Characterization of spexin (SPX) in chickens: molecular cloning, functional analysis, tissue expression and its involvement in appetite regulation

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ABSTRACT Spexin (**SPX**) is a conservative tetradecapeptide which has been proven to participate in multiple physiological processes, including anxiety, feed intake, and energy metabolism in fish and mammals. However, whether SPX exists and functions in birds remain largely unknown. Using chicken (c-) as a model, the full-length cDNA encoding cSPX precursor was cloned, and it was predicted to generate a mature peptide with 14 amino acids conserved across vertebrates. The pGL4-SRE-luciferase reporter system-based functional analysis demonstrated that cSPX was effective in activating chicken galanin type Ⅱ receptor (**cGALR2**), cGALR2-like receptor (**cGALR2L**) and galanin type Ⅲ receptor (**cGALR3**), thus to stimulate intracellular MAPK/ERK signaling pathway. Quantitative real-time PCR revealed that *SPX* was widely expressed in chicken tissues, especially abundant in the central nervous system, pituitary, testes, and pancreas. Interestingly, it was noted that chicken hypothalamic SPX mRNA could be up-regulated by 24-h and 36-h fasting, heralding its latent capacity in appetite regulation. In accordance with this speculation, peripheral injection of cSPX was proved to be functional in reducing feed intake of 3-wk-old chicks. Furthermore, we found that cSPX could reduce the expression of *AgRP* and *MCH*, with a concurrent rise in *CART1* mRNA level in the hypothalamic of chicks. Collectively, our findings not only provide the evidences that SPX can act as a satiety factor by orchestrating the expression of key feeding regulators in the chicken hypothalamus but also help to facilitate a better understanding of its functional evolution across vertebrates.

Key words: chicken, *SPX*, galanin receptors, hypothalamus, appetite regulation

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INTRODUCTION

Spexin (**SPX**) is a novel neuropeptide which was initially discovered in the human genome by a bioinformatics search strategy, based on a hidden Markov model, in 2007 (Mirabeau et al., 2007). In human, SPX is encoded by *C12ORF39* gene consisting of 6 exons and 5 introns (Wan et al., 2009; Kim et al., 2014). Since the SPX precursor contains 2 dibasic prohormone cleavage sites, it is capable of generating a mature peptide with 14 amino acid (-aa) residues after proteolytic processing (Sonmez et al., 2009). In less than 2 decades, *SPX* gene has been identified in human, rat, mouse, and some fishes, and the generated mature peptide is evolutionally conserved from fish to mammals (Wong et al., 2013; Kim et al., 2014).

To explore the biological significance of *SPX*, its tissue distribution has been examined in several species. Previous reports showed that *SPX* was widely expressed in the central nervous system (**CNS**) and peripheral tissues, suggesting it may take part in many physiological processes across vertebrates (Mirabeau et al., 2007; Porzionato et al., 2010; Wong et al., 2013; Wong et al., 2021a). In mammals, SPX is relevant to adrenocortical cell proliferation (Rucinski et al., 2010), stomach contraction (Mirabeau et al., 2007), bowel movement (Lin et al., 2015), cardiovascular and renal modulation (Toll et al., 2012; Kumar et al., 2018). Recent research demonstrated that SPX could reduce adipocyte uptake of long-chain fatty acids and lose weight in rodents with diet-induced obesity (Walewski et al., 2014). Moreover, peripheral SPX suppresses feed intake in mice (Lv et al., 2020). Because its level correlates with human obesity and diabetes (Gu et al., 2015; Kumar et al., 2016; Karaca et al., 2018; Kolodziejski et al., 2018), SPX was believed to regulate feeding and energy regulation as

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well, in mammals. Analogously, previous results proved SPX also modulates feed intake in fish. In goldfish (Wong et al., 2013) and Siberian sturgeon (Tian et al., 2020), feed consumption was observably decreased by injection of SPX. Recently, Zheng et al. found SPX suppressed feed intake in zebrafish because SPX-knockout individuals ate more than the wild-type ones (Zheng et al., 2017). Interesting, starvation could induce *SPX* mRNA expression in the hypothalamus of ya-fish (Wu et al., 2016), half-smooth tongue sole (Wang et al., 2018), and orange-spotted grouper (Li et al., 2016), clearly stating SPX is a pivotal factor in the appetite regulation of teleosts. In addition, SPX also can affect the activity of the reproductive axis by inhibiting gonadotrophin release or synthesis (Liu et al., 2013; Cohen et al., 2020).

SPX exerts its biological functions via a specific membrane-anchored receptor which has not yet been clearly identified. In 2014, the coevolution relationship of *SPX, Galanin* (*GAL*), *Kisspeptin* (*KISS*) with their receptor genes was confirmed (Kim et al., 2014). GAL, a 29-aa peptide (30 in human), is identified as the ligand for G protein-coupled receptors (GPCR), termed galanin receptors (GALR1-3) (Fang et al., 2020). After binding GAL, GALRs could modulate intracellular MAPK/ERK signaling pathways (Liu and Hokfelt, 2002; Sipkova et al., 2017). Ligand-receptor interaction study showed that SPX could activate human GALR2 and GALR3, zebrafish and tilapia GALR2a and GALR2b, as well as GALR2b and GALR3 in *Xenopus*, but not GALR1 (Kim et al., 2014; Cohen et al., 2020). Remarkably, the potency of SPX in activating GALR2 and GALR3 is similar or even higher than GAL, raising a possibility that SPX is the endogenous ligand for GALR2/3, which are in charge of SPX-mediated physiological significance (Kim et al., 2014). Soon afterward, this hypothesis was affirmed, since SPX-dependent GALR2 activation could enhance bowel movement in mouse (Lin et al., 2015). Moreover, SPX-based specific agonists of GALR2 exhibited an anxiolytic effect and could decrease feed intake and body weight in a dose-dependent manner in mice (Yun et al., 2019). In tilapia, SPX-inhibiting gonadotrophins secretion was achieved through GALR2b (Cohen et al., 2020).

Although the research regarding SPX-related physiological roles and its endogenous receptor have been constantly documented in fish and mammals, whether SPX exists in birds remains unknown until now. Apart from GALR1-3, there are 2 additionally novel GALR-like receptors (GALR1L and GALR2L) subtypes in chicken (Ho et al., 2011, 2012), whether they can be effectively activated by SPX is also an open question. Therefore, using chicken as a model organism, our study aims to: 1) clone the full-length cDNA of *SPX* and examine its tissue expression; 2) explore the capacity of cSPX in activating chicken galanin receptors; 3) investigate whether SPX is a potential appetite-regulatory factor in chicken. The results will not only contribute to uncovering the physiological roles of SPX in birds but also provide a clue to understanding its functional evolution across vertebrates.

MATERIALS AND METHODS

Animals

Chicks (Lohmann Sandy) used in this study were purchased from a local commercial company. All animal experiments were performed under the guidelines provided by the Animal Ethics Committee of Sichuan Agricultural University.

Chemicals, Peptides, and Primers

All chemicals were purchased from Sigma-Aldrich (Shanghai, China), and restriction enzymes were obtained from Takara (Dalian, China) unless stated otherwise. Chicken spexin (cSPX-14, NWTPQAMLYLKGAQ) and galanin (cGAL -29, GWTLNSAGYLLGPHAVDNHRS FNDKHGFT) with the amidated C-terminus were synthesized by GL Biochem Ltd (Shanghai, China). The purity of synthesized peptides is more than 95% (analyzed by HPLC), and their structure was verified by mass spectrometry. All primers used in this study were synthesized by Chengdu Tsingke Biotechnology and listed in Supplementary Table 1.

RNA Extraction and Reverse Transcription

Three roosters and three hens (Lohmann Sandy, 31-wk-old) were individually stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 and sacrificed by decapitation to collect various tissue samples including the whole brain, spinal cord, heart, adrenal, duodenum, kidney, liver, lung, muscle, skin, pituitary, spleen, pancreas, ovary, testes, abdominal fat, telencephalon, midbrain, cerebellum, hindbrain, and hypothalamus. Total RNA was extracted with RNAzol (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and dissolved in RNase-free water. Reverse transcription (**RT**) was employed to prepare the cDNA samples by using the PrimeScript RT reagent kit with a gDNA eraser (Takara, Dalian, China) and 1 μg total RNA as a template. After that, the cDNA samples were used for subsequent PCR or quantitative real-time PCR (qRT-PCR) for detecting mRNA expression of the target gene.

Cloning the Full-Length cDNA of Chicken Spexin and Galanin Receptors

According to the predicted cDNA sequence of *cSPX* (XM_015290798), we designed gene-specific primers and amplified the 5'-cDNA and 3'-cDNA ends of *cSPX* from the chicken brain by using SMART-RACE cDNA amplification kit (Clontech, Palo Alto, CA). The PCR products were inserted into pTA2 vector (TOYOBO,

Shanghai, China) for sequencing. To investigate the potency and specificity of cSPX in activating galanin receptors, 5 complete ORFs corresponding to distinct chicken galanin receptor subtypes (cGALR1, cGALR1L, cGALR2, cGALR2L, and GLAR3) were cloned into pcDNA3.1(+) expression vector (Invitrogen) for subsequent functional assays. The sequences of DNA plasmids were determined by Chengdu Tsingke Biotechnology.

Luciferase Reporter Assays

According to the previously established method (Gao et al., 2017), the functionality and signaling properties of cSPX in activating galanin receptors were evaluated by pGL4-SRE-Luciferase system. In brief, 1,000 ng of pGL4-SRE-luciferase reporter construct and 200 ng of pcDNA3.1 expression plasmid (or empty vector) were transfected into human embryonic kidney 293 (**HEK293**) cells. HEK293 cells were then sub-cultured into a 96-well plate for 24 h culture, and cells were treated by peptide for an additional 6 h at 37°C. Afterward, the cells were lysed by 1 × Passive lysis buffer (Promega) per well, and the luciferase activity was determined with the luciferase assay kit (Promega). The luciferase activities of peptide treatment groups were expressed as the relative fold increase compared with the control group (without peptide treatment).

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was conducted on the CFX96 Real-time PCR Detection System (Bio-Rad) in a volume of 20 μL by using EvaGreen (Biotium Inc., Hayward, CA) and a previously established method (Gao et al., 2017). 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 the specificity of PCR amplification. Finally, the mRNA levels of target genes were calculated relative to levels of *β-actin* and then expressed as the fold change compared to the chosen tissue or control.

Investigation of Fasting-modulated SPX Expression in Chick Hypothalamus

Two-wk-old male chicks were maintained at 30°C on a 12 h light and 12 h dark (12 L: 12 D) photoperiod and fed a commercial mixed diet (CP Group, Shijiazhuang, China) with free access to water. The feed components and chemical composition of the diets are shown in Supplementary Table 2. To determine whether fasting modifies the expression of *cSPX* mRNA in the hypothalamus, 24 chicks were randomly divided into 2 groups (N = 8). In the control group, chicks could access feed and water freely. In experimental groups, chicks drank water freely but were deprived of feed for 24 h or 36 h. And then, hypothalami were collected for RNA extraction and measuring the changes of c*SPX* mRNA by qRT-PCR.

Effect of cSPX on Feed Intake

Three-wk-old male chicks (with body weights of 160 −200 g) were randomly assigned to four groups (N ≥ 10). After feed deprivation for 12 h, chicks were intravenously injected with PBS (as control) or different dosages (10 ng/g, 50 ng/g, and 100 ng/g body weight) of cSPX respectively. After that, all chicks were free to drink and eat pre-weighed food. The leftover feed pellets were harvested and measured at 6 h, 12 h, and 24 h after injection. The mass difference between the leftover and total input of feed was used as an index for feed consumption.

Regulation of cSPX on the Expression of Appetite-related Genes

In this experiment, 3-wk-old male chicks were randomly divided into four groups (N ≥ 3). Chicks were intravenously injected with PBS or distinct doses of cSPX, and the hypothalami were sampled after 6 h administration. The mRNA levels of cocaine- and amphetamine-regulated transcript I (*CART1*), agouti gene-related protein (*AgRP*), proopiomelanocortin (*POMC*), melanin-concentrating hormone (*MCH*), and neuropeptide Y (*NPY*) were detected by qRT-PCR. The changes of the above appetite-related gene expression were also examined in the chick hypothalamus after 12 h and 24 h with cSPX injection by using the same treatment procedure.

Data Analysis

The data were analyzed by one-way ANOVA followed by Dunnett's test with the use of GraphPad Prism 7 (GraphPad Software, San Diego, CA). Data were expressed as means ± SEM, and statistical significance was set at $P < 0.05$.

RESULTS

Cloning the Full-length cDNA of cSPX

Using RT- and RACE-PCR, we cloned the full-length cDNA of *cSPX* from the adult chicken brain. As shown in Figure 1A, the cloned *cSPX* is 751-bp in length which is composed of 214-bp 5'-UTR, 351-bp ORF and 186-bp 3'-UTR with a 30-bp long polyA tail terminal. *cSPX* encodes a precursor of 116 amino acids with a 26-aa signal peptide and can produce a 14-aa mature peptide after proteolysis at 2 dibasic processing sites. A comparison of the *cSPX* cDNA sequence with the chicken genome (http://www.ensembl.org/Gallus_gallus) revealed that *cSPX* consists of 6 coding exons separated by 5 introns (Figure 1B). Amino acid sequence alignment shows the N- and C-terminal regions of SPX-precursors are highly variable among examined species (Figure 2A). In contrast, the 14-aa mature SPXs are conserved in the vertebrate lineage, since there is only

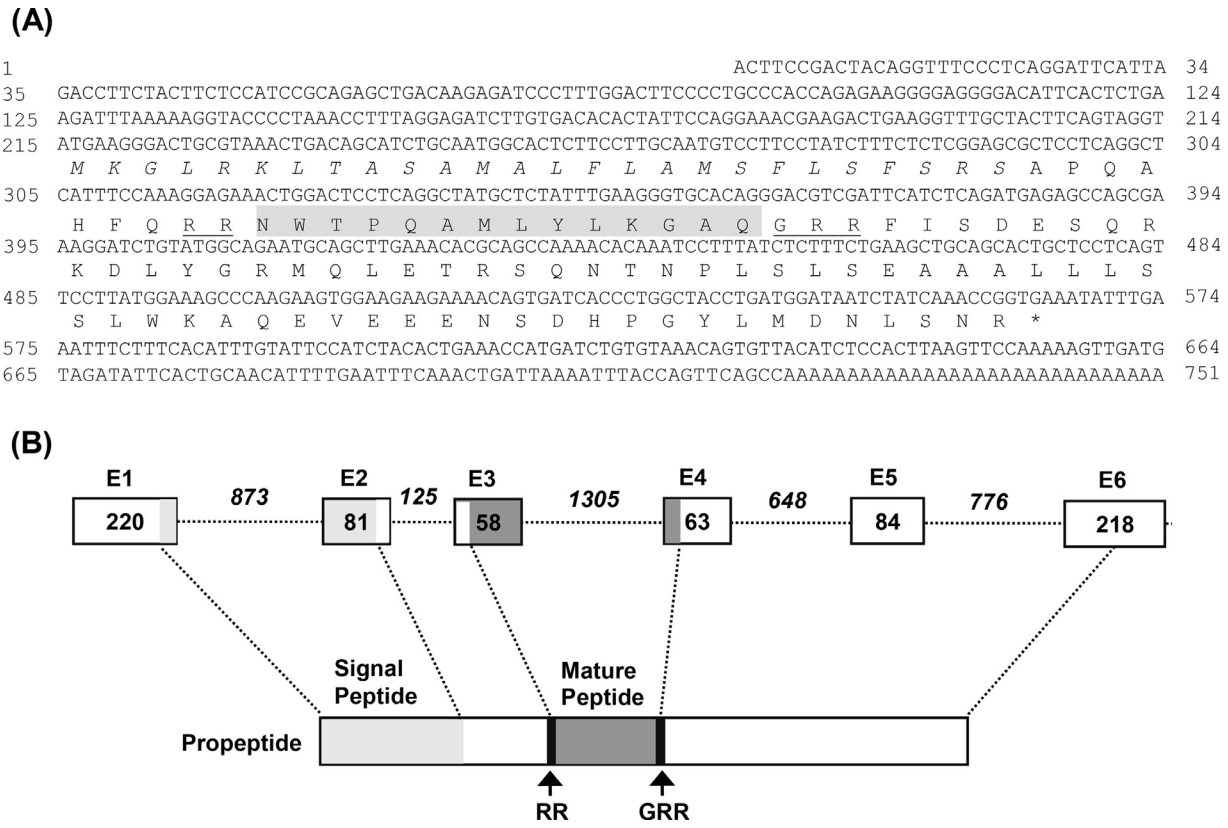

Figure 1. (A) The full-length cDNA sequence and deduced amino acid of chicken spexin (cSPX). The putative signal peptide is indicated by italic letters, and the mature peptide is shaded in gray, which is flanked by two dibasic cleavage sites (RR and GRR) dashed underline. (B) Genomic organization of *cSPX* gene. Six exons (E1-E6) are labeled, and the numbers in the boxes indicate the sizes (bp). The signal peptide is encoded by exon 1-2, and the coding sequences of the mature peptide are located in the exon 3 and 4, which contain dibasic cleavage sites indicated by arrows.

one substitution in their amino acid composition at most (Figure 2B).

The Potency of cSPX in Activating Chicken Galanin Receptors

To investigate whether cSPX is functional in arousing chicken galanin receptors, each galanin receptor subtype expressed in HEK293 cells was treated by chemically synthetic peptides. Receptor-regulated MAPK/ERK signaling pathway was then examined by the pGL4-SRE-luciferase reporter system.

As shown in Figure 3, cGAL could dose-dependently stimulate luciferase activity in HEK293 cells expressing cGALR1 and cGALR1L, but only a high concentration of cSPX treatment slightly altered the luciferase activity of cGALR1L expressed in HEK293 cell, suggesting that cGALR1 and cGALR1L are not functional receptors for cSPX. Instead, both cGAL and cSPX could initiate cGALR2, cGALR2L, and cGALR3 activation in HEK293 cells (Figure 3C–E). Although the potency of cGAL in triggering cGALR2 is much greater than cSPX, cSPX has a higher affinity with cGALR2L and cGALR3 than cGAL. Moreover, SPX is more likely to bind GALR2L among the 5 galanin receptor subtypes. The EC_{50} values of cSPX and cGAL for distinct receptors are summarized in Table 1.

Tissue Expression of SPX in Adult Chickens

In order to explore the physiological role of *SPX* in chicken, its mRNA expression profile was detected in various adult chicken tissues. qRT-PCR showed *cSPX* was widely expressed in all tissues examined, with abundant expression in the whole brain, spinal cord, pituitary, testes, and pancreas (Figure 4A). Additionally, weak *SPX* mRNA level was also noted in other tissues, including the heart, adrenal gland, duodenum, kidney, liver, lung, muscle, skin, spleen, ovary, and abdominal fat. Since *SPX* is highly expressed in the chicken's whole brain, we further detected its expression in discrete brain regions. As shown in Figure 4B, the expression signal of *cSPX* was ubiquitous in various brain regions, especially abundant in the hypothalamus and hindbrain.

Relationship between cSPX and Feeding Status

Since SPX can act as a satiety factor in goldfish, considering the abundant *SPX* mRNA level in chicken hypothalamus, we investigated whether fasting could

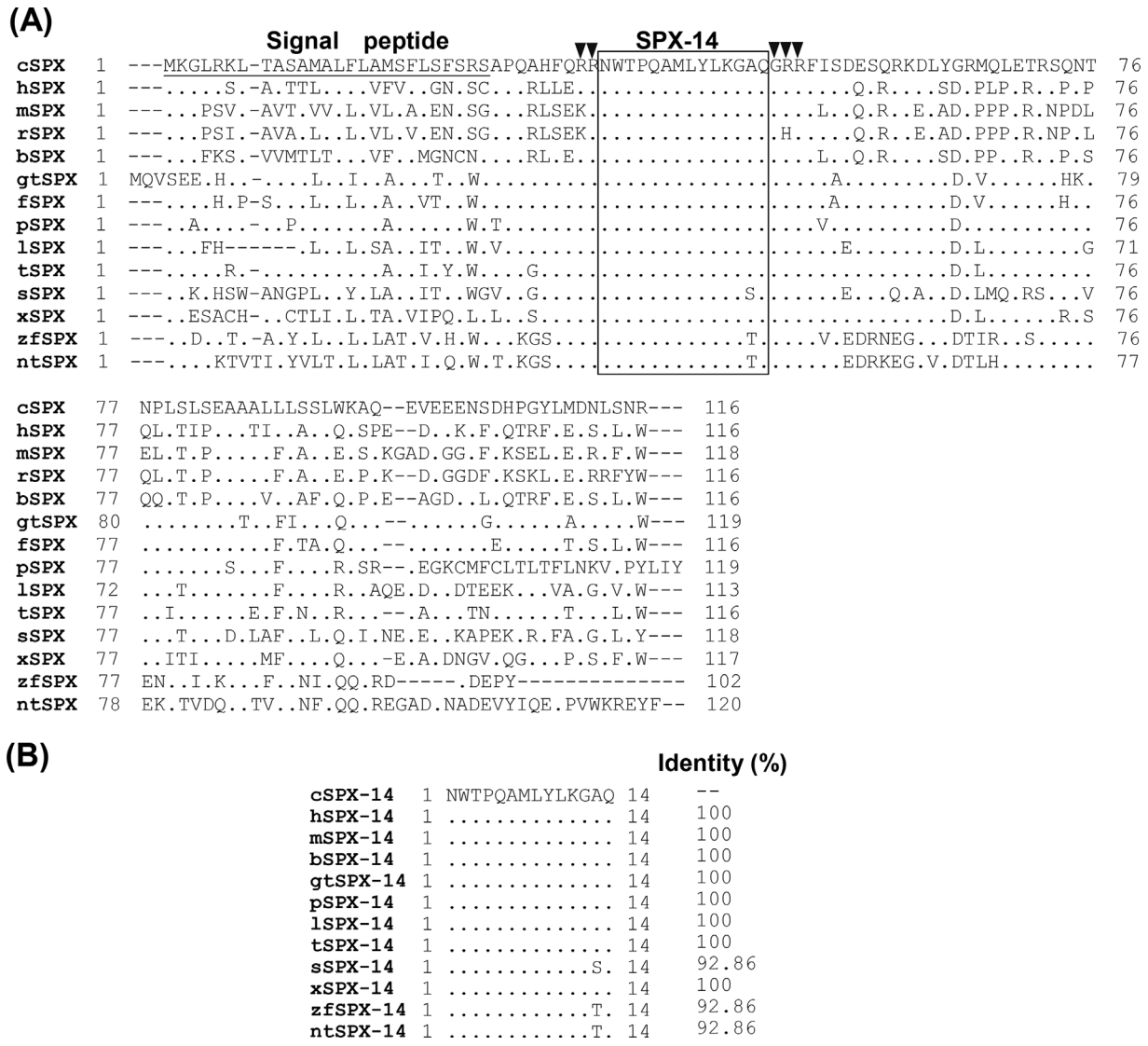

Figure 2. (A) Amino acid alignment of chicken spexin precursor (cSPX) with that of different species, inducing human (hSPX, NM_001083933), mouse (mSPX, NM_ 001242345), rat (rSPX, NM_001083933), bovine (bSPX, NM_001075407), great tit (gtSPX, XP_015482461), medium ground finch (fSPX, XP_005429821), pigeon (pSPX, XP_013223962), bearded dragon lizards (lSPX, XP_020656732), goodes thornscrub tortoise (tSPX, XP_030405032), eastern brown snake (sSPX, XP_026558826), *Xenopus tropicalis* (xSPX, XM_031898436), zebrafish (zfSPX, XM_005164774), and nile tilapia (ntSPX, XP_005475162). The predicted signal peptide is underlined, and arrows indicate the putative cleavage sites.The mature SPX peptide (SPX-14) is boxed. (B) Sequence conservation of SPX-14 between chicken and other vertebrate species from human (h), mouse (m), bovine (b), great tit (gt), pigeon (p), bearded dragon lizards (l), goodes thornscrub tortoise (t), eastern brown snake (s), *Xenopus tropicalis* (x), zebrafish (zf), and nile tilapia (nt). Note: Dots indicate amino acids identical to cSPX in (A) or cSPX-14 in (B), and dashes represent gaps in the sequence.

alter hypothalamic *SPX* expression in 2-wk-old chicks. As shown in Figure 5, it was noted that *SPX* mRNA expression was significantly up-regulated in the chicken hypothalamus after fasting for 24 h and 36 h. Afterward, cSPX injection on feed intake was also explored in the present study. After drug administration, cumulative feed intake of chicks exhibited downwards tendencies (Table 2), whereas no significant difference was examined after 6 h and 24 h postinjection of low dose of cSPX (10 ng/g BW). Nonetheless, when moderate (50 ng/g BW) and high (100 ng/g BW) cSPX dosage was injected into chicks, the inhibitory effect of feed intake was significant at all checking time points (6 h, 12 h, and 24 h). Moreover, after 12 h post-injection, even a low amount (10 ng/g BW) of cSPX could trigger a marked reduction of food intake in chicks.

Effect of cSPX on the mRNA Expression of Appetite-related Genes

To verify whether cSPX is an appetite modulator, a time course experiment was initiated to examine intravenous injection of cSPX-14 on hypothalamic appetite-related genes in 3-wk-old chicks. As shown in Figure 6, cSPX significantly stimulated *CART1* mRNA levels in the chick hypothalamus at 6 h, 12 h, and 24 h post-injection. Oppositely, *MCH* expression was negatively regulated at 12 h and 24 h after 100 ng/g cSPX intravenous administration, and its expression was also reduced at 12 h when a dose of 50 ng/g treatment was used. Meanwhile, a high dosage (100 ng/g BW) of cSPX could obviously inhibit *AgRP* mRNA level from 6 h to 24 h after administration. Nevertheless, cSPX is unavailable for

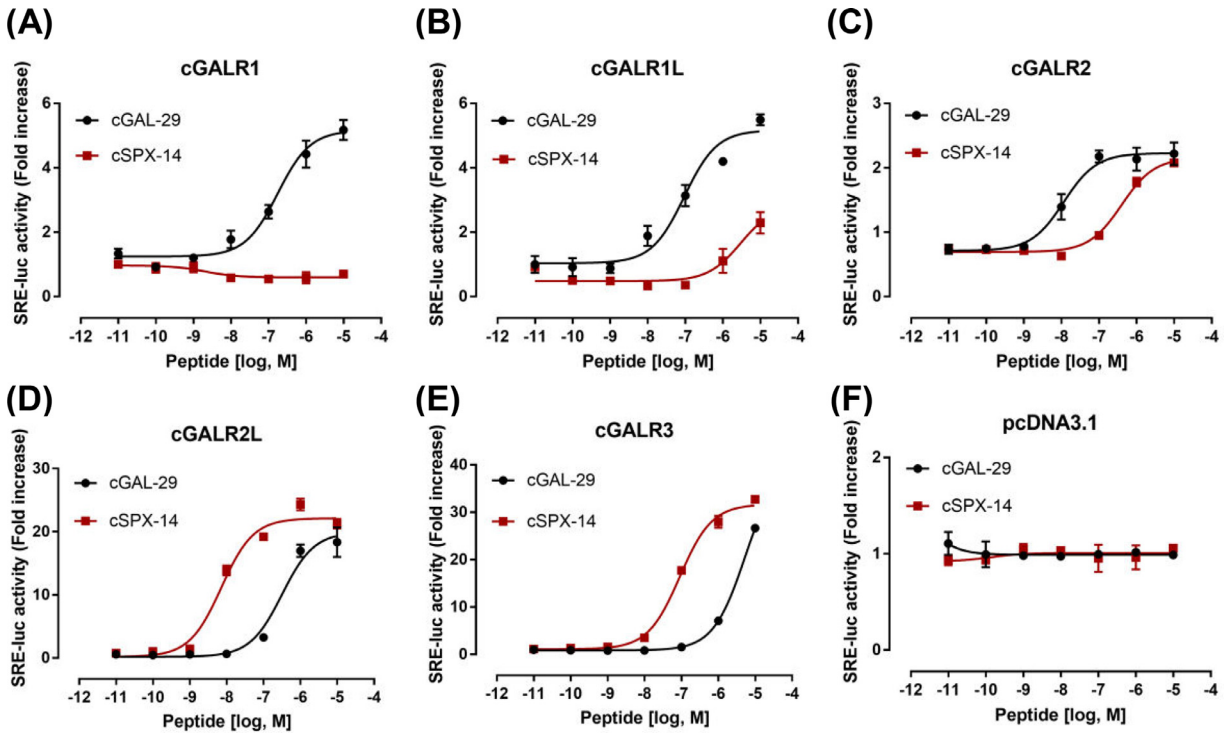

Figure 3. Effects of cSPX-14 and cGAL-29 (10^{-11}-10^{-5} M, 6 h) on activation of the intracellular MAPK/ERK signaling pathway in HEK293 cells expressing cGALR1 (A), cGALR1L (B), cGALR2 (C), cGALR2L (D) and cGALR3 (E), monitored by pGL4-SRE-luciferase reporter system. Co-transfection of the pGL4-SRE-luciferase reporter construct and empty pcDNA3.1(+) vector was used as an internal control (F). Each data point represents the mean ± SEM of three replicates (N = 3).

Table 1. EC_{50} values of cSPX-14 and cGAL-29 in activating MAPK/ERK signaling pathway in HEK293 cells expressing chicken galanin receptors.

Peptides	EC_{50} (nM)				
	cGALR1	cGALR1L	cGALR2	cGALR2L	cGALR3
cGAL-29	166.40 ± 5.69	96.68 ± 2.72	12.86 ± 1.30	277.20 ± 124.40	6685 ± 1339
cSPX-14	-	-	277.20 ± 124.40	18.39 ± 7.06	62.13 ± 19.08

Data are expressed as mean ± SEM (N = 3).

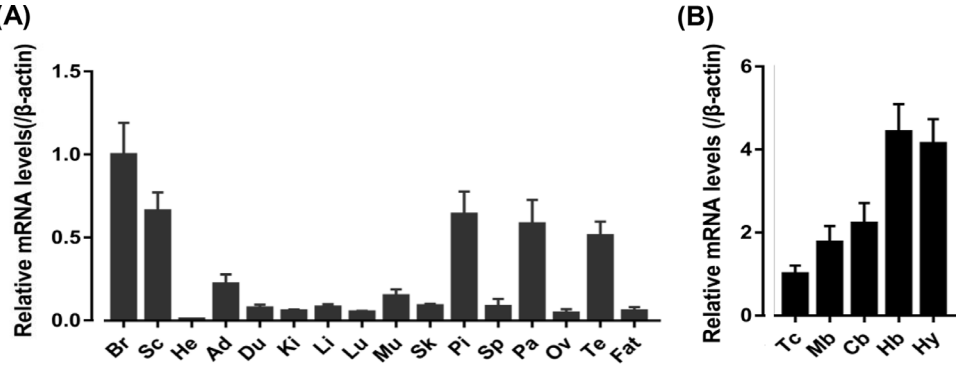

Figure 4. Tissue distribution of *cSPX* in adult chickens. (A) Quantitative real-time PCR detection the mRNA level of *cSPX* in multiple adult chicken tissues, including brain (Br), spinal cord (Sc), heart (He), adrenal (Ad), duodenum (Du), kidney (Ki), liver (Li), lung (Lu), muscle (Mu), skin (Sk), pituitary (Pi), spleen (Sp), pancreas (Pa), ovary (Ov), testis (Te), and abdominal fat (Fat). (B) The *cSPX* expression in adult chicken brain regions including telencephalon (Tc), midbrain (Mb), cerebellum (Cb), hindbrain (Hb), and hypothalamus (Hy). The mRNA expression of *cSPX* was normalized to that of *β-actin* and expressed as the fold difference compared with that of the brain (Br) or telencephalon (Tc). Each data point represents the mean ± SEM of 6 adult chickens (N = 6).

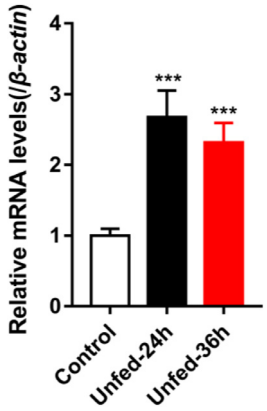

Figure 5. Changes of *cSPX* mRNA expression in the hypothalami of 2-wk-old chicks which were deprived of food for 24 h and 36 h. The control group chicks were provided adequate feed, and *β-actin* was used to normalize all samples. Each data point represents means ± SEM of 8 individuals (N = 8). $^{***}P < 0.001$ vs. control.

Table 2. Feed intake of chicks after peripheral administration of cSPX for 6 h, 12 h, and 24 h.

Time post-injection (hours)	Groups	Cumulative feeding (g)
6	Control	14.05 ± 0.8581
	cSPX-14 (10 ng/g, BW)	11.85 ± 0.6884 (P = 0.0525)
	cSPX-14 (50 ng/g, BW)	10.75 ± 0.6099*
	cSPX-14 (100 ng/g, BW)	10.58 ± 0.9122*
12	Control	45.62 ± 4.44
	cSPX-14 (10 ng/g, BW)	33.73 ± 2.361*
	cSPX-14 (50 ng/g, BW)	29.93 ± 2.452**
	cSPX-14 (100 ng/g, BW)	29.53 ± 1.7**
24	Control	52.22 ± 2.521
	cSPX-14 (10 ng/g, BW)	46.13 ± 3.144
	cSPX-14 (50 ng/g, BW)	42.68 ± 2.829*
	cSPX-14 (100 ng/g, BW)	39.84 ± 2.023**

Each data point represents the mean ± SEM.
*$P < 0.05$.
**$P < 0.01$ vs. respective control.

modulating *cNPY* and *cPOMC* mRNA levels at all checking time points.

DISCUSSION

In mammal and fish species, numerous pieces of research were invested to identify the physiological significance of SPX and got great achievements. However, whether SPX exists in avians is not clear, which hugely impedes us to clarify its functional evolution across vertebrates. In this study, the full-length cDNA of chicken *SPX* was cloned and found to be composed of 6 exons and 5 introns, which is identical to that in orange-spotted grouper, tilapia, mice and humans (Li et al., 2016). However, zebrafish and goldfish *SPX* consist of 5 exons and 4 introns (Wong et al., 2013; Zheng et al., 2017), indicating the genomic organization of *SPX* is not highly conserved in vertebrates. Nor is this all, the SPX-precursors are highly variable in their amino acid sequence among species. Nonetheless, apart from residue at position 13, its mature peptide is well conserved from fish to mammals, suggesting the physiological roles of SPX might be still conserved and essential for the survival of vertebrates.

Like other neuropeptides, the SPX-related biological roles were triggered by binding membrane-bound GPCR and subsequent mobilization of intracellular signaling pathways (Wong et al., 2013). Before 2014, SPX remained to be an orphan ligand. Since *SPX, KISS,* and *GAL* were demonstrated to arise from a common ancestor, it raised doubt on whether SPX could activate GAL or KISS receptors. Then, SPX-14 was proved to have higher affinity with GALR2 and GALR3 than GAL, but was incompetent in activating GALR1 (Kim et al., 2014), indicating GALR2-3 can serve as the functional receptors for SPX-14. However, whether chicken SPX could activate GALRs remains to be uncovered. Besides, the discovery of two novel GALR-like members (Ho et al., 2011, 2012) makes it more complicated to understand the ligand-receptor interaction manner. After binding ligands, GALRs were generally considered to be responsible for the stimulation of the intracellular MAPK/ERK cascade (Liu and Hokfelt, 2002; Sipkova et al., 2017). Herein, the capacities of cSPX-14 in activating several cGALRs were examined by using a pGL4-SRE-luciferase reporter assay. Similar to previous reports from mammals and fish (Kim et al., 2014; Cohen et al., 2020), we found cSPX-14 failed to efficiently activate cGALR1, as well as its analogous receptor (GALR1L), but successfully aroused cGALR2, cGALR2L, and cGALR3 activation. Meanwhile, we mentioned that GAL is more potent than SPX in activating GALR2. However, upon ligand treatment, cGALR2L and cGALR3 are much more sensitive to recognize cSPX-14, rather than cGAL, suggesting that SPX might be a natural ligand for GALR2L and GALR3 in chicken. Thus, we speculate that SPX-involved physiological functions including appetite regulation may attribute to these two receptors, especially GALR2L-stimulated MAPK/ERK cascade.

To explore the physiological roles of *cSPX*, we detected its tissues distribution in adult chickens by utilizing qRT-PCR. As expected, *cSPX* mRNA was found to be highly expressed in the CNS including the hypothalamus and hindbrain, which is consistent with the findings in the rat (Porzionato et al., 2010), goldfish (Wong et al., 2013), Siberian sturgeon (Tian et al., 2020), orange-spotted grouper (Li et al., 2016), Ya-fish (Wu et al., 2016), and half-smooth tongue sole (Wang et al., 2018). The abundant expression of *SPX* in the CNS supports the notion that SPX may act as a neurotransmitter or neuromodulator involved in the regulation of anxiety behaviors and feed intake, which has been confirmed in fish (Jeong et al., 2019) and mammals (Reyes-Alcaraz et al., 2016; Zhuang et al., 2020), and further demonstrated in chicken by our present study. Apart from CNS, *cSPX* mRNA was also detected in several peripheral tissues including the pituitary and gonad, stating it may participate in the modulation of reproduction mediated by the "hypothalamic-pituitary-gonad" axis in chicken. Coincidentally, the roles of *SPX* in regulating reproduction have been confirmed in some fish

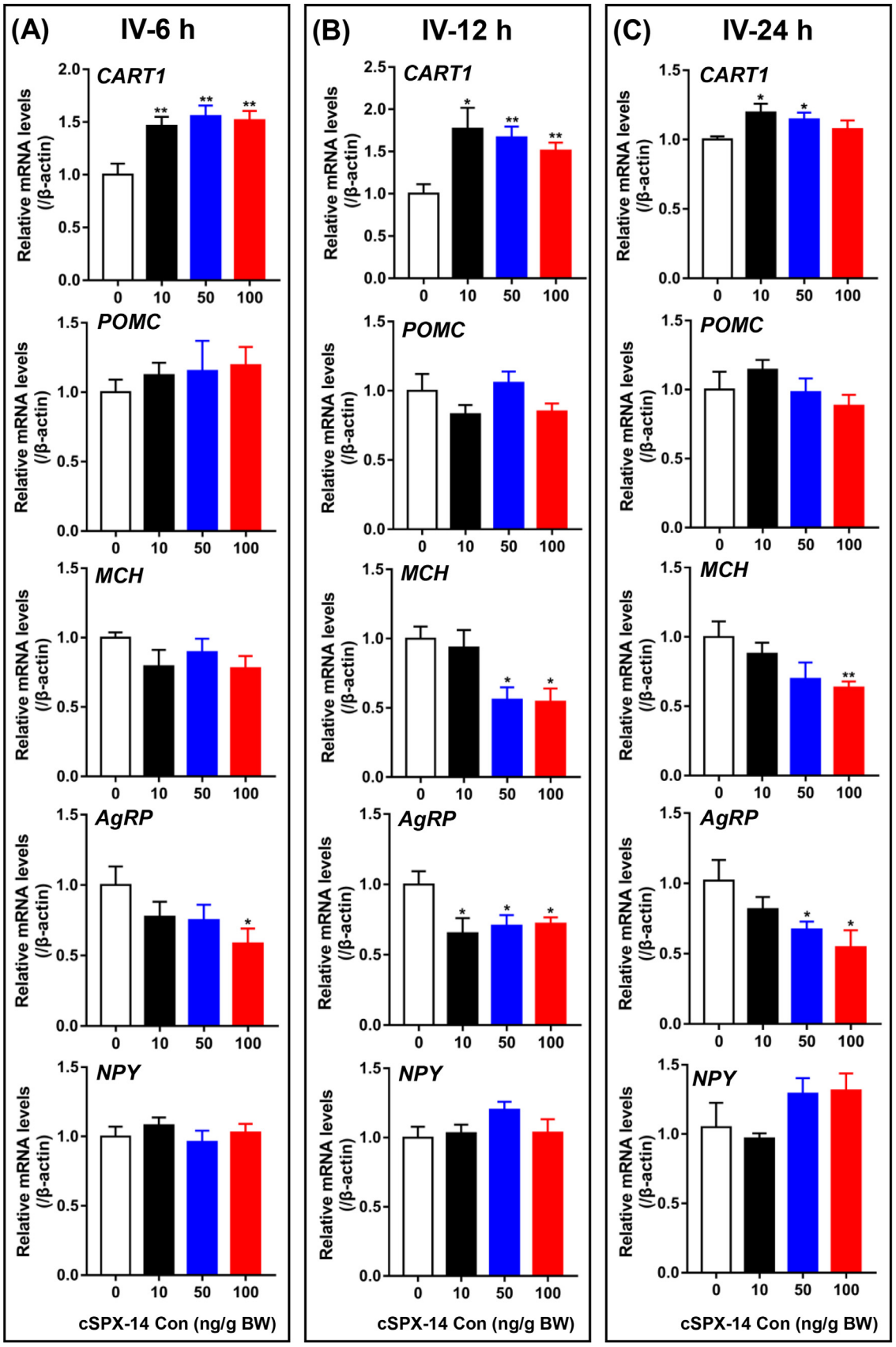

Figure 6. In vivo effects of cSPX on the expression of *CART1, POMC, MCH, AgRP*, and *NPY* in the hypothalami of 3-wk-old chicks. Chicks were intravenously injected with different doses of cSPX (10 ng/g, 50 ng/g, and 100 ng/g BW). The hypothalami were collected at 6 h (A), 12 h (B), and 24 h (C) post-injection, and then the mRNA expressions of appetite-regulating factors were detected by qRT-PCR. All relative expressions were first calculated as the ratio to that of *β-actin* and as the fold compared to that of control group chicks injected with PBS. Each data point represents as the mean ± SEM of at least 3 individuals (N ≥ 3). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. respective control.

species. For instance, SPX was demonstrated to inhibit the release or synthesis of gonadotrophins in sexually matured female goldfish (Liu et al., 2013), adult female tilapia (Cohen et al., 2020), and half-smooth tongue sole (Wang et al., 2018). However, SPX does not impact the basal and KISS-induced luteinizing hormone (**LH**) release in the ewe (Lomet et al., 2020), indicating the functional difference of SPX in regulating reproduction may be related to species. Therefore, whether SPX can act as a reproductive modulator in other species needs to be further verified, such as in chicken. In addition, our results showed that the chicken pancreas had a high level of *SPX* expression, which is in accordance with previous findings from rodents (Porzionato et al., 2010; Wong et al., 2021a) and humans (Gu et al., 2015). In obese women, serum SPX had a negative correlation with insulin and glucagon content (Kołodziejski et al., 2018), and the release of SPX in the pancreas was stimulated by glucose and inhibited by insulin in pigs (Sassek et al., 2019). Accordingly, it is logical to speculate that SPX might be also implicated in insulin resistance and glycemic control in chicken. The ubiquitous expression of *SPX* is corroborating its pleiotropic biological roles in vertebrates, but the physiological functions of SPX in birds are still largely unknown and remain to be elucidated.

In mammals and fish, it has been well-acknowledged that SPX is an appetite regulator. In this study, we found chicken *SPX* was abundantly expressed in discrete brain regions including the hypothalamus, raising a possibility that SPX can modulate feed intake in birds as well. In agreement with this hypothesis, *SPX* mRNA was significantly up-regulated in chicks after food deprivation, which is analogous to that in fish species including orange-spotted grouper (Li et al., 2016), half-smooth tongue sole (Wang et al., 2018), and spotted scat (Deng et al., 2018). In goldfish, intracerebroventricular (**ICV**) injection of SPX inhibited both feeding behavior and feed consumption (Wong et al., 2013). Likewise, intraperitoneal (**IP**) injection assay demonstrated that SPX could also lead to feeding inhibition in Siberian sturgeon (Tian et al., 2020), mice (Lv et al., 2020; Wong et al., 2021b), and diet-induced obese rat (Walewski et al., 2014). Meanwhile, SPX-caused weight loss was also noted in rodents (Kolodziejski et al., 2021). These achievements suggest that SPX is a satiety factor, which is consistent with the result from our present study since intravenous injection of cSPX was confirmed to suppress feed intake in 3-wk-old chicks. Taken together, these findings clearly proclaim that the role of SPX in regulating animals feeding behavior is well conserved from fish to mammals.

As the crucial center controlling energy intake and expenditure, the hypothalamus can secrete orexigenic and anorexigenic neuropeptides, which are implicated in the modulation of feeding and energy homeostasis (Parker et al., 2012). As of now, a series of neuropeptides including SPX were affirmed to participate in appetite regulation (Mills et al., 2021). Although the exact mechanism is not clear, SPX-modulated feed intake has been proven to be associated with the expression and secretion change of hypothalamic neuropeptides (Wong et al., 2013). After cSPX-14

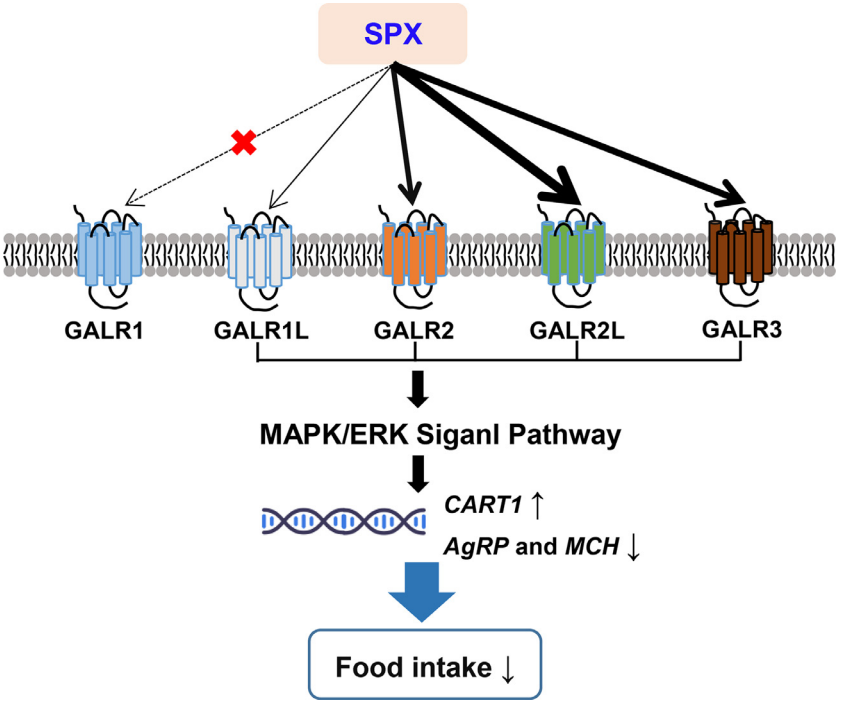

Figure 7. Proposed model for SPX in control of chicken food intake. cSPX can effectively activate cGALR2, cGALR2L, and cGALR3, and mediate intracellular MAPK/EKR signal pathways. Whereafter, SPX acts as a satiety factor by decreasing the expressions of *AgRP* and *MCH*, along with the concurrent rise in *CART1* mRNA level in the chicken hypothalamus. The thickness of the lines represents the capacity of SPX in activating chicken galanin receptors.

injection, *AgRP*, the well-known orexigenic gene, was reduced in the chick hypothalamus, which is tally with the findings from goldfish (Wong et al., 2013), zebrafish (Zheng et al., 2017) and mouse (Lv et al., 2020). Unexpectedly, although SPX could elevate *POMC* expression in goldfish (Wong et al., 2013), zebrafish (Zheng et al., 2017), and orange-spotted grouper (Li et al., 2016), it failed to change the mRNA level of *POMC* in mouse (Lv et al., 2020) and chicken. In chicken, *CART1* is an energy status-dependent gene and its expression could be induced by fasting, hinting at its involvement in the feeding inhibition of chicken (Cai et al., 2015). This speculation was confirmed by our observation that cSPX, a putative satiety factor, could stimulate *CART1* expression in the chicken hypothalamus, which is similar to that in Siberian sturgeon (Tian et al., 2020), but not the mouse (Lv et al., 2020) or zebrafish (Zheng et al., 2017). In chicken, *MCH* expression could be induced by feed deprivation (Cui et al., 2017), as that in the rat (Qu et al., 1996) and winter flounders (Tuziak and Volkoff, 2012), indicating its potential role in promoting feed intake. Coincidentally, SPX-mediated anorexia is accompanied by decreased *MCH* expression, which is opposite to that in goldfish (Wong et al., 2013). Surprisingly, as a well-documented appetite-suppressive factor (Mercer et al., 2011), expression of *NPY* could not be altered by cSPX injection in chicken hypothalamus, which is consistent with the findings in zebrafish (Zheng et al., 2017) and orange-spotted grouper (Li et al., 2016). To sum up, these pieces of evidence suggest the working mechanism of the SPX-mediated anorexic effect might be similar, but not identical, to that in mammal and fish.

In summary, chicken *SPX*, which is capable of generating a conserved mature peptide with 14-aa, has been successfully identified in our present study. Luciferase assays showed cSPX-14 might be the endogenous ligand for GALRL2L and GALR3. Using qRT-PCR, we found *SPX* distributed widely in various tissues of adult chickens, especially abundantly in the hypothalamus, the feeding regulatory center. Expectedly, *SPX* was proved to be a potential satiety gene in chicken, which may play roles via modulating other hypothalamic appetite-related neuropeptides expression (Figure 7). Collectively, these findings will lead to a better understanding of SPX-involved physiological roles in birds, as well as its functional evolution across vertebrates.

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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.2022.102279.

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