric analysis, normalized by that of cellular β-actin, and expressed as fold difference compared to the control (0 min). Data points represent the mean ± SEM of three independent experiments performed in triplicate. The representative set of Western blot is shown at the bottom. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ vs. control (0 min). Abbreviation: CHO, Chinese hamster ovary.

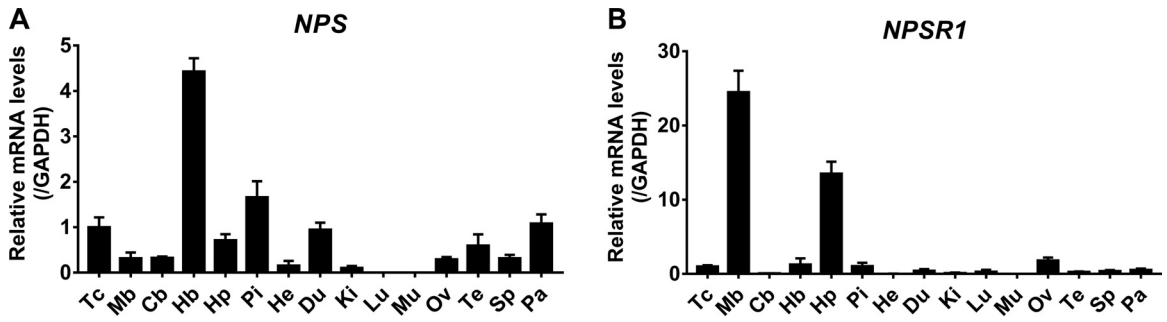

Figure 5. Quantitative real-time PCR assay of *NPS* (A) and *NPSR1* (B) mRNA levels in chicken tissues, including the telencephalon (Tc), midbrain (Mb), cerebellum (Cb), hindbrain (Hb), hypothalamus (Hp), anterior pituitary (Pi), heart (He), duodenum (Du), kidneys (Ki), lung (Lu), breast muscle (Mu), ovary (Ov), testes (Te), spleen (Sp), and pancreas (Pa). The mRNA levels of target genes were normalized to that of GAPDH and expressed as the fold difference compared with that of the telencephalon (Tc). Each data point represents the mean ± SEM of six adult chickens ($N = 6$, 3 males and 3 females, 1 -yr-old), except for that of ovary and testes, which represent the mean ± SEM of three adult chickens ($N = 3$).

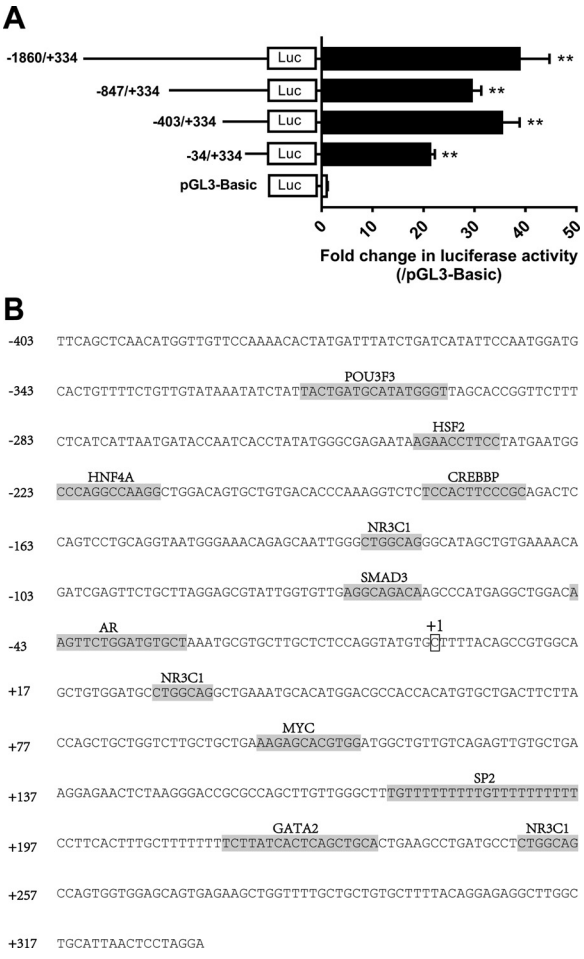

Figure 6. Promoter analysis of chicken *NPSR1*. (A) Detection of the promoter activities of the 5'-flanking regions of chicken *NPSR1* gene in cultured DF-1 cells. Various stretches of the 5'-flanking regions of chicken *NPSR1* were cloned into a pGL3-Basic vector for the generation of four promoter-luciferase constructs (−1,860/+334-Luc; −847/+334-Luc; −403/+334-Luc; −34/+334-Luc). These promoter-luciferase constructs were then co-transfected into DF-1 cells along with a pRL-TK vector and their promoter activities were determined by the Dual-Luciferase Reporter (DLR) assay. All experiments were performed in triplicate and represent at least three independent biological repeats. Data shown represent mean ± SEM. **, $P < 0.001$ vs. promoter-less pGL3-Basic vector; (B) Partial sequence (−403/+317) of the chicken *NPSR1* promoter region. The predicted binding sites for transcriptional factors, such as POU3F3, HSF2, HNF4A and CREBBP, NR3C1, SMAD3, and AR were shaded. The transcriptional start site 'C' identified by 5'-RACE was boxed and designated as '+1'.

DISCUSSION

In this study, *NPS* and *NPSR1* cDNAs were cloned from the chicken brain. Functional assay demonstrated that cNPSR1 is a functional receptor for cNPS and its activation can stimulate multiple signaling pathways. qPCR revealed that *NPS* and *NPSR1* are widely expressed in chicken tissues. To our knowledge, our study represents the first to report that an NPS-NPSR1 system functions in a non-mammalian vertebrate species.

Although *NPS* and *NPSR1* genes have been predicted to exist in birds, their functionality and tissue expression have not been examined in any non-mammalian vertebrate species including birds. Here, from the chicken brain, we cloned the cDNAs of *NPS* and *NPSR1*, which are orthologous human *NPS* and *NPSR1*, respectively, as revealed by synteny analysis (Figure 3). Like human NPS, chicken NPS consists of 3 exons and predicted to produce a mature NPS peptide of 20 amino acids after proteolytic processing, which is highly conserved between chicken and other avian species or with humans. Moreover, the conserved amino acids, such as "Phe2, Arg3, and Asn4" known to be essential for NPSR binding and activation and "Gly5-Val6-Gly7" motif important for bioactive confirmation, also identified in chickens, suggesting that NPS is bioactive in birds. Using the RACE-PCR, we also found that like humans, NPSR1 consists of 9 exons. The putative cNPSR1 has 373 amino acids, which shows high similarity to mammalian NPSR1 in terms of amino acid sequence. As shown in the multisequence alignment, chicken NPSR1 shares 71 to 73% amino acid sequence identities with its orthologues in humans (Feldman et al., 2002) and mice (Vassilatis et al., 2003). Amino acid residues in the 7 transmembrane helices of chicken NPSR1 are highly conserved to those in the mammalian NPSR1s, while the N- and C-termini of NPSR1 are less conserved across species. Various structural features conserved among mammalian NPSR1s are also present in cNPSR1. These features include 2 cysteine residues for disulfide bond formation and protein stabilization, a DRY motif in the second intracellular domain essential for receptor function (Rovati et al., 2007). Interestingly, chicken NPSR1

Table 1. EC_{50} values of chicken NPS in activating different signaling pathways in CHO cells expressing chicken NPSR1.

Signaling pathways	cNPS (nM)
Calcium signaling	0.23 ± 0.07 nM
cAMP/PKA signaling	0.90 ± 0.07 nM
MAPK/ERK signaling	5.9 ± 0.61 nM

Abbreviation: CHO, Chinese hamster ovary; NPS, neuropeptide S.

only contains a DXXCR motif, one of the 2 well-conserved motifs (WRFTG and DXXCR) linking to mammalian NPSR signaling (Clark et al., 2010), while the other motif 'WRFTG' of mammalian NPSR1 is replaced by "WRYTG" in chicken NPSR1 (Figure 2A).

The structural similarity or difference between birds and mammals led us to further examine whether cNPS-cNPSR1 is functional. Using the cell-based luciferase reporter assays, we proved that cNPSR1 could be potently activated by chicken NPS and its activation can stimulate intracellular calcium mobilization, cAMP/PKA, and MAPK/ERK signaling pathways (Table 1). These findings indicate that cNPS is an endogenous ligand for cNPSR1. Our finding is consistent with the previous reports in humans and mice (Camarda et al., 2008; Erdmann et al., 2015). It was reported that human or mouse NPSR1 expressed in human embryonic kidney 293 (**HEK293**) cells (or CHO cells) were detected to trigger Ca^{2+} mobilization in response to NPS challenge. Moreover, activation of human or mouse NPSR1 can induce cAMP formation, measured by CRE-luciferase reporter system (Reinscheid et al., 2005). Using a time-resolved fluorescence resonance energy transfer assay or Western blot analysis, human NPSR1 expressed in HEK 293 cells and CHO cells could also elicit ERK phosphorylation (Clark et al., 2010; Thorsell et al., 2013). These findings, together, support that as in mammals, NPSR1 is likely coupled to Gαq and Gαs proteins, and thus can stimulate multiple signaling pathways after binding NPS in chickens (Figure 7).

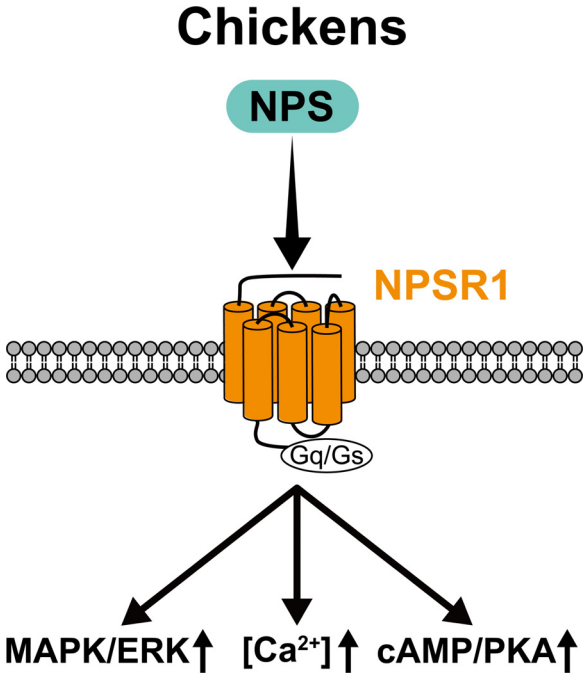

Figure 7. Signaling pathways coupled to cNPSR1. cNPS can potently activate cNPSR1, which is likely to be coupled to $G\alpha_s$-AC/cAMP/PKA and Gαq-PLC/IP3/Ca^{2+} signaling pathways. In addition, cNPSR1 activation can enhance ERK phosphorylation.

Although pioneering studies show that intracerebroventricular (**ICV**) injection of mammalian NPS can inhibit food intake, decrease plasma corticosterone levels, and increase time spent in deep rest in chicks (Cline et al., 2007, 2008), implying an NPS-NPSR1 system functions in vivo, the tissue expression of NPS and NPSR1 is unknown in chickens and other avian species. In mammals, the tissue expression of NPS and NPSR1 was well-documented. In rats, NPS precursor mRNA is expressed in a group of neurons located between the locus coeruleus and Barrington's nucleus, the principle sensory trigeminal nucleus, and the lateral parabrachial nucleus (Xu et al., 2004, 2007). In mice, *NPS* mRNA is strictly expressed in only 2 regions of the brainstem: the pericoerulear area and the Kölliker-Fuse nucleus (Clark et al., 2011). On the contrary, *NPSR1* is widely expressed in rat and mouse brain, including the amygdala, thalamus, and hypothalamus (Xu et al., 2007; Clark et al., 2011). Since LC neurons are reported to play important roles in regulating arousal and anxiety (Berridge and Waterhouse, 2003), administration of NPS contributes to anxiolytic, and antinociceptive effects in rats (Zhang et al., 2014). In the present study, we found that within CNS, chicken *NPS* mRNA is highly expressed in the hindbrain and moderately and weakly expressed in other brain regions including telencephalon and hypothalamus, while *NPSR1* is highly expressed in midbrain and moderately expressed in the hypothalamus, therefore, whether avian NPS and NPSR1 play roles similar to those mentioned above awaits further studies. It was reported that NPS can stimulate the hypothalamus-pituitary-adrenal axis and inhibit food intake in rats and chickens (Smith et al., 2006). Notably, a moderate expression of *NPSR1* was detected in chicken hypothalamus. This finding supports the anorexigenic action of cNPS is likely mediated by cNPSR1 expressed in this region (Dridi et al., 2005). Apart from CNS, cNPS and cNPSR1 has also been found to be widely expressed in all peripheral tissues examined. This finding is also consistent with the finding in rats, in which NPS and NPSR1 is also widely expressed in many peripheral tissues. Chicken *NPS* has a moderate expression level in the anterior pituitary, duodenum, testes, and pancreas, while *NPSR1* has a weak expression in all peripheral tissues examined. This finding hint that NPS acts more likely as an autocrine/paracrine factor in these peripheral tissues via NPSR1, a topic which needs to be clarified in birds and mammals.

In this study, we also found that chicken *NPSR1* expression is likely controlled by a functional promoter near exon 1 (within −1,870 to +334), which contains putative binding sites for many transcriptional factors (such as SP2, NR3C1, AR, GATA2, and SMAD3) and

displays a strong promoter activity in DF-1 cells. Similar to our finding, Anedda et al., also identified a promoter region of human NPSR1 near exon 1, which displays a promoter activity in cultured colo-205 cells (Anedda et al., 2011). This finding suggests that the promoter near exon 1 is likely responsible for the wide expression of *NPSR1* detected in chicken and mammals.

In summary, we cloned *NPS* and *NPSR1* genes from the chicken brain. Functional assays proved the cNPSR1 can be potently activated by its endogenous ligand NPS and trigger multiple downstream signaling pathways (Figure 7). qPCR assay revealed that both *NPS* and *NPSR1* are widely expressed in chicken tissues with a relatively high expression level noted in the brain. Our data provide clear evidence that as in mammals, an NPS-NPSR1 system function in birds, which may play roles similar to those in mammals, such as central regulation of food intake in birds.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101445.

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