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Research paper

Molecular characterization of neuropeptide Y (NPY) receptors (Y1, Y4 and Y6) and investigation of the tissue expression of their ligands (NPY, PYY and PP) in chickens

Shunyu Gao [a,b,1], Jiannan Zhang [a,1], Chen He [a], Fengyan Meng [a], Guixian Bu [a], Guoqiang Zhu [a], Juan Li [a], Yajun Wang [a,*]

[a] Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610064, PR China
[b] College of Chemistry and Life Sciences, Chuxiong Normal University, Chuxiong 675000, PR China

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ABSTRACT

Neuropeptide Y (NPY) receptors and its ligands, NPY, peptide YY (PYY) and pancreatic polypeptide (PP), are suggested to regulate many physiological processes including food intake in birds. However, our knowledge regarding this avian NPY system remains rather limited. Here, we examined the tissue expression of *NPY*, *PYY* and *PP* and the gene structure, expression and signaling of three NPY receptors (cY1, cY4 and cY6) in chickens. The results showed that 1) *NPY* is widely expressed in chicken tissues with abundance noted in the hypothalamus via quantitative real-time PCR, whereas *PYY* is highly expressed in the pancreas, gastrointestinal tract and various brain regions, and *PP* is expressed almost exclusively in the pancreas; 2) *cY1*, *cY4* and *cY6* contain novel non-coding exon(s) at their 5′-UTR; 3) The wide tissue distribution of *cY1* and *cY4* and *cY6* were detected in chickens by quantitative real-time PCR and their expression is controlled by the promoter near exon 1, which displays strong promoter activity in DF-1 cells as demonstrated by Dual-luciferase reporter assay; 4) Monitored by luciferase reporter assays, activation of cY1 and cY4 expressed in HEK293 cells by chicken NPY_{1-36}, PYY_{1-37}, and PP_{1-36} treatment inhibits cAMP/PKA and activates MAPK/ERK signaling pathways, while cY6-expressing cells show little response to peptide treatment, indicating that cY1 and cY4, and not cY6, can transmit signals *in vitro*. Taken together, our study offers novel information about the expression and functionality of cY1, cY4, cY6 and their ligands in birds, and helps to decipher their conserved roles in vertebrates.

1. Introduction

Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) are structurally and functionally related peptides belonging to the NPY family (Cerdá-Reverter and Larhammar, 2000). These peptides are composed of 36 (or 37) amino acids and characterized by the presence of a PP-fold and an amidated C-terminus (Blundell et al., 1981; Larhammar, 1996). In this family, NPY is the most conserved peptide among vertebrate species, while PYY and PP peptides show more divergence (Blomqvist et al., 1992; Cerdá-Reverter and Larhammar, 2000). In mammals, NPY is reported to be expressed in the central and peripheral nervous systems at all levels of the gut-brain axis and involved in the regulation of many physiological processes, including food intake, stress, circadian rhythm, vasoconstriction, gastrointestinal activity, reproduction and bone mass (Baldock et al., 2009; Holzer et al., 2012; Pedrazzini et al., 2003). PYY is mainly expressed in intestinal endocrine L cells and regulates food intake, gastric emptying, intestinal motility, and pancreatic functions (Persaud and Bewick, 2014). PP is predominantly expressed in endocrine F cells of pancreatic islets and involved in the control of gastrointestinal motility and secretion, and food intake (Holzer et al., 2012).

In mammals, the biological actions of the three peptides are reported to be mediated by five NPY receptors (Y), namely Y1, Y2, Y4, Y5, and Y6, respectively (Michel et al., 1998). Y1, Y2 and Y5 preferentially bind to NYP and PYY, while Y4 preferentially binds to PP, hence generally viewed as a PP-specific receptor (Cerdá-Reverter and Larhammar, 2000). *Y6* is a pseudogene in some mammalian species, *e.g.* primates, while it seems to be functional in mice and rabbits (Gregor et al., 1996a; Matsumoto et al., 1996; Starbäck et al., 2000). As in mammals, 7 or 8 NPY receptors (named Y1, Y2, Y4, Y5, Y6, Y7, Y8a, and Y8b) have been identified in

* Corresponding author.
E-mail address: cdwyjhk@gmail.com (Y. Wang).
[1] Authors contributed equally to this work.

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cartilaginous fish, teleosts, and frogs (Larhammar and Bergqvist, 2013; Larsson et al., 2008, 2009; Salaneck et al., 2008; Sundström et al., 2013, 2012). Based on the amino acid sequence similarity and hypothesized evolutionary origin of Y receptors, these receptors have been subdivided into three subfamilies: Y1-subfamily (Y1, Y4, Y6, and Y8), Y2-subfamily (Y2 and Y5) and Y5-subfamily (Y5) (Larsson et al., 2008, 2009). All Y receptors are reported to be coupled to $G_{i/o}$ protein and their activation can inhibit cAMP/protein kinase A (PKA) signaling pathway. Moreover, there is evidence showing that activation of Y receptors also causes intracellular inositol trisphosphate (IP3) accumulation and calcium mobilization, and activates ERK signaling pathway (Michel et al., 1998; Mullins et al., 2002; Pedrazzini et al., 2003; Persaud and Bewick, 2014).

In birds, NPY, PYY and PP peptides were identified more than two decades ago (Blomqvist et al., 1992; Conlon and O'Harte, 1992; Kimmel et al., 1975), however, our knowledge regarding their expression and physiological roles remains rather scarce. More intriguingly, *PYY* gene still has not been cloned in any avian species to date. Although NPY, PYY and PP are also suggested to be involved in the regulation of food intake, body weight, adipogenesis, gastrointestinal activity, and pancreatic function in birds (Denbow and Cline, 2015; Denbow et al., 1988; Hazelwood et al., 1973; Kuenzel, 1994; Zhang et al., 2015), their actions seem to be similar, but not identical, to those reported in mammals. For instance, in chickens, NPY, PYY and PP can stimulate food intake via central administration (Denbow et al., 1988; Kuenzel et al., 1987; Pedrazzini et al., 2003), whereas in mammals, NPY has a powerful orexigenic effect after ICV injection, and PYY and PP mainly act as appetite suppressors (Holzer et al., 2012). Undoubtedly, more in-depth studies are required to reveal the functional difference of NPY family members between birds and mammals, for instance, whether NPY, PYY, PP and their receptors are expressed and function in birds in ways similar to, or distinct from, that in mammals. Although the pioneering studies identified the 6 Y receptors (Y1, Y2, Y4, Y5, Y6, and Y7) and demonstrated that some of them can bind to mammalian (or chicken) NPY, PYY and PP peptides (Bromée et al., 2006; Holmberg et al., 2002; Lundell et al., 2002; Salaneck et al., 2000), the gene structure and signaling properties of these Y receptors have not been fully characterized in birds. Therefore, using chicken as an animal model, the present study aims to: 1) clone *PYY* gene and examine the tissue expression patterns of *NPY*, *PYY* and *PP*; 2) investigate the gene structure, tissue expression and signaling properties of three NPY receptors (Y1, Y4 and Y6) belonging to the Y1-subfamily, while the remaining three NPY receptors (Y2, Y5 and Y7) has been reported in another study (He et al., 2016). Results from the present study will not only facilitate a better understanding of the physiological roles of NPY, PYY and PP and their receptors in birds, but also help to decipher the conserved roles of the NPY system in vertebrates.

2. Materials and methods

2.1. Chemicals, reagents, antibodies, and primers

All the chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and the restriction enzymes were obtained from Takara (Takara, Dalian, China) unless stated otherwise. Chicken neuropeptide Y ($cNPY_{1-36}$), peptide YY ($cPYY_{1-37}$) and pancreatic polypeptide (cPP_{1-36}) were synthesized with solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). The purity of the synthesized chicken peptides is >95% (analyzed by HPLC), and their structures were verified by mass spectrometry (GL Biochem). Antibodies for β-actin and phosphorylated ERK1/2 (Cat. no: 4370S) were purchased from Cell Signaling Technology Inc. (CST, Beverly, MA). All primers used in this study are listed in Table 1 and were synthesized by Beijing Genome Institute (BGI, Shanghai).

2.2. Total RNA extraction

Adult chickens were purchased from local commercial companies or local markets. Different tissues including heart, gastrointestinal tract (crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, and rectum), kidneys, liver, lung, muscle, ovary, testes, anterior pituitary, spleen, adipose tissue, pancreas, and various brain regions (telencephalon, midbrain, cerebellum, hindbrain and hypothalamus) were collected. Total RNA was extracted using RNAzol (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. All experiments were performed according to the guidelines provided by the Animal Ethics Committee of Sichuan University.

2.3. Reverse transcription (RT)

Two μg of total RNA isolated from different tissues and 0.5 μg oligodeoxythymidine were mixed in a total volume of 5 μL, incubated at 70 °C for 10 min and 4 °C for 2 min. Then, 5 × M-MLV buffer containing 0.5 mM each deoxynucleotide triphosphate and 100 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Takara) were added into the cold mixture to a final total volume of 10 μL. Reverse transcription (RT) was performed at 42 °C for 90 min and diluted by 50 μL H_2O. These RT samples were then used for quantitative real-time PCR assay (qPCR) of mRNA expression of target genes in chicken tissues.

2.4. Cloning the full-length cDNA of chicken PYY gene

Based on the predicted turkey *PYY* cDNA sequence deposited in GenBank (XM_010728076), several pairs of primers were designed to amplify a cDNA fragment of *PYY* from chicken small intestine by PCR. Then, the amplified PCR product was cloned into pTA2 vector (TOYOBO, Osaka, Japan) and sequenced. According to the sequence of the cloned chicken *PYY* cDNA fragment, new gene-specific primers were designed to amplify the 5′ and 3′-cDNA ends of *cPYY* by using SMART-RACE kit according to the manufacturer's instruction (Clontech, Palo Alto, CA). The amplified PCR products were then cloned into pTA2 vector through T/A cloning and sequenced. Finally, the full-length cDNA of *cPYY* was determined based on the sequences of 5′- and 3′-cDNA ends with an overlapping region.

2.5. Quantitative real-time PCR assay of NPY, PYY, PP, Y1, Y4, and Y6 mRNA expression in chicken tissues

To examine the mRNA levels of *NPY*, *PYY*, *PP*, *Y1*, *Y4*, and *cY6* in chicken tissues, quantitative real-time PCR assay (qPCR) was used in this experiment. The real-time PCR was conducted on the CFX96 Real-time PCR Detection System (Bio-Rad), as described in our recent study (Cai et al., 2015). The mRNA levels of target genes were first calculated as the ratio to that of *β-actin* and then expressed as the fold difference compared to that of telencephalon or hypothalamus.

2.6. Isolation of the 5′-untranslated region (5′-UTR) of chicken Y1, Y4, and Y6

To determine whether chicken *Y1 (cY1)*, *Y4 (cY4)* and *Y6 (cY6)* contain additional exons at their 5′-UTR, 5′-RACE PCR was performed to amplify the 5′-UTR of c*Y1*, c*Y4* and c*Y6* using SMART-RACE Kit (Clontech) according to manufacturer's instruction. The PCR products were cloned into pTA2 vector (TOYOBO) and sequenced (BGI). The 5′-UTR of each gene was determined by

Table 1
Primers used in this study.[a]

Gene/construct name	Sense/antisense	Primer sequence (5′-3′)	Size (bp)
Primers used for 5′-RACE or 3′-RACE			
cPYY	Sense	CCTGTGCTGTCACCCCACTGCCTG	
	Sense	ACTGCCTGGCACCATGGCGCTGTC	
	Antisense	ACCAGGTTGATGTAATGGCGCAGCG	
	Antisense	GCGCCGAGAAGTACTGCGCGATCTC	
cY1	Antisense	CCGAGACGGTGATCGAGGCACATTG	
	Antisense	CAGGGCCAGATTCCCAGAGACTCCC	
cY4	Antisense	GACACTGTCACAGATGTGCACTG	
	Antisense	CCCACAATGCCTAGAACAGTCTCC	
cY6	Antisense	CGGAGACTGTGACAGACATACTTTG	
	Antisense	GCAGGGAAGGAAGGTTGGCATGTAT	
Primers used for quantitative real-time RT-PCR assay			
cNPY	Sense	AGCCCAGAGACACTGATCTCAG	184
	Antisense	TGCATGCACTGGGAATGACGCT	
cPYY	Sense	CCGCGTATCCACCGAAGCCCG	125
	Antisense	GCTGCGCTTCCCATACCGCTGC	
cPP	Sense	GCTCCCGTGGAGGACCTCAT	152
	Antisense	AGCGCTGCATCCCGTGCTCA	
cY1	Sense	CGGATAGGCGGAGCAGTTG	274
	Antisense	CACATGGCAGTCCTCATCTTC	
cY4	Sense	ACAGAAACGTGCAGCAACAG	118
	Antisense	ATGCAATCTGGGTCTTCTGG	
cY6	Sense	AACAAAGTCCTTTTCTGGCTAG	338
	Antisense	GAAGGAAGGTTGGCATGTATC	
β-actin	Sense	CCCAGACATCAGGGTGTGATG	123
	Antisense	GTTGGTGACAATACCGTGTTCAAT	
[b]*Primers for construction of the expression plasmids*			
cY1	Sense	cggggtacCATGAATGCTTCGGTTCTTGA	1334
	Antisense	ccggaATTCCACCTGTGGGCACGCT	
cY4	Sense	cggggtaCCAGGATGAATAAGACAAGGGCA	1225
	Antisense	ccggaaTTCAGCTTTGGCATCACCAGT	
cY6	Sense	cccaaGCTTCACAATGGATAAAGCCAT	1221
	Antisense	ccggaaTTCAGTTAGGTGAGGTACCACGA	
[b]*Primers for preparation of the promoter-luciferase constructs*			
Y1(−2076/+312Luc)	Sense	ggtaccGCAGTTCATTAGTAGA	2388
Y1(−859/+312Luc)	Sense	CGGggtaccATAACAATGAGCATCCT	1171
Y1(−279/+312Luc)	Sense	CGGggtaccTGGACTTCTGTACTGGCTA	591
Y1(−128/+312Luc)	Sense	CGGggtaccTGTATGAAGAAGTCTGCT	440
Y1(−57/+312Luc)	Sense	CGGggtaccGCTGCTCCACTTCA	369
	Antisense	ctcgagGATCCTCCGATGTGCCT	
Y1(−2076/−188Luc)	Sense	ggtaccGCAGTTCATTAGTAGA	1867
	Antisense	CCGCTCGAGAACAACTGCTGCTGCAGACCT	
Y4(−1808/+19Luc)	Sense	gctagcCAAATAGTCATGGTCAGGTC	1827
Y4(−156/+19Luc)	Sense	CGGggtaccACCTCCTCTATCTGTA	175
Y4(−73/+19Luc)	Sense	CGGggtaccGGGATTCATTGGCTA	92
	Antisense	CCGCTCGAGCTCGCTGCTTCTGTAGGTA	
Y4(−1808/+126Luc)	Sense	gctagcCAAATAGTCATGGTCAGGTC	1934
	Antisense	ctcgagGAAACGACCCCTGTTGCTGCA	
Y6(−1745/+19Luc)	Sense	ggtaccTTGTTTGCAAATAGACACAAC	1764
Y6(−248/+19Luc)	Sense	CGGggtaccTGATGAATTTAATTGCAATAGA	267
Y6(−116/+19Luc)	Sense	CGGggtaccAGAACAAATAAGATAACTGA	135
Y6(−38/+19Luc)	Sense	CGGggtaccATCCCCATGACTACTGGT	57
	Antisense	CCGCTCGAGAAGAGGCAAACAGCAGTGA	

[a] All primers were synthesized by Beijing Genome Institute (Shanghai, China).
[b] Restriction sites added at the 5′-end of the primers are underlined.

sequencing at least three independent positive clones and the exon-intron organization of each receptor was then determined by comparing the 5′UTR sequence with the chicken genome database.

2.7. Identification of the promoter regions of chicken Y1,Y4 and Y6

To determine whether 5′ flanking region (near exon 1) of chicken *Y1*, *Y4* and *Y6* genes display promoter activities, we cloned the 5′-flanking regions into a pGL3-Basic vector and tested its promoter activities in cultured DF-1 cells (transformed chicken embryonic fibroblast cells) [purchased from American Type Culture Collection (ATCC, Manassas, VA)]. In brief, according to the genomic sequence of *cY1*, *cY4* and *cY6* genes (http://www.ensembl.org/gallus_gallus), we designed gene-specific primers and amplified the 5′-flanking region (near or upstream of exon 1) of *cY1*, *cY4* and *cY6* with high-fidelity Taq DNA polymerase (TOYOBO). The PCR products were cloned into pGL3-Basic vector (Promega, Madison, WI) and subjected to sequencing analysis. Finally, a series of promoter-luciferase reporter constructs for *cY1*

(−2076/+312Luc, −859/+312Luc, −279/+312Luc, −128/+312Luc, −57/+312Luc, and −2076/−188Luc), c*Y4* (−1808/+126Luc, −1808/+19Luc, −962/+19Luc, −156/+19Luc, and −73/+19Luc) and c*Y6* genes (−1745/+19Luc, −248/+19Luc, −116/+19Luc, and −38/+19Luc) were prepared. In this experiment, transcription start site (TSS) on exon 1 of each gene determined by 5′-RACE was designated as '+1', and the first nucleotide upstream of TSS was designed as '−1'.

To test whether these constructs display promoter activities, these plasmids were transfected into cultured chicken DF-1 cells and their promoter activities were analyzed by the Dual-luciferase Reporter assay (Promega), as described in our previous study (Wang et al., 2010). The DF-1 cells transfected with empty pGL3-Basic vector were used as internal controls.

2.8. Functional characterization of chicken Y1, Y4 and Y6 in cultured HEK293 cells

According to the coding sequence of chicken Y1 (NM_001031535.1), Y4 (NM_001031555.1), and Y6 (NM_001044687.1) deposited in GenBank, gene-specific primers (Table 1) were designed to amplify the open reading frame (ORF) of c*Y1*, c*Y4* and c*Y6* using high-fidelity *Taq* DNA polymerase (TOYOBO). The amplified PCR products were cloned into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA) and sequenced.

To examine whether cY1, cY4 and cY6 could be activated by chicken NPY_{1-36}, PYY_{1-37} and PP_{1-36} and are capable of signal transduction, the receptor expression plasmids were used to transfect human embryonic kidney 293 (HEK293) cells (purchased from ATCC) and the receptor signaling properties were monitored by the pGL3-CRE-luciferase reporter system and pGL4-SRE-luciferase reporter assay system, which were shown to be capable of monitoring receptor-activated (or -inhibited) cAMP-PKA and MAPK/ERK signaling pathways respectively, as described in our recent studies (Meng et al., 2014; Mo et al., 2015). In brief, HEK293 cells were cultured in Dulbecco minimal Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (Thermo Fisher Scientific Inc, Waltham, MA), 100 U/mL penicillin G, and 100 μg/mL streptomycin (Life Technologies Inc., Grand Island, NY) in a 90-cm culture dish (Nunc, Rochester, NY) and incubated at 37 °C with 5% CO_2. Cells were then plated in a 6-well plate at a density of 3×10^5 cells per well 18 h before transfection. A mixture containing 700 ng of pGL3-CRE-luciferase construct (or pGL4-SRE-lucfierase construct), 200 ng of pcDNA3.1 expression plasmid (or empty vector), and 2 μL of jetPRIME were prepared in 100 μL of jetPRIME solution. Transfection was performed according to the manufacturer's instruction when cells reached 70% confluence. After 24-h incubation, HEK293 cells were subcultured onto a 96-well plate at 37 °C for 24 h before treatment. After removal of medium from 96-well plate, 100 μL of peptide containing medium (or peptide-free medium) was added in the presence or absence of forskolin (5 μM). The cells were incubated for an additional 6 h at 37 °C before being harvested for luciferase assay. After removal of culture medium, HEK293 cells were lysed by adding 50 μL of 1 × Passive Lysis Buffer (Promega) per well, and the luciferase activity of 15 μL cellular lysates was determined with the luciferase assay kit (Promega). The luciferase activities in peptide treatment groups were expressed as percentage of the control group, or relative fold increase compared with the control group (without peptide treatment).

2.9. Western blot

To investigate whether activation of cY1 can enhance ERK1/2 phosphorylation, HEK293 cells transfected with 500 ng of cY1 expression plasmid or an empty pcDNA3.1 vector (+) were cultured for 24 h in a 24-well plate and treated with $cNPY_{1-36}$ (100 nM) for 10 min. Then, Western blot was performed to examine the phosphorylated ERK1/2 in whole cellular lysates according to the manufacturer's instructions (CST). In parallel, β-actin levels were also examined and used as internal controls to monitor protein loading.

2.10. Data analysis

Luciferase activity of promoter-luciferase construct in DF-1 cells was normalized to *Renilla* luciferase activity derived from the pRL-TK vector (Promega) and then expressed as relative fold increase to that of the control group (pGL3-Basic vector). The reporter assay data were analyzed by one-way ANOVA followed by Dunnett's test. The dose-responsive curves were constructed using nonlinear regression models, and the corresponding half-maximal effective concentration (EC_{50}) values were evaluated with GraphPad Prism 5 (GraphPad Software, San Diego, CA). All experiments were repeated at least twice to validate our results.

3. Results

3.1. Cloning the full-length cDNA of PYY from chicken small intestine

cPYY peptide was purified from chicken intestine in 1992 (Conlon and O'Harte, 1992), however, *PYY* gene has not been cloned in any avian species even to date. Therefore, using RACE PCR, we first amplified and cloned the full-length cDNA of *PYY* from chicken small intestine. The cloned chicken *PYY* cDNA is 659 bp long and encodes a precursor of 90 amino acids (accession No. KU059551), which shows a high degree of amino acid sequence identity with that of turkeys (91%, XP_010726378), hummingbirds (84%), ground tits (69%), common cuckoos (65%), saker falcons (67%), American alligators (60%), green sea turtles (64%), western painted turtles (64%), green anoles (58%), mummichogs (49%), zebrafish (PYYa, 48%; PYYb: 55%) (Sundström et al., 2013), grass carp (49%), amazon molly (PYYa, 56%; PYYb, 54%), *Xenopus tropicalis* (54%), Nile tilapia (55%), sea lamprey (62%), humans (69%), mice (69%), and rats (69%) (Figs. 1 and S1).

The deduced amino acid sequence of $cPYY_{1-37}$ peptide is identical to that of PYY peptide purified from chicken intestine in a previous report (Conlon and O'Harte, 1992). $cPYY_{1-37}$ shares a remarkable degree of amino acid sequence identity with putative PYY_{1-37} of turkeys (97%), or PYY_{1-36} from other avian species (89–92%), with only one to four amino acid substitutions noted between them (Fig. 1). Moreover, $cPYY_{1-37}$ shares a high identity (89%) with PYY_{1-36} of reptiles including green anoles, but a comparatively low identity with PYY_{1-36} of mammals (69%), *Xenopus tropicalis* (78%) and teleosts (69–78%) (Fig. 1).

Notably, chicken PYY_{1-37} also shares a considerably high degree of identity with chicken and human NPY_{1-36} (67–69%), but a limited identity with PP_{1-36} of chickens (42%) and humans (56%) (Fig. 1).

3.2. Tissue expression of NPY, PYY and PP in chickens

Since information regarding the tissue expression of *NPY* family members, particularly *PYY* and *PP*, is limited in birds (Blomqvist et al., 1992), therefore, in this study, we examined the mRNA levels of *cNPY*, *cPYY* and *cPP* in 24 chicken tissues, including the gastrointestinal (GI) tract and various brain regions, using quantitative real-time RT-PCR (qPCR).

As shown in Fig. 2A, *cNPY* was detected to be widely expressed in the adult chicken tissues examined. In the central nervous system (CNS), *cNPY* was abundantly expressed in the telencephalon, midbrain and hypothalamus, while its mRNA level is relatively

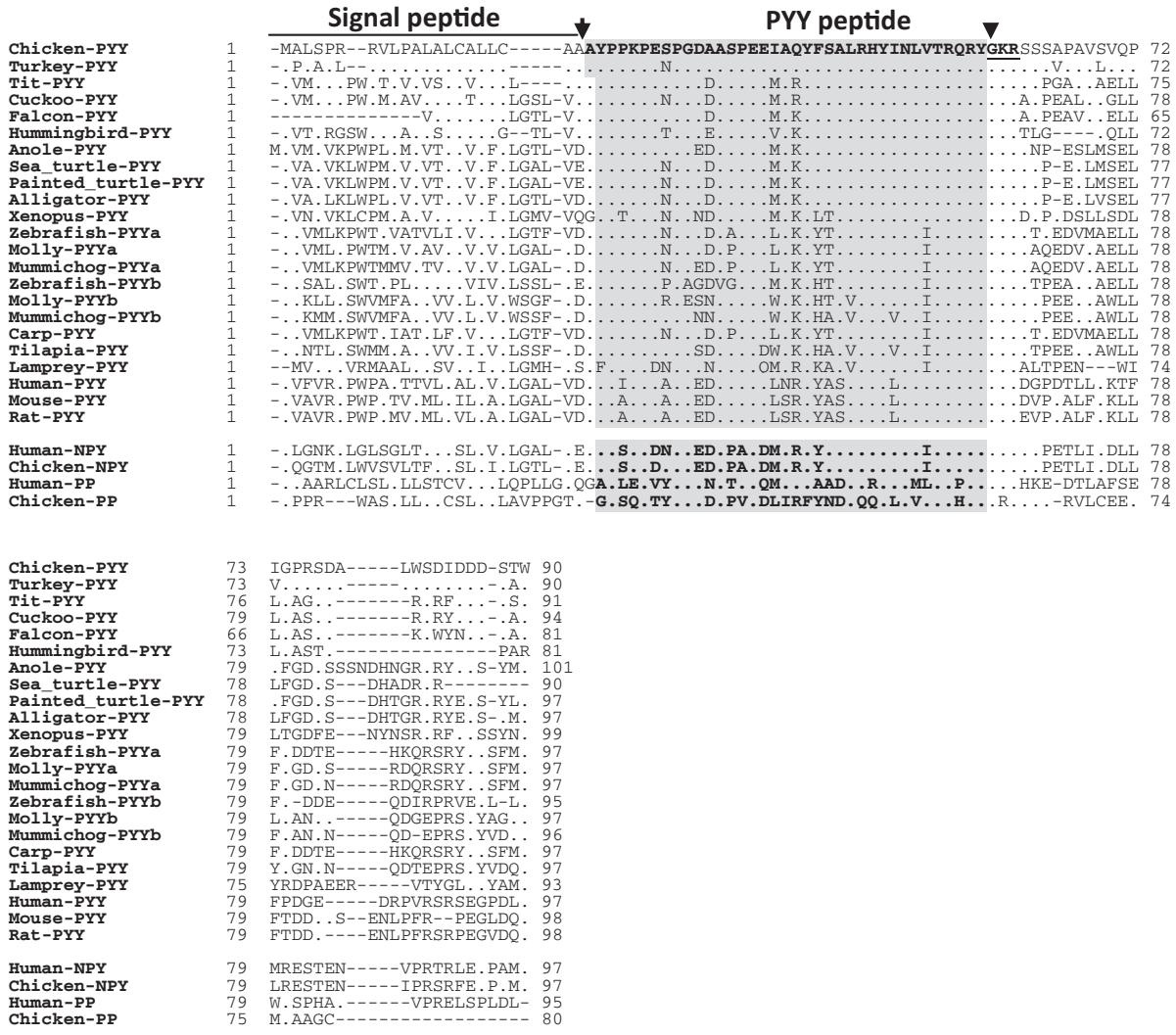


Fig. 1. Alignment of chicken PYY precursor (KU059551) with that of turkeys (XP_010726378), Tibet ground tits (XM_005532483), common cuckoos (XM_009571156), saker falcons (XM_005439463), hummingbirds (XM_008492091), green anoles (XM_003222643), green sea turtles (XM_007061902), western painted turtles (XM_005301945), American alligators (XM_006271543), *Xenopus tropicalis* (NM_001078959), zebrafish (PYYa, NM_001164371; PYYb, XM_001333186), amazon molly (PYYa, XM_007575601; PYYb, XM_007559608), mummichogs (XM_012881986), grass carp (JQ247041), Nile tilapia (XM_003438748), sea lamprey (AY823513), humans (NM_004160), mice (NM_145435) and rats (NM_001034080), or with NPY/PP precursors from humans (*NPY*, NM_000905; *PP*, NM_002722) and chickens (*NPY*, NM_205473; *PP*, NM_204786). The putative mature peptides (PYY_{1-37} or PYY_{1-36}, NPY_{1-36}, PP_{1-36}) were shaded. *Note:* chicken and turkey PYY is 37 amino acids in length, while PYY from other avian species is 36 amino acids in length. Arrows indicate the putative cleavage sites; arrowheads indicate the conserved amidation site (G) noted in PYY, NPY and PP precursors of all species. All sequences were retrieved from the GenBank. Dots indicate amino acids identical to the chicken PYY precursor; dashes denote gaps in the alignment.

low in the cerebellum and hindbrain. In peripheral tissues, *cNPY* was abundantly expressed in the anterior pituitary and moderately expressed in the testes, while *cNPY* mRNA level is low in the heart, kidneys, lung, muscle, ovary, spleen, subcutaneous fat, pancreas and various parts of GI tract. In the liver, *cNPY* expression is undetectable.

Like *cNPY*, *cPYY* was also widely expressed in chicken tissues. In the CNS, *cPYY* was highly expressed in various brain regions including the hypothalamus. In peripheral tissues, it is highly expressed in the proventriculus, duodenum, jejunum, ileum, heart, and pancreas, and weakly expressed in the other tissues examined except liver, in which it is undetectable. Notably, among all tissues examined, the highest mRNA level of *cPYY* was noted in the pancreas, where it was at least 8-fold higher than that in the GI tract and CNS (Fig. 2B).

Unlike *cNPY* and *cPYY*, *cPP* was detected to be expressed in the pancreas almost exclusively. Only an extremely low mRNA level of *cPP* (~4000-fold lower than the pancreatic level) was detected in the heart, lung, muscle, subcutaneous fat, testes, pituitary, and various brain regions except telencephalon. In the GI tract, kidneys, liver, ovary and spleen, *cPP* mRNA is undetectable (Fig. 2C).

3.3. Characterization of the 5′-UTR of chicken Y1, Y4, and Y6

To determine whether additional novel exon(s) are located upstream of the translation start site (ATG) of *cY1*, *cY4* and *cY6*, 5′-RACE was performed to amplify and clone the 5′-untranslated region (5′-UTR) of *cY1*, *cY4* and *cY6* from adult chicken brain (Fig. S2). Comparison of the cloned 5′-UTR sequences with the chicken genome database revealed that: (1) *cY1* contains three exons including a non-coding exon (exon 1, 379-bp long) upstream of the ATG codon, and its 5′-UTR is 525 bp in length (accession No. KU059552) (Fig. S3); (2) c*Y4* contains two exons, including a non-coding exon (exon 1, 138-bp long) upstream of the ATG codon, and its 5′-UTR is 251 bp long (accession No. KU059554) (Fig. S3); (3) c*Y6* has four exons, including three non-coding exons (exon 1, 277-bp; exon 2, 90-bp; exon 3, 35-bp long) upstream of the ATG codon, and its 5′-UTR is 600 bp in length (accession No.

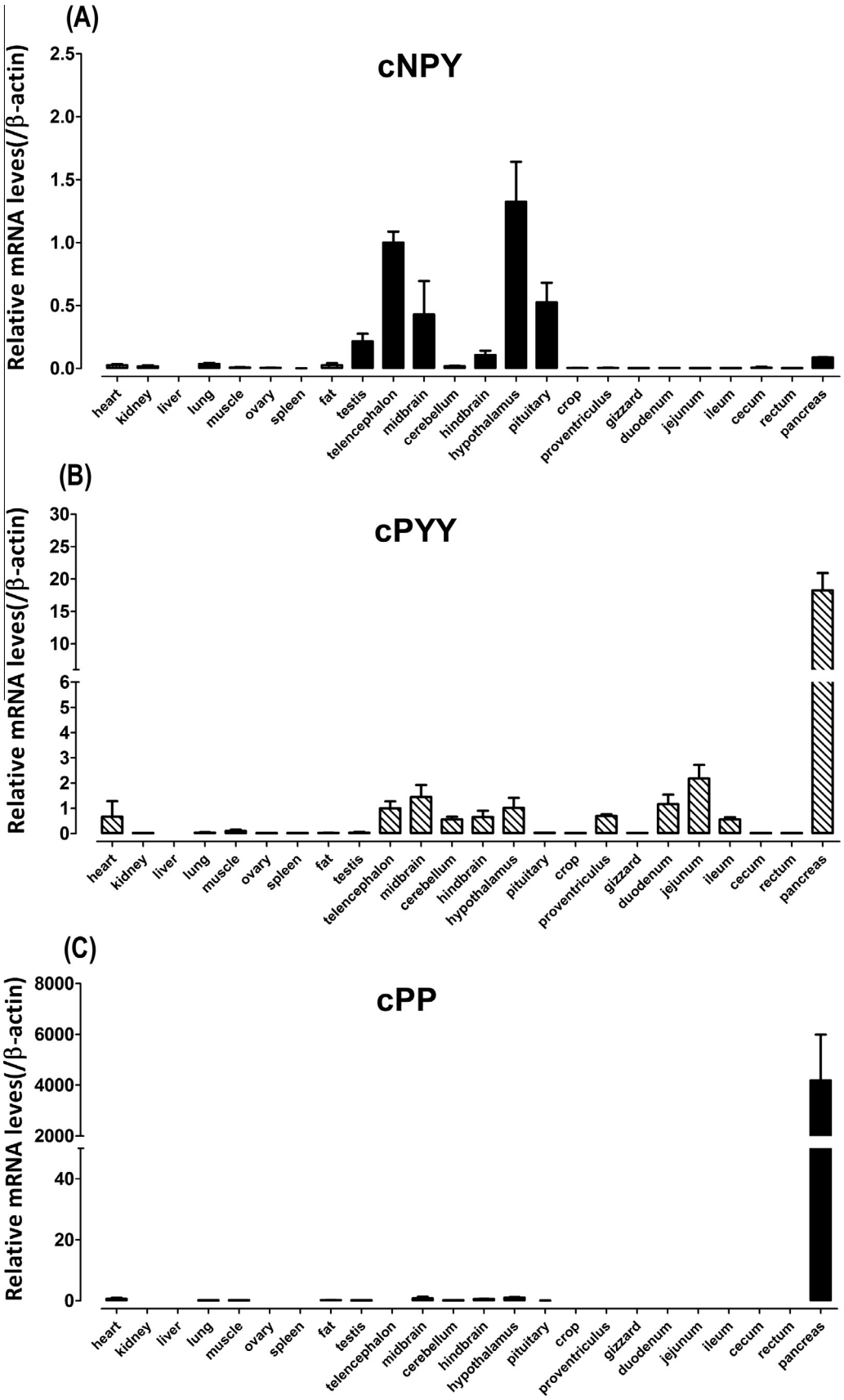


Fig. 2. Quantitative real-time PCR assay of c*NPY* (A), *cPYY* (B) and c*PP* (C) mRNA expression in chicken tissues. The mRNA levels of each target gene were normalized to that of *β-actin* and expressed as the fold difference compared with that of the telencephalon (A, B) or hypothalamus (C). Each data point represents the mean ± SEM of 4 adult chickens ($N = 4$).

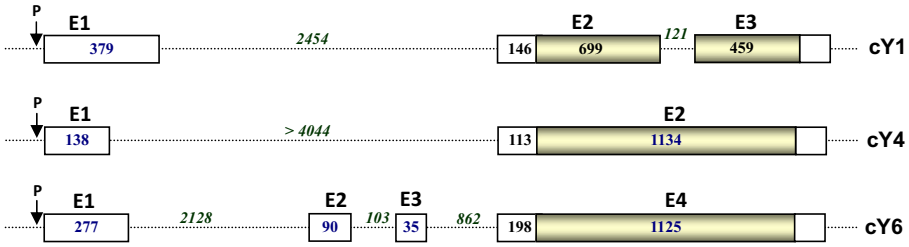


Fig. 3. Gene structure of chicken *Y1*, *Y4* and *Y6*. Exon-intron organization of *cY1*, *cY4* and *cY6*. The coding region of each receptor gene is shaded. Boxes represent exons (E), with their respective sizes labeled inside in term of base pair (bp); while numbers in italic denote the sizes of intron(s) (bp). The promoter region (P) of each receptor gene is located near exon 1, which was proved to be functional (please refer to Figs. 5–7 for details).

KU059556) (Fig. S3). The gene structure of c*Y1*, c*Y4* and c*Y6* is schematically depicted in Fig. 3.

3.4. Tissue expression of Y1, Y4 and Y6 in chickens

To further evaluate the mRNA levels of *Y1*, *Y4* and *Y6* in adult chicken tissues, quantitative real-time PCR assay was performed using primers spanning intron(s), which avoid any amplification of contaminating genomic DNA. As shown in Fig. 4, *cY1* is widely expressed in all tissues examined. *cY1* is abundantly expressed in various brain regions (including the hypothalamus and telencephalon), anterior pituitary, muscle and ovary, and moderately expressed in the spinal cord, heart, kidneys, liver and testes, whereas only an extremely low expression level of *cY1* was noted in other tissues, including the GI tract and pancreas. Like *cY1*, *cY4* is widely expressed in all tissues examined including various brain regions. *cY4* is highly expressed in the pancreas, subcutaneous fat, cerebellum, hindbrain, and pituitary, moderately expressed in kidneys, muscle and spleen, and weakly expressed in other tissues examined. Similar to the *cY1* and *cY4*, *cY6* is also widely expressed in all tissue examined. *cY6* is highly expressed in the duodenum, moderately in the hypothalamus, kidneys, muscle, testes, proventriculus, gizzard, and jejunum, and weakly expressed in other tissues examined.

3.5. Characterization of the promoter regions of chicken Y1, Y4, and Y6

To determine whether the 5′-flanking regions near exon 1 of c*Y1*, c*Y4* and c*Y6* (Fig. 3) display promoter activities, we cloned the 5′-flanking regions of c*Y1*, c*Y4* and c*Y6* into the promoter-less pGL3-Basic vector and examined their promoter activities in cultured chicken DF-1 cells.

As shown in Fig. 5, the 5′-flanking region (from −2076 to +312; accession No. KU059553) of *cY1* displays a strong promoter activity in DF-1 cells and causes a ~60-fold increase in luciferase activity compared to cells transfected with the promoter-less pGL3-Basic vector. Using the deletion approach, we also noted that the removal of the 5′-end (from −2076 to −279) of this fragment reduced its promoter activity, suggesting that this region is critical for the full promoter activity of *cY1* in DF-1 cells. Further deletion from −279 to −57 did not reduce the promoter activity significantly. Since the 5′-flanking region from −57 to +312 of *cY1* still has a strong promoter activity, and the construct (−2076/−188Luc) without this region displays no promoter activity, indicating that a core promoter region of *cY1* is likely located within this region (−57/+312). Using the online softwares, TFBind (http://tfbind.hgc.jp/) and JASPAR (http://jaspar.genereg.net), the putative binding sites for transcription factors, such as MyoD, Sp1, NF-kB, P53, Nkx2-5, C/EBPb, Pdx1, USF2, and GATA3, were predicted to be located within or near this region (−57/+312) (Fig. 5B), however, whether they are functional *cis*-regulatory elements requires further investigation.

Like *cY1*, *cY4* promoter region was also identified to be located near exon 1 (Fig. 6). The 5′-flanking region (from −1808 to +126; accession No. KU059555) of *cY4* displays the maximal promoter activity in DF-1 cells. Deletion of its 5′-end (from −1808 to −157) or 3′ end (from +20 to +126) significantly reduced its promoter activity in DF-1 cells, suggesting that these regions are required for the full promoter activity of c*Y4*. Since the construct containing a region from −156 to +19 still has a strong promoter activity in DF-1 cells and further deletion of its 5′-end (from −156 to −74) abolished its promoter activity, it is suggested that a core promoter region is likely located within this region (−156 to +19). Using the online softwares, the potential binding sites for transcriptional factors, such as NF-kB, Sp1, GATA4, Nkx2-5, Nr5a2, and C/EBPα, were also predicted within (or near) this region.

In this study, *cY6* promoter region was also found to be located near exon 1. The 5′-flanking region (from −1745 to +19; accession No. KU059557) of *cY6* displayed a strong promoter activity in DF-1 cells (Fig. 7A). Interestingly, removal of its 5′-end (from −1745 to −249) further enhanced the promoter activity significantly, implying that negative regulatory elements may be located within this region (−1745 to −249). Since the 5′-flanking region from −248 to +19 of *cY6* could display the maximal promoter activity and further deletion of its 5′-end (from −248 to −117) resulted in loss of promoter activity, therefore, a core promoter region of *cY6* is likely to be located within this region (−248 to +19), where the predicted binding sites for many transcriptional factors, such as Nkx2-5, GATA2, Tcf3, GATA3, Sp1, and Pax2, were also found (Fig. 7B).

3.6. Functional analysis of chicken Y1, Y4 and Y6 in cultured HEK293 cells

To determine the signaling pathways coupled to cY1, cY4 and cY6, each of the receptor under test was transiently expressed in HEK293 cells and treated by various concentrations of $cNPY_{1\text{-}36}$, $cPYY_{1\text{-}37}$ and $cPP_{1\text{-}36}$. Then, the receptor-activated (or -inhibited) cAMP/PKA and MAPK/ERK signaling pathways were examined by pGL3-CRE-luciferase and pGL4-SRE-luciferase reporter systems established in our previous studies (Meng et al., 2014; Mo et al., 2015).

As shown in Fig. 8, using pGL3-CRE-luciferase reporter system, we found that cNPY, cPYY and cPP treatment (10^{-12} to 10^{-6} M, 6 h) dose-dependently inhibited forskolin (5 μM)-stimulated luciferase activities of cY1-expressing HEK293 cells, and the order of potencies is, cNPY (EC_{50}: 0.9 ± 0.12 nM) > cPYY (EC_{50}: 4.67 ± 0.62 nM) ≫ cPP (EC_{50}: >230 nM) (Table 2). These findings clearly indicate that as in mammals, Y1 is functionally coupled to $G_{i/o}$ protein and capable of inhibiting cAMP/PKA signaling pathway upon activation.

Similarly, cPP, cPYY and cNPY treatment inhibited forskolin (5 μM)-stimulated luciferase activities of HEK293 cells expressing

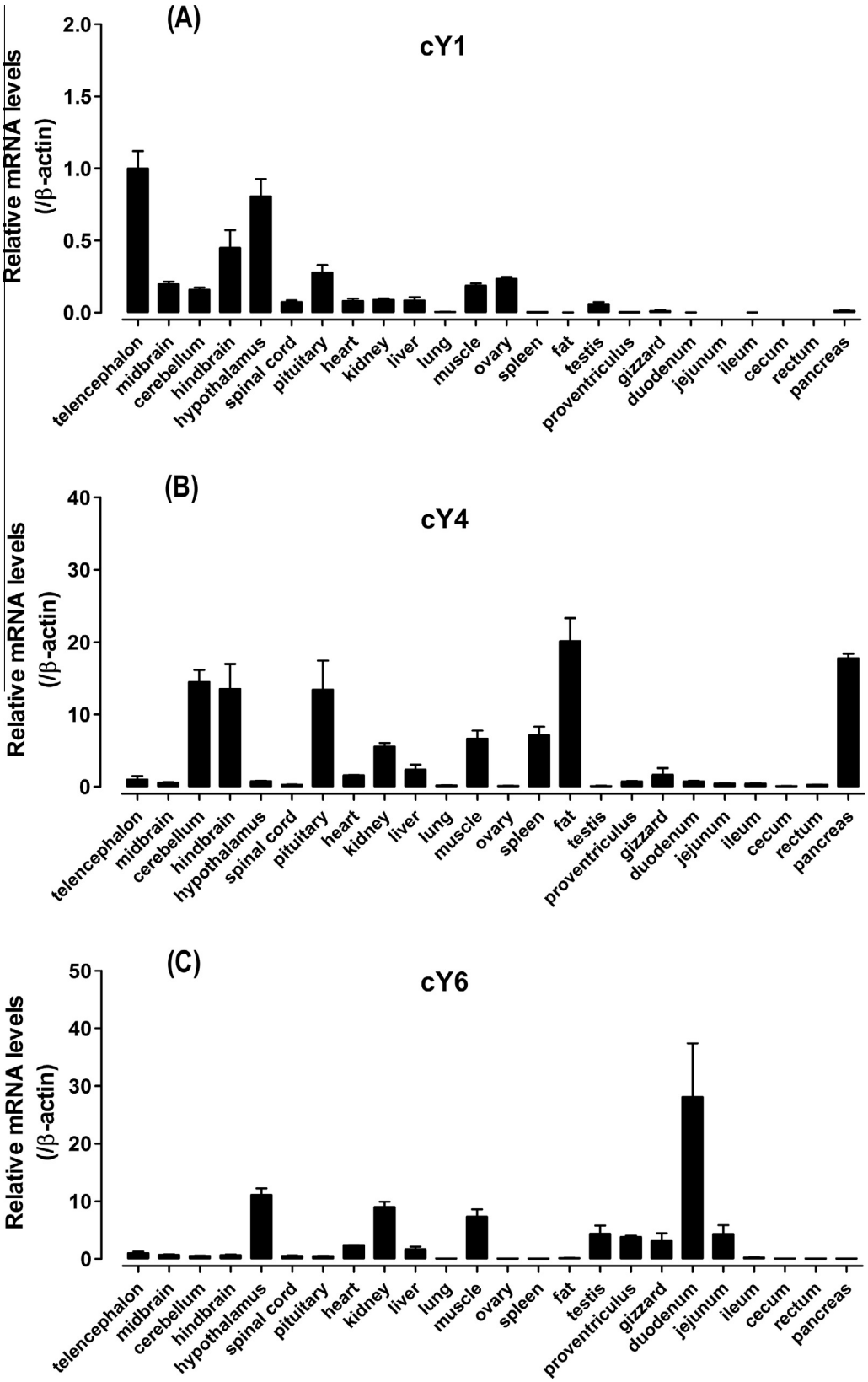
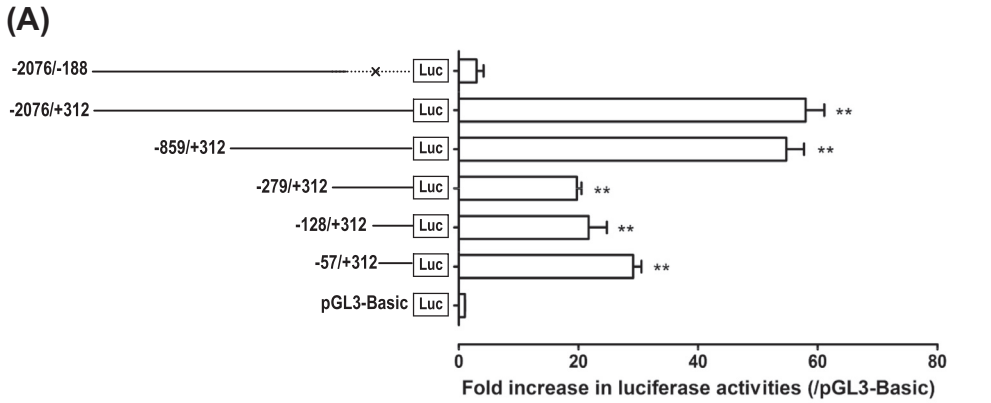


Fig. 4. Quantitative real-time PCR assay of *Y1* (A), *Y4*, (B) and *Y6* (C) mRNA expression in adult chicken tissues. The mRNA levels of each target gene were normalized to that of *β-actin* and expressed as the fold difference compared with that of the telencephalon. Each data point represents the mean ± SEM of 4–6 adult chickens ($N = 4–6$).



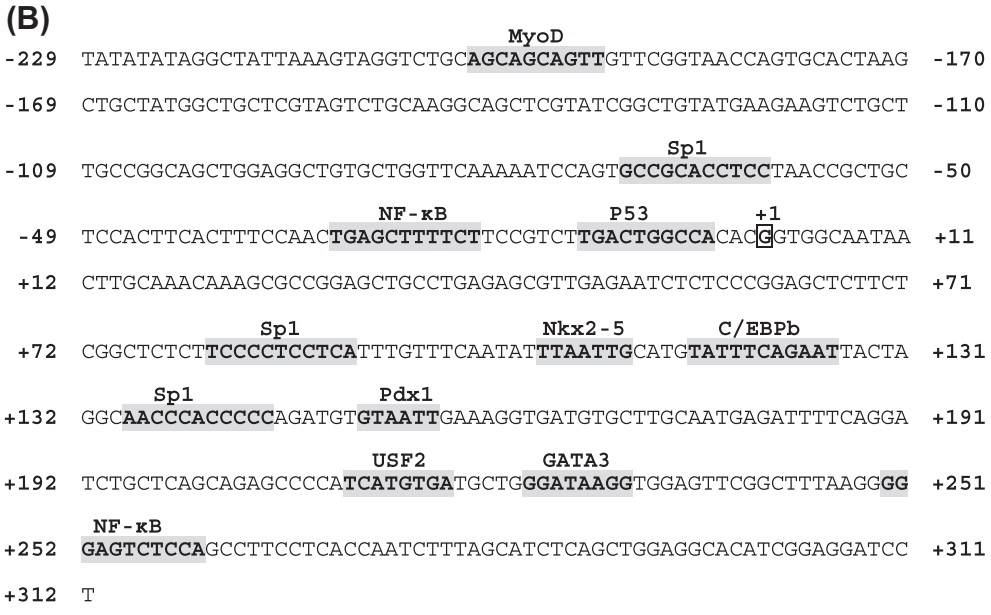


Fig. 5. Promoter analysis of chicken *Y1*. (A) Detection of the promoter activities of 5′-flanking region of *cY1* gene in cultured DF-1 cells. The 5′-flanking regions of *cY1* gene were cloned into pGL3-Basic vector to make promoter-luciferase constructs (−2076/−188Luc; −2076/+312Luc; −859/+312Luc; −279/+312Luc; −128/+312Luc; −57/+312Luc. The transcription start site 'G' on exon 1 determined by 5′-RACE is designated as '+1'). The promoter-luciferase reporter constructs were then co-transfected into DF-1 cells along with pRL-TK vector, and their promoter activities were determined by the Dual-luciferase reporter assay, as described in our previous studies (Wang et al., 2010). Each value represents the means ± SEM of three replicates ($N = 3$). **, $P < 0.001$ *vs* pGL3-Basic; (B) Partial sequence (−229/+312) of *cY1* promoter region. The predicted binding sites for transcriptional factors, including MyoD, Sp1, NF-kB, P53, Nkx2-5, C/EBPb, Pdx1, USF2 and GATA3, are shaded.

cY4 in a dose-dependent manner, indicating that cY4 activation inhibited the cAMP/PKA signaling pathway. However, we found that the potencies of the three peptides in activating cY4 are much low (EC_{50} of cPP: 13.8 ± 3.77 nM; EC_{50} of cPYY: 48.01 ± 5.08 nM; EC_{50} of cNPY: 687.5 ± 148.1 nM).

Unlike cY1 and cY4, cY6-expressing HEK293 cells (Fig. 8) or cY6-expressing Chinese hamster ovary (CHO) cells (*data not shown*) show no significant response to peptide treatment, even at high dosage ($\geqslant 10^{-7}$ M).

Using a pGL4-SRE-luciferase reporter system, cNPY and cPYY treatment stimulated luciferase activities of cY1-expressing HEK293 cells dose-dependently (Fig. 8 and Table 2), whereas cPP was ineffective, suggesting that cY1 is also coupled to intracellular MAPK/ERK signaling pathway. This finding was substantiated by the observation that $cNPY_{1\text{-}36}$ treatment (100 nM) enhanced ERK1/2 phosphorylation of cY1-expressing HEK293 cells via Western blot (Fig. 9). Similarly, cPP, cPYY and cNPY also stimulated luciferase activities of cY4-expressing HEK293 cells at a relatively higher dosage (10^{-6} M), indicating that cY4 is also likely to be coupled to the MAPK/ERK signaling pathway. In contrast, cY6-expressing HEK293 cells show no response to peptide treatment at any dosage tested.

In parallel with the above experiments, HEK293 cells co-transfected with the empty pcDNA3.1 (+) vector and pGL3-CRE-luciferase (or pGL4-SRE-luciferase) construct were used as controls, and peptide treatment did not alter the luciferase activities of HEK293 cells at any concentration tested (*data not shown*), confirming the specific effects of the peptides on receptor activation.

4. Discussion

In this study, c*PYY* gene was first cloned from chicken intestine and quantitative real-time PCR assay revealed that *cPYY, cNPY* and c*PP* are differentially expressed in chicken tissues. Moreover, the gene structures, promoter regions, tissue expression, and signaling properties of *cY1*, *cY4* and *cY6* were also characterized. Clearly, the results from our present study will help to uncover the physiological roles of NPY, PYY and PP peptides and their receptors in avian species.

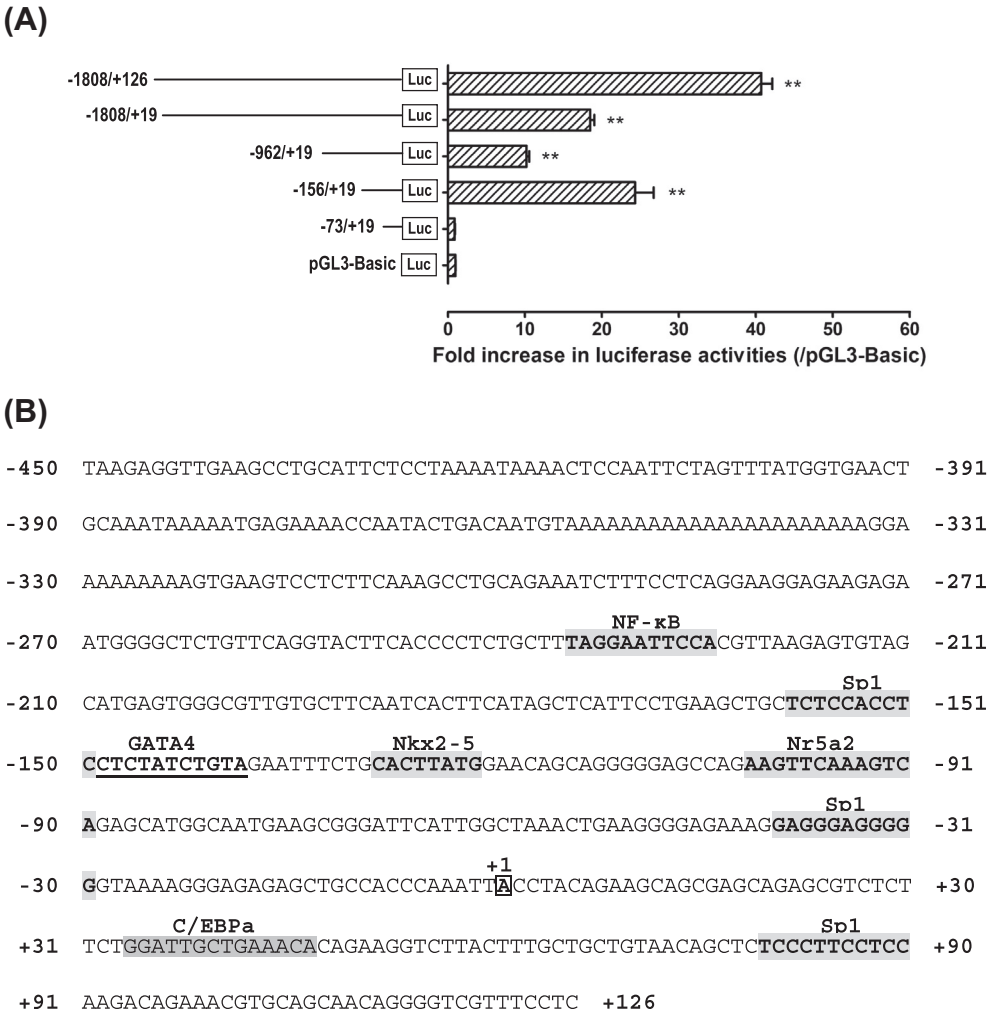


Fig. 6. Promoter analysis of chicken *Y4*. (A) Detection of the promoter activities of 5′-flanking region of *cY4* gene in cultured DF-1 cells. The 5′-flanking regions of *cY4* gene were amplified and cloned into pGL3-Basic vector to generate promoter-luciferase constructs (−1808/+126Luc; −1808/+19Luc; −962/+19Luc; −156/+19Luc; −73/+19Luc. The transcription start site 'A' on exon 1 is designated as '+1'). The promoter activities of these constructs were then determined by the Dual-luciferase reporter assay. Each value represents the means ± SEM of three replicates ($N = 3$). **, $P < 0.001$ *vs* pGL3-Basic; (B) Partial sequence (−450/+126) of *cY4* promoter. The predicted binding sites for transcriptional factors, including Sp1, NF-kB, GATA4, Nkx2-5, Nr5a2 and C/EBPa, are shaded.

4.1. Identification of PYY gene from chicken and other avian species

In this study, we cloned the full-length cDNA of *PYY* from chicken intestine. As in chickens, predicted sequences of *PYY* are found in all avian species examined. All these putative avian PYY peptides ($PYY_{1\text{-}37}$ or $PYY_{1\text{-}36}$) show a remarkable degree of structural conservation and possess an amidated C-terminus (Cerdá-Reverter and Larhammar, 2000). Moreover, they show high amino acid sequence identities (69–89%) with PYY of mammals, reptiles, frogs and teleosts. Our findings, for the first time, clearly indicate the presence and expression of a conserved PYY peptide in birds.

As previously reported (Conlon and O'Harte, 1992), the mature chicken PYY peptide is 37 amino acids ($cPYY_{1\text{-}37}$) in length after the removal of the 21-amino acid signal peptide at the N-terminus (Fig. 1). Likewise, the predicted turkey PYY peptide is 37 amino acids in length. Interestingly, the putative PYY peptides from other avian species are only 36 amino acids in length, same as $PYY_{1\text{-}36}$ identified in reptiles, frogs, teleosts and mammals (Cerdá-Reverter and Larhammar, 2000). Whether the presence of an extra alanine residue (A) at the N-terminus of chicken and turkeys' $PYY_{1\text{-}37}$ confers difference in biological activities observed in $PYY_{1\text{-}36}$ remains to be clarified (Fig. 1).

4.2. Differential expression of NPY, PYY and PP in chicken tissues

The identification of *PYY* gene promoted us to further examine and compare the tissue expression of *PYY*, *NPY* and *PP* in chickens, an issue which has not been depicted in any avian species.

In this study, we found that *cNPY* is expressed in nearly all tissues examined, with the highest mRNA level being noted in the hypothalamus (Fig. 2). Our finding coincides with the previous reports in chickens and rats, in which *NPY* mRNA or protein were detected to be highly expressed in hypothalamic nuclei (Allen et al., 1983; Kameda et al., 2001; Liu et al., 2012; Wang et al., 2001; Yuan et al., 2009; Zhou et al., 2005). The abundant expression of *cNPY* in the hypothalamus, together with the expression of multiple Y receptors in the chicken hypothalamus detected in present and previous studies (Fig. 4) (Bromée et al., 2006; Holmberg et al., 2002; Saneyasu et al., 2011; Yi et al., 2015; He et al., 2016), strongly suggest that NPY plays crucial roles in chicken hypothalamus, such as regulation of food intake and reproduction (Contijoch et al., 1993; Denbow and Cline, 2015; Kuenzel et al., 1987; Tachibana et al., 2006). It is well documented that the expression and actions of NPY, the potent orexigenic peptide, in the hypothalamus are tightly controlled by both peripheral metabolic signals *e.g.* adipocyte-derived leptin (LEP) (Schwartz

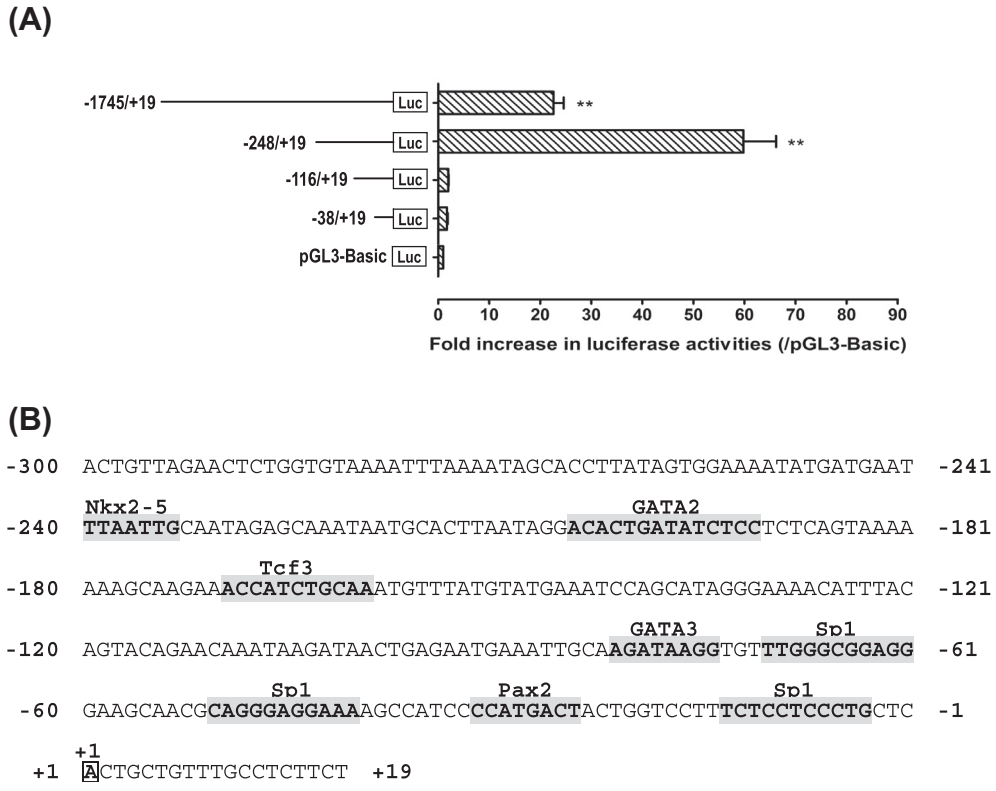


Fig. 7. Promoter analysis of chicken *Y6*. (A) Detection of the promoter activities of 5′-flanking region of *cY6* gene in cultured DF-1 cells. The 5′-flanking regions of *cY6* gene were amplified and cloned into pGL3-Basic vector to generate promoter-luciferase constructs (−1745/+19Luc, −248/+19Luc, −116/+19Luc, −38/+19Luc. The transcription start site 'A' on exon 1 is designated as '+1'). The promoter activities of these constructs were then determined by the Dual-luciferase reporter assay. Each value represents the means ± SEM of three replicates ($N = 3$). **, $P < 0.001$ *vs* pGL3-Basic; (B) Partial sequence (−300/+19) of *cY6* promoter. The predicted binding sites for transcriptional factors, including Nkx2-5, GATA2, Tcf3, GATA3, Sp1 and Pax2, are shaded.

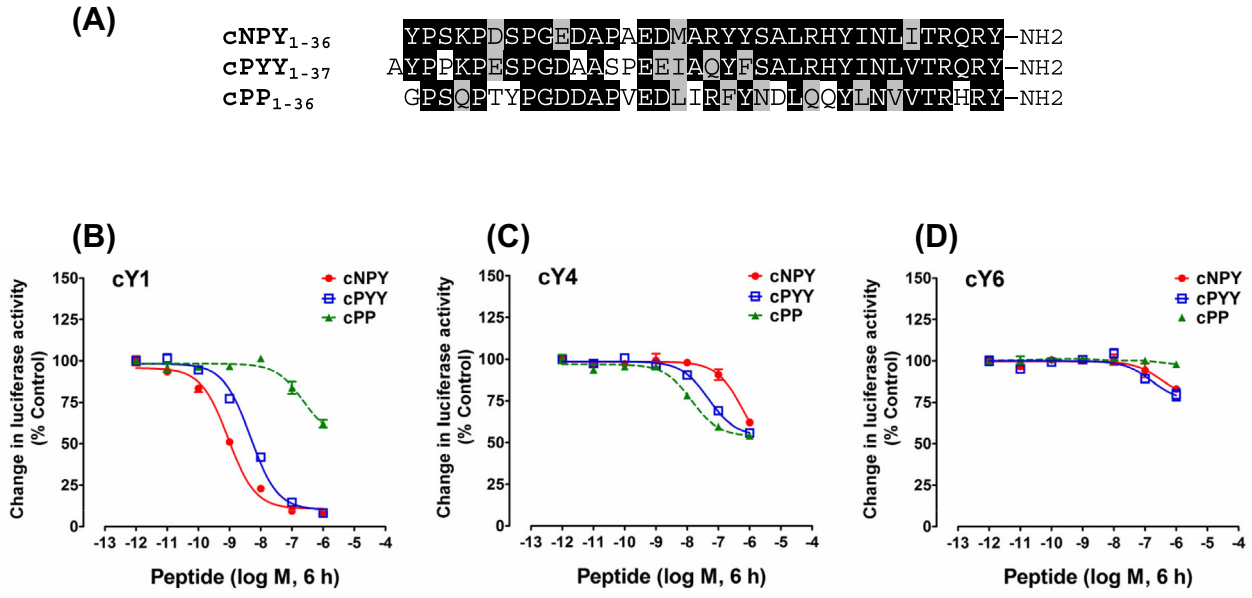


Fig. 8. Functional analysis of chicken Y1, Y4 and Y6. (A) Amino acid sequences of chicken NPY_{1-36}, PYY_{1-37}, and PP_{1-36} peptides used in the experiment. (B–D) Inhibition of forskolin (5 μM)-induced luciferase activities of HEK293 cells expressing cY1 (B), cY4 (C), and cY6 (D) in response to various concentrations of peptide, monitored by a pGL3-CRE-luciferase reporter system. HEK293 cells treated by forskolin only were set as the control. Each data point represents the mean ± SEM of 3 replicates ($N = 3$). Each dose-response curve shows one representative experiment performed in triplicates.

et al., 2000) and local neuropeptides such as cocaine and amphetamine regulated transcript (CART) and α-melanocyte-stimulating hormone (α-MSH) in mammals (Cone, 2005; Kristensen et al., 1998; Mercer et al., 2011; Rogge et al., 2008); however, unlike mammals, no expression of LEP was noted in the adipose tissue of avian species (Huang et al., 2014). Moreover, chicken CART1 peptide, an ortholog of mammalian CART, is predominantly expressed in the anterior pituitary, but not in the hypothalamus,

Table 2
EC_{50} values of $cNPY_{1-36}$, $cPYY_{1-37}$, and cPP_{1-36} in activation of various signaling pathways in HEK293 cells expressing chicken Y receptors.

EC_{50} (nM)			
	cY1	cY4	cY6
Inhibition of cAMP/PKA signaling pathway			
$cNPY_{1-36}$	0.90 ± 0.12	687.5 ± 148.1[a]	–
$cPYY_{1-37}$	4.67 ± 0.62	48.01 ± 5.08[*]	–
cPP_{1-36}	>230.2[a]	13.80 ± 3.77[*]	–
Activation of MAPK/ERK signaling pathway			
$cNPY_{1-36}$	114.6 ± 20.8[a]	–	–
$cPYY_{1-37}$	497.5[a]	–	–
cPP_{1-36}	–	–	–

Notes: Data shown are the mean ± SEM of two (for cY1/cY6, n = 2) or three (for cY4, n = 3) independent experiments performed in triplicates. '–' means that the EC_{50} value could not be calculated based on the experimental data.

[a] Indicates that EC_{50} value was roughly estimated based on the experimental data, which requires additional experimental verification.

[*] $P < 0.01$ between the two groups (cPYY *vs* cPP) (analyzed by the Student *t*-test using GraphPad Prism 5 software).

which contrasts to the findings in mammals (Cai et al., 2015; Mo et al., 2015). These fundamental differences noted between birds and mammals also raise an open question whether NPY and other signals, such as LEP, CART1 and α-MSH, could form a regulatory network and thus control food intake in a way similar to or distinct from that present in mammals (Boswell and Dunn, 2015; Friedman-Einat et al., 2014; Higgins et al., 2010; Huang et al., 2014; Yi et al., 2015).

Besides the hypothalamus, *cNPY* is widely expressed in other brain regions examined, with particularly high expression levels noted in the telencephalon, midbrain and hindbrain (Fig. 2). Our finding is consistent with the previous reports in chickens, rats and humans, in which NPY is widely distributed across various brain regions (Adrian et al., 1983; Allen et al., 1983; Wang et al., 2001). This finding also implies NPY may have diverse actions beyond appetite regulation, such as regulation of anxiety, memory and learning, neuronal cell survival and proliferation, in the avian CNS, as demonstrated in mammals (Hansel et al., 2001; Holzer et al., 2012; Xapelli et al., 2006).

In peripheral tissues, *cNPY* is abundantly expressed in chicken anterior pituitary. This finding, together with the expression of multiple Y receptors in the anterior pituitary (Fig. 4) (He et al., 2016), also suggests that cNPY can regulate pituitary functions in an autocrine/paracrine manner. Future studies of NPY actions on the expression/secretion of pituitary hormone(s) will substantiate this hypothesis. In addition to the pituitary, a relatively low mRNA level of *cNPY* was detected in most peripheral tissues examined including the testes and adipose tissue. These findings also support the notion that cNPY exerts local actions on these tissues (Pedrazzini et al., 2003), such as regulation of adipogenesis and

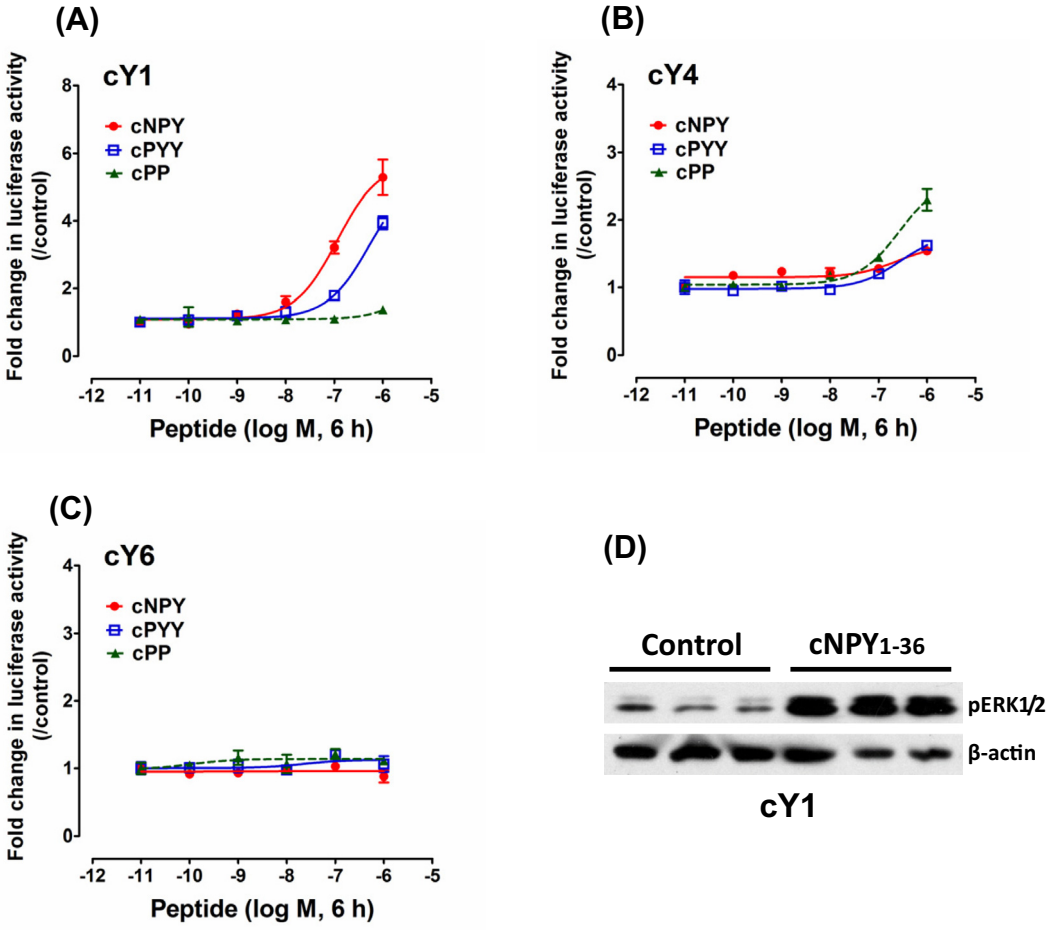


Fig. 9. (A–C) Effects of $cNPY_{1-36}$, $cPYY_{1-36}$ and cPP_{1-36} (10^{-11} to 10^{-6} M, 6 h) on activation of the MAPK signaling pathway of HEK293 cells expressing cY1 (A), cY4 (B), and cY6 (C), assayed by a pGL4-SRE-luciferase reporter system. HEK293 cells without peptide treatment were set as the control. Each data point represents the mean ± SEM of 3 replicates ($N = 3$). Each dose-response curve shows one representative experiment performed in triplicates. (D) The representative set of experimental triplicates showing that $cNPY_{1-36}$ (100 nM, 10 min) treatment strongly enhances ERK1/2 phosphorylation (pERK1/2) of HEK293 cells transfected with cY1 expression plasmid. As a negative control, $cNPY_{1-36}$ treatment causes no change in ERK1/2 phosphorylation of HEK293 cells transfected with empty pcDNA3.1 vector (*data not shown*).

gonadal steroidogenesis, as reported in chickens and mammals (Korner et al., 2011; Zhang et al., 2015).

Like *cNPY*, *cPYY* is widely expressed across chicken tissues examined including the GI tract and CNS (Fig. 2). Our finding is consistent with the findings in elephant sharks (Larsson et al., 2009) and *Takifugu rubripes* (Sundström et al., 2008), but contrasts to that in mammals, in which *cPYY* is mainly expressed in intestinal endocrine L cells (Holzer et al., 2012). The comparatively high mRNA levels of *cPYY* in the proventriculus, duodenum, jejunum and ileum suggest that intestinal PYY may be released to the circulation postprandially and thus control GI tract activities such as inhibition of gastric emptying and intestinal peristalsis in chickens, similar to its mammalian ortholog (Holzer et al., 2012). Since *cPYY* is also highly expressed in various brain regions including the hypothalamus, thus, brain-derived PYY, like NPY, may control neuronal activities, such as stimulating food intake in chickens (Kuenzel et al., 1987).

It is of particular interest to note that *cPYY* is abundantly expressed in the pancreas and its expression level is at least 8-fold higher than that in the GI tract or hypothalamus. Our finding partially agrees with a previous report in chickens, in which PYY-immunoreactive cells were found in the pancreas (Ding et al., 1997). Our data imply that chicken pancreatic PYY may not only regulate pancreatic development and functions in an autocrine/-paracrine manner (Persaud and Bewick, 2014), but also contributes to a large fraction of the circulating PYY pool, which may control other physiological processes of extra-pancreatic tissues via an endocrine route.

Unlike *cNPY* and *cPYY*, *cPP* is expressed in the pancreas nearly exclusively. This is consistent with the finding in mammals, in which *PP* is expressed in the pancreas almost exclusively (Ekblad and Sundler, 2002). However, it contrasts the observation in frogs, in which *PP* is abundantly expressed in many extra-pancreatic tissues including the intestine, and weakly expressed in the pancreas (Sundström et al., 2012). The striking difference in spatial expression patterns of *PP* between frogs and birds/mammals may hint of a functional change of PP during vertebrate evolution.

4.3. Signaling pathways coupled to chicken Y1, Y4 and Y6

Pioneering studies show that chicken Y1 and Y4 can bind NPY, PYY, or PP peptide from pigs or chickens *in vitro*, however, their downstream signaling pathways remains obscure (Holmberg et al., 2002; Lundell et al., 2002). In this study, we proved that chicken Y1 is potently activated by cNPY and cPYY, and their activation leads to the inhibition of intracellular cAMP/PKA signaling pathway, as reported in mammals (Michel et al., 1998). Our finding contrasts a previous study, in which activation of chicken Y1 was unable to inhibit forskolin-induced cAMP synthesis when expressed in HEK293 EBNA-1 cells, though chicken Y1 was shown to bind pig NPY_{1-36} and chicken PYY_{1-37} with much higher affinity than cPP_{1-36} (Holmberg et al., 2002).

Like cY1, cY4 activation inhibits forskolin-induced luciferase activities in HEK293 cells upon ligand treatment (Fig. 7), indicating that cY4 is coupled to $G_{i/o}$ protein and capable of inhibiting cAMP signaling pathway, as reported in mammals (Michel et al., 1998). Intriguingly, we noted that $cNPY_{1-36}$, $cPYY_{1-37}$ and cPP_{1-36} activate cY4 with low potencies (EC_{50}, >10 nM), and cPP_{1-36} appears to be only ~3-fold and ~50-fold more potent than $cPYY_{1-37}$ and $cNPY_{1-36}$, respectively. This experiment was repeated three times with similar results (Table 2). These data suggest that cY4 displays a weak selectivity for cPP_{1-36}. Our finding contrasts to the finding in mammals, in which Y4 receptor was potently activated by PP, but not by NPY and PYY, hence Y4 receptor is commonly viewed as a PP-receptor (Bard et al., 1995; Gregor et al., 1996b; Lundell et al., 1995). Interestingly, Lundell et al. demonstrated that cY4 binds cPP, cPYY and pig NPY (pNPY) with equally high affinities (IC_{50}: ~0.1 nM) when expressed in COS-7 cells (Lundell et al., 2002). The discrepancy between our study and the previous study is indefinite, though a possible explanation may lie in the difference of cell lines used.

In addition to the inhibition of the cAMP signaling pathway, we also demonstrated that the activation of Y1 and Y4 lead to the activation of the MAPK/ERK signaling pathway (Fig. 8D). All these findings coincide with the observation in mammals (Mullins et al., 2002).

Unlike cY1 and cY4, the signaling pathway(s) coupled to cY6 remains elusive. Peptide treatment could neither significantly inhibit the cAMP/PKA signaling pathway, nor activate the MAPK/ERK signaling pathway of Y6-expressing HEK293 cells at any concentrations tested. Our finding agrees with a previous report in chickens, in which pNPY, cPYY and cPP treatment could neither inhibit the cAMP signaling pathway, nor up-regulate the intracellular IP3 and Ca^{2+} concentrations of Y6-expressing HEK293 EBNA cells (Bromée et al., 2006). Therefore, the question remains as to whether cY6 is a functional receptor, or a pseudogene.

4.4. Gene structure and tissue expression of chicken Y1, Y4 and Y6

The coding regions of chicken *Y1*, *Y4* and *Y6* have been determined in previous studies (Bromée et al., 2006; Holmberg et al., 2002; Lundell et al., 2002) (Fig. S4), however, their detailed gene structures have not been characterized in any avian species. In this study, novel non-coding exon(s) upstream of the ATG codon were identified in chicken *Y1*, *Y4* and *Y6*. Similar to our finding in chickens, multiple non-coding exon(s) have also been identified in rat or human *Y1*, *Y4* and *Y6* genes (Ball et al., 1995; Eva et al., 1992).

Based on the novel gene structures of *cY1*, *cY4* and *cY6* revealed in this study (Fig. 3), we re-analyzed their tissue expression in chickens. *cY1* mRNA was detected to be widely distributed across all tissues examined. Our finding is partially consistent with the wide tissue expression of *Y1* reported in frogs (Sundström et al., 2012) and elephant sharks (Larsson et al., 2009). Moreover, *cY1* is abundantly expressed in various brain regions including the hypothalamus. This finding coincides with an early report in chickens, in which *cY1* mRNA was detected to be highly expressed in the infundibular nucleus of the hypothalamus by *in situ* hybridization (Holmberg et al., 2002). Similar to our finding in chickens, *Y1* is also widely expressed throughout rat CNS, including the supraoptic and arcuate nuclei of the hypothalamus (Parker and Herzog, 1999). The wide expression of both cY1 and its ligands (cNPY/cPYY) in the CNS (Fig. 2) suggests that cY1 is able to mediate the NPY(/PYY) actions such as orexigenic action in the avian CNS (Denbow and Cline, 2015; Kameda et al., 2001). In addition to the CNS, *cY1* shows at least moderate expression in the pituitary, muscle, ovary, heart, kidneys, liver, spinal cord and testes, implying that cY1 can mediate the actions of cNPY(or cPYY), such as regulation of pituitary functions demonstrated in mammals (Chabot et al., 1988; Pedrazzini et al., 2003). Since an extremely low mRNA level of *cY1* was noted in other tissues examined including the GI tract, it is questionable whether cY1 plays a physiologically relevant role in these tissues. In this study, we further revealed that *cY1* expression is likely controlled by a promoter (−2076/+312) near exon 1, which displays strong promoter activities in three different types of cell lines including chicken DF-1 (Fig. 5), HEK293, and CHO cells (*data not shown*). Like mouse *Y1*, no typical TATA box was identified within the core promoter region (−57/+312) of cY1 (Eva et al., 1992), however, predicted binding sites for many transcriptional factors such as Sp1, P53, and NF-kB have been found (Eva et al., 1992; Musso et al., 1997), hinting that these *cis*-regulatory elements may be involved in driving *Y1* transcription in chicken tissues.

Like *cY1*, *cY4* is widely expressed in all tissues examined including various brain regions and its expression is also controlled by a functional promoter region (−1808/+126) upstream of exon 1, which contains putative binding sites for many transcriptional factors. The tissue expression pattern of *cY4* reported here is slightly different from a previous report in chickens, in which *cY4* was detected to be expressed in most peripheral tissues and three brain regions (including brainstem, cerebellum and hippocampus) by RT-PCR, but not in the other 5 brain regions assayed including the hypothalamus (Lundell et al., 2002). On the other hand, similar to our finding in chickens, *Y4* has been reported to be widely expressed in the CNS and peripheral tissues of frogs (Sundström et al., 2012), elephant sharks (Larsson et al., 2009), rats (Parker and Herzog, 1999) and humans (Bard et al., 1995), hinting that *cY4* may mediate actions of its ligands, such as PP/PYY action on food intake, GI tract motility, and pancreatic secretion (Dupont, 2015). However, it should be reminded that cY4 is activated preferentially by cPP and cPYY at a considerably high concentration beyond the normal physiological range (Fig. 8, Table 2), hence, it remains to be clarified whether cY4 alone is effective in mediating the actions of cPP/cPYY on target tissues *in vivo*, or cY4 has a less significant role in chickens.

Despite the little or no responsiveness of cY6 to peptide treatment (Figs. 8 and 9), *cY6* mRNA was detected in all tissues examined including the GI tract and various brain regions, and its expression is controlled by a functional promoter (−1745/+19) upstream of exon 1. Our finding is slightly different from a previous report in chickens, in which *cY6* mRNA was only detected in the hypothalamus among various brain regions tested, as well as in several peripheral tissues including the liver, kidneys, adipose tissue and proventriculus (Bromée et al., 2006). As in chickens, *Y6* is also expressed in human tissues though it is a pseudogene in humans (Gregor et al., 1996a; Matsumoto et al., 1996). Although it is unclear whether *cY6* is functional, the expression of *Y6* in elephant sharks (Larsson et al., 2009), chickens (Fig. 4) (Bromée et al., 2006), and mammals (Gregor et al., 1996a; Matsumoto et al., 1996) provides us a good example to study the structural and functional change of Y6 during vertebrate evolution.

In summary, in this study, the gene encoding a PYY peptide of 37 (or 36) amino acids was first identified in chicken and all other avian species examined. qPCR assay revealed a differential expression pattern of *PYY*, *NPY* and *PP* in chicken tissues. Moreover, the gene structure, expression, and signaling properties of cY1, cY4 and cY6 were also characterized. These findings, together with our recent characterization of the other three Y receptors (Y2, Y5 and Y7) in chickens (He et al., 2016), will not only establish a molecular basis to interpret the physiological roles of NPY, PYY and PP and their receptors in birds, but also help to uncover the structural and functional changes of all these ligand-receptor pairs during vertebrate evolution, in particular their actions on food intake and body weight control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.09.005.

References

Adrian, T.E., Allen, J.M., Bloom, S.R., Ghatei, M.A., Rossor, M.N., Roberts, G.W., Crow, T.J., Tatemoto, K., Polak, J.M., 1983. Neuropeptide Y distribution in human brain. Nature 306, 584–586.
Allen, Y.S., Adrian, T.E., Allen, J.M., Tatemoto, K., Crow, T.J., Bloom, S.R., Polak, J.M., 1983. Neuropeptide Y distribution in the rat brain. Science 221, 877–879.
Baldock, P.A., Lee, N.J., Driessler, F., Lin, S., Allison, S., Stehrer, B., Lin, E.J., Zhang, L., Enriquez, R.F., Wong, I.P., McDonald, M.M., During, M., Pierroz, D.D., Slack, K., Shi, Y.C., Yulyaningsih, E., Aljanova, A., Little, D.G., Ferrari, S.L., Sainsbury, A., Eisman, J.A., Herzog, H., 2009. Neuropeptide Y knockout mice reveal a central role of NPY in the coordination of bone mass to body weight. PLoS ONE 4, e8415.
Ball, H.J., Shine, J., Herzog, H., 1995. Multiple promoters regulate tissue-specific expression of the human NPY-Y1 receptor gene. J. Biol. Chem. 270, 27272–27276.
Bard, J.A., Walker, M.W., Branchek, T.A., Weinshank, R.L., 1995. Cloning and functional expression of a human Y4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. J. Biol. Chem. 270, 26762–26765.
Blomqvist, A.G., Söderberg, C., Lundell, I., Milner, R.J., Larhammar, D., 1992. Strong evolutionary conservation of neuropeptide Y: sequences of chicken, goldfish, and *Torpedo marmorata* DNA clones. Proc. Natl. Acad. Sci. U.S.A. 89, 2350–2354.
Blundell, T.L., Pitts, J.E., Tickle, I.J., Wood, S.P., Wu, C.W., 1981. X-ray analysis (1.4-A resolution) of avian pancreatic polypeptide: small globular protein hormone. Proc. Natl. Acad. Sci. U.S.A. 78, 4175–4179.
Boswell, T., Dunn, I.C., 2015. Regulation of the avian central melanocortin system and the role of leptin. Gen. Comp. Endocrinol. 221, 278–283.
Bromée, T., Sjödin, P., Fredriksson, R., Boswell, T., Larsson, T.A., Salaneck, E., Zoorob, R., Mohell, N., Larhammar, D., 2006. Neuropeptide Y-family receptors Y6 and Y7 in chicken. Cloning, pharmacological characterization, tissue distribution and conserved synteny with human chromosome region. FEBS J. 273, 2048–2063.
Cai, G., Mo, C., Huang, L., Li, J., Wang, Y., 2015. Characterization of the two CART genes (CART1 and CART2) in chickens (*Gallus gallus*). PLoS ONE 10, e0127107.
Cerdá-Reverter, J.M., Larhammar, D., 2000. Neuropeptide Y family of peptides: structure, anatomical expression, function, and molecular evolution. Biochem. Cell. Biol. 78, 371–392.
Chabot, J.G., Enjalbert, A., Pelletier, G., Dubois, P.M., Morel, G., 1988. Evidence for a direct action of neuropeptide Y in the rat pituitary gland. Neuroendocrinology 47, 511–517.
Cone, R.D., 2005. Anatomy and regulation of the central melanocortin system. Nat. Neurosci. 8, 571–578.
Conlon, J.M., O'Harte, F., 1992. The primary structure of a PYY-related peptide from chicken intestine suggests an anomalous site of cleavage of the signal peptide in preproPYY. FEBS Lett. 313, 225–228.
Contijoch, A.M., Malamed, S., McDonald, J.K., Advis, J.P., 1993. Neuropeptide Y regulation of LHRH release in the median eminence: immunocytochemical and physiological evidence in hens. Neuroendocrinology 57, 135–145.
Denbow, D.M., Cline, M.A., 2015. Food intake regulation. In: Scanes, C.G. (Ed.), Sturkie's Avian Physiology. sixth ed. Academic Press, New York, pp. 469–485.
Denbow, D.M., Duke, G.E., Chaplin, S.B., 1988. Food intake, gastric secretion, and motility as affected by avian pancreatic polypeptide administered centrally in chickens. Peptides 9, 449–454.
Ding, W.G., Kimura, H., Fujimura, M., Fujimiya, M., 1997. Neuropeptide Y and peptide YY immunoreactivities in the pancreas of various vertebrates. Peptides 18, 1523–1529.
Dupont, J., 2015. Endocrine pancreas. In: Scanes, C.G. (Ed.), Sturkie's Avian Physiology. sixth ed. Academic Press, New York, pp. 613–631.
Ekblad, E., Sundler, F., 2002. Distribution of pancreatic polypeptide and peptide YY. Peptides 23, 251–261.
Eva, C., Oberto, A., Sprengel, R., Genazzani, E., 1992. The murine NPY-1 receptor gene. Structure and delineation of tissue-specific expression. FEBS Lett. 314, 285–288.
Friedman-Einat, M., Cogburn, L.A., Yosefi, S., Hen, G., Shinder, D., Shirak, A., Seroussi, E., 2014. Discovery and characterization of the first genuine avian leptin gene in the rock dove (*Columba livia*). Endocrinology 155, 3376–3384.
Gregor, P., Feng, Y., DeCarr, L.B., Cornfield, L.J., McCaleb, M.L., 1996a. Molecular characterization of a second mouse pancreatic polypeptide receptor and its inactivated human homologue. J. Biol. Chem. 271, 27776–27781.
Gregor, P., Millham, M.L., Feng, Y., DeCarr, L.B., McCaleb, M.L., Cornfield, L.J., 1996b. Cloning and characterization of a novel receptor to pancreatic polypeptide, a member of the neuropeptide Y receptor family. FEBS Lett. 381, 58–62.
Hansel, D.E., Eipper, B.A., Ronnett, G.V., 2001. Neuropeptide Y functions as a neuroproliferative factor. Nature 410, 940–944.
Hazelwood, R.L., Turner, S.D., Kimmel, J.R., Pollock, H.G., 1973. Spectrum effects of a new polypeptide (third hormone?) isolated from the chicken pancreas. Gen. Comp. Endocrinol. 21, 485–497.
He, C., Zhang, J., Gao, S., Meng, F., Bu, G., Li, J., Wang, Y., 2016. Molecular characterization of three NPY receptors (Y2, Y5 and Y7) in chickens: gene structure, tissue expression, promoter identification, and functional analysis. Gen. Comp. Endocrinol. 236, 24–34.
Higgins, S.E., Ellestad, L.E., Trakooljul, N., McCarthy, F., Saliba, J., Cogburn, L.A., Porter, T.E., 2010. Transcriptional and pathway analysis in the hypothalamus of newly hatched chicks during fasting and delayed feeding. BMC Genomics 11, 162.

Holmberg, S.K., Mikko, S., Boswell, T., Zoorob, R., Larhammar, D., 2002. Pharmacological characterization of cloned chicken neuropeptide Y receptors Y1 and Y5. J. Neurochem. 81, 462–471.
Holzer, P., Reichmann, F., Farzi, A., 2012. Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. Neuropeptides 46, 261–274.
Huang, G., Li, J., Wang, H., Lan, X., Wang, Y., 2014. Discovery of a novel functional leptin protein (LEP) in zebra finches: evidence for the existence of an authentic avian leptin gene predominantly expressed in the brain and pituitary. Endocrinology 155, 3385–3396.
Kameda, Y., Miura, M., Nishimaki, T., 2001. Localization of neuropeptide Y mRNA and peptide in the chicken hypothalamus and their alterations after food deprivation, dehydration, and castration. J. Comp. Neurol. 436, 376–388.
Kimmel, J.R., Hayden, L.J., Pollock, H.G., 1975. Isolation and characterization of a new pancreatic polypeptide hormone. J. Biol. Chem. 250, 9369–9376.
Korner, M., Waser, B., Thalmann, G.N., Reubii, J.C., 2011. High expression of NPY receptors in the human testis. Mol. Cell Endocrinol. 337, 62–70.
Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N., Larsen, P.J., Hastrup, S., 1998. Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393, 72–76.
Kuenzel, W.J., 1994. Central neuroanatomical systems involved in the regulation of food intake in birds and mammals. J. Nutr. 124, 1355S–1370S.
Kuenzel, W.J., Douglass, L.W., Davison, B.A., 1987. Robust feeding following central administration of neuropeptide Y or peptide YY in chicks, *Gallus domesticus*. Peptides 8, 823–828.
Larhammar, D., 1996. Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. Regul. Pept. 62, 1–11.
Larhammar, D., Bergqvist, C.A., 2013. Ancient grandeur of the vertebrate neuropeptide Y system shown by the coelacanth latimeria chalumnae. Front. Neurosci. 7, 27.
Larsson, T.A., Olsson, F., Sundström, G., Lundin, L.G., Brenner, S., Venkatesh, B., Larhammar, D., 2008. Early vertebrate chromosome duplications and the evolution of the neuropeptide Y receptor gene regions. BMC Evol. Biol. 8, 184.
Larsson, T.A., Tay, B.H., Sundström, G., Fredriksson, R., Brenner, S., Larhammar, D., Venkatesh, B., 2009. Neuropeptide Y-family peptides and receptors in the elephant shark, *Callorhinchus milii* confirm gene duplications before the gnathostome radiation. Genomics 93, 254–260.
Liu, L., Song, Z., Sheikhahmadi, A., Jiao, H., Lin, H., 2012. Effect of corticosterone on gene expression of feed intake regulatory peptides in laying hens. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 162, 81–87.
Lundell, I., Blomqvist, A.G., Berglund, M.M., Schober, D.A., Johnson, D., Statnick, M.A., Gadski, R.A., Gehlert, D.R., Larhammar, D., 1995. Cloning of a human receptor of the NPY receptor family with high affinity for pancreatic polypeptide and peptide YY. J. Biol. Chem. 270, 29123–29128.
Lundell, I., Boswell, T., Larhammar, D., 2002. Chicken neuropeptide Y-family receptor Y4: a receptor with equal affinity for pancreatic polypeptide, neuropeptide Y and peptide YY. J. Mol. Endocrinol. 28, 225–235.
Matsumoto, M., Nomura, T., Momose, K., Ikeda, Y., Kondou, Y., Akiho, H., Togami, J., Kimura, Y., Okada, M., Yamaguchi, T., 1996. Inactivation of a novel neuropeptide Y/peptide YY receptor gene in primate species. J. Biol. Chem. 271, 27217–27220.
Meng, F., Huang, G., Gao, S., Li, J., Yan, Z., Wang, Y., 2014. Identification of the receptors for somatostatin (SST) and cortistatin (CST) in chickens and investigation of the roles of cSST28, cSST14, and cCST14 in inhibiting cGHRH1-27NH2-induced growth hormone secretion in cultured chicken pituitary cells. Mol. Cell. Endocrinol. 384, 83–95.
Mercer, R.E., Chee, M.J., Colmers, W.F., 2011. The role of NPY in hypothalamic mediated food intake. Front. Neuroendocrinol. 32, 398–415.
Michel, M.C., Beck-Sickinger, A., Cox, H., Doods, H.N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., Westfall, T., 1998. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacol. Rev. 50, 143–150.
Mo, C., Cai, G., Huang, L., Deng, Q., Lin, D., Cui, L., Wang, Y., Li, J., 2015. Corticotropin-releasing hormone (CRH) stimulates cocaine- and amphetamine-regulated transcript gene (CART1) expression through CRH type 1 receptor (CRHR1) in chicken anterior pituitary. Mol. Cell. Endocrinol. 417, 166–177.
Mullins, D.E., Zhang, X., Hawes, B.E., 2002. Activation of extracellular signal regulated protein kinase by neuropeptide Y and pancreatic polypeptide in CHO cells expressing the NPY Y(1), Y(2), Y(4) and Y(5) receptor subtypes. Regul. Pept. 105, 65–73.
Musso, R., Grilli, M., Oberto, A., Gamalero, S.R., Eva, C., 1997. Regulation of mouse neuropeptide Y Y1 receptor gene transcription: a potential role for nuclear factor-kappa B/Rel proteins. Mol. Pharmacol. 51, 27–35.
Parker, R.M., Herzog, H., 1999. Regional distribution of Y-receptor subtype mRNAs in rat brain. Eur. J. Neurosci. 11, 1431–1448.
Pedrazzini, T., Pralong, F., Grouzmann, E., 2003. Neuropeptide Y: the universal soldier. Cell. Mol. Life Sci. 60, 350–377.
Persaud, S.J., Bewick, G.A., 2014. Peptide YY: more than just an appetite regulator. Diabetologia 57, 1762–1769.
Rogge, G., Jones, D., Hubert, G.W., Lin, Y., Kuhar, M.J., 2008. CART peptides: regulators of body weight, reward and other functions. Nat. Rev. Neurosci. 9, 747–758.
Salaneck, E., Holmberg, S.K., Berglund, M.M., Boswell, T., Larhammar, D., 2000. Chicken neuropeptide Y receptor Y2: structural and pharmacological differences to mammalian Y2(1). FEBS Lett. 484, 229–234.
Salaneck, E., Larsson, T.A., Larson, E.T., Larhammar, D., 2008. Birth and death of neuropeptide Y receptor genes in relation to the teleost fish tetraploidization. Gene 409, 61–71.
Saneyasu, T., Honda, K., Kamisoyama, H., Ikura, A., Nakayama, Y., Hasegawa, S., 2011. Neuropeptide Y effect on food intake in broiler and layer chicks. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 159, 422–426.
Schwartz, M.W., Woods, S.C., Porte Jr., D., Seeley, R.J., Baskin, D.G., 2000. Central nervous system control of food intake. Nature 404, 661–671.
Starbäck, P., Wraith, A., Eriksson, H., Larhammar, D., 2000. Neuropeptide Y receptor gene y6: multiple deaths or resurrections? Biochem. Biophys. Res. Commun. 277, 264–269.
Sundström, G., Larsson, T.A., Brenner, S., Venkatesh, B., Larhammar, D., 2008. Evolution of the neuropeptide Y family: new genes by chromosome duplications in early vertebrates and in teleost fishes. Gen. Comp. Endocrinol. 155, 705–716.
Sundström, G., Xu, B., Larsson, T.A., Heldin, J., Bergqvist, C.A., Fredriksson, R., Conlon, J.M., Lundell, I., Denver, R.J., Larhammar, D., 2012. Characterization of the neuropeptide Y system in the frog *Silurana tropicalis* (Pipidae): three peptides and six receptor subtypes. Gen. Comp. Endocrinol. 177, 322–331.
Sundström, G., Larsson, T.A., Xu, B., Heldin, J., Larhammar, D., 2013. Interactions of zebrafish peptide YYb with the neuropeptide Y-family receptors Y4, Y7, Y8a, and Y8b. Front. Neurosci. 7, 29.
Tachibana, T., Sato, M., Oikawa, D., Takahashi, H., Boswell, T., Furuse, M., 2006. Intracerebroventricular injection of neuropeptide Y modifies carbohydrate and lipid metabolism in chicks. Regul. Pept. 136, 1–8.
Wang, X., Day, J.R., Vasilatos-Younken, R., 2001. The distribution of neuropeptide Y gene expression in the chicken brain. Mol. Cell. Endocrinol. 174, 129–136.
Wang, Y., Li, J., Yan Kwok, A.H., Ge, W., Leung, F.C., 2010. A novel prolactin-like protein (PRL-L) gene in chickens and zebrafish: cloning and characterization of its tissue expression. Gen. Comp. Endocrinol. 166, 200–210.
Xapelli, S., Agasse, F., Ferreira, R., Silva, A.P., Malva, J.O., 2006. Neuropeptide Y as an endogenous antiepileptic, neuroprotective and pro-neurogenic peptide. Recent Pat. CNS Drug Discov. 1, 315–324.
Yi, J., Gilbert, E.R., Siegel, P.B., Cline, M.A., 2015. Fed and fasted chicks from lines divergently selected for low or high body weight have differential hypothalamic appetite-associated factor mRNA expression profiles. Behav. Brain Res. 286, 58–63.
Yuan, L., Ni, Y., Barth, S., Wang, Y., Grossmann, R., Zhao, R., 2009. Layer and broiler chicks exhibit similar hypothalamic expression of orexigenic neuropeptides but distinct expression of genes related to energy homeostasis and obesity. Brain Res. 1273, 18–28.
Zhang, W., Bai, S., Liu, D., Cline, M.A., Gilbert, E.R., 2015. Neuropeptide Y promotes adipogenesis in chicken adipose cells *in vitro*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 181, 62–70.
Zhou, W., Murakami, M., Hasegawa, S., Yoshizawa, F., Sugahara, K., 2005. Neuropeptide Y content in the hypothalamic paraventricular nucleus responds to fasting and refeeding in broiler chickens. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 141, 146–152.