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Molecular characterization of three NPY receptors (Y2, Y5 and Y7) in chickens: Gene structure, tissue expression, promoter identification, and functional analysis

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ABSTRACT

Six neuropeptide Y (NPY) receptors are suggested to mediate the biological actions of NPY, peptide YY (PYY), and pancreatic polypeptide (PP), such as food intake in birds, however, information regarding the structure and signaling of avian NPY receptors are rather limited. In this study, we investigated the gene structure, tissue expression and signaling property of three NPY receptors (cY2, cY5 and cY7) in chickens. The results showed that 1) *cY2*, *cY5* and *cY7* contain novel non-coding exons upstream of their start codon and alternative mRNA splicing in their 5′-UTR results in the formation of multiple transcript variants; 2) *cY2*, *cY5* and *cY7* transcripts were detected to be widely expressed in adult chicken tissues including various brain regions by RT-PCR, and their expression is controlled by a promoter(s) near exon 1, which display promoter activity in DF-1 cells as demonstrated by Dual-luciferase reporter assay; 3) cY2, cY5 and cY7 expressed in HEK293 cells were preferentially (or potently) activated by $cNPY_{1-36}$ and $cPYY_{1-37}$, but not by cPP_{1-36}, and their activation led to the inhibition of cAMP/PKA signaling pathway and activation of MAPK/ERK signaling pathway, monitored by the cell-based luciferase reporter systems or western blots, indicating that the three NPY receptors are functional and capable of transmitting signals effectively. On the whole, our data establishes a molecular basis to elucidate the actions of three functional NPY receptors (cY2, cY5 and cY7) and their ligands in birds, which helps to uncover the conserved roles of these ligand-receptor pairs in vertebrates.

1. Introduction

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) are structurally related peptides which are 36 or 37 amino acids in length in vertebrates (Cerdá-Reverter and Larhammar, 2000). Constituting the NPY family, these peptides are encoded by separate genes, which have arisen by gene or genome duplication (Cerdá-Reverter and Larhammar, 2000). In mammals, NPY is reported to be widely expressed in the central and peripheral nervous systems and regulate many physiological processes, such as food intake, stress, neurogenesis, blood pressure, memory retention, circadian rhythm and gastrointestinal activity (Hansel et al., 2001; Pedrazzini et al., 2003). Unlike NPY, PYY is mainly expressed in intestinal L cells of the lower gastrointestinal tract, while PP is almost exclusively expressed in the pancreas. Like NPY, both PYY and PP are suggested to be involved in the control of many physiological processes, such as food intake, gastrointestinal tract activity and pancreatic functions (Ekblad and Sundler, 2002; Holzer et al., 2012; McGowan and Bloom, 2004; Persaud and Bewick, 2014; Ueno et al., 2008).

The diverse actions of NPY, PYY and PP are suggested to be mediated through multiple NPY receptors (Michel et al., 1998), all of which belong to G protein-coupled receptors and share high structural similarity. To date, five to eight NPY receptors, namely Y1, Y2, Y4, Y5, Y6, Y7 and Y8 (Y8a and Y8b), have been identified in vertebrates (Larhammar and Bergqvist, 2013; Salaneck et al., 2008). These receptors can be divided into three subfamilies, namely Y1-subfamily (Y1, Y4, Y6, and Y8), Y2-subfamily (Y2 and Y7), and Y5-subfamily (Y5), based on their amino acid sequence identities and evolutionary origins (Larhammar and Salaneck, 2004). NPY receptors are reported to couple to $G_{i/o}$ and thus able to reduce cAMP synthesis upon ligand binding (Michel et al., 1998). In addition, NPY receptors are reported to be capable of activating phospholipase C (PLC) (Pedrazzini et al., 2003; Persaud and

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Bewick, 2014) and MAPK/ERK signaling pathways (Mullins et al., 2002).

In chickens, 6 NPY receptors, namely cY1, cY2, cY4, cY5, cY6 and cY7, have been identified, and some of them have been shown to bind pig NPY, and chicken PYY and PP with distinct affinities *in vitro* (Bromee et al., 2006; Holmberg et al., 2002; Lundell et al., 2002; Salaneck et al., 2000). In spite of these pioneering studies, our knowledge regarding the gene structure and signaling property of these NPY receptors remains rather scarce in birds. As in mammals, three NPY family peptides, NPY, PYY and PP, have been identified in chickens (Blomqvist et al., 1992; Conlon and O'Harte, 1992; Kimmel et al., 1975). However, their actions in chickens appear to be similar, but not identical, to those in mammals. For instance, NPY, PYY and PP can increase food intake in chickens by central administration (Ando et al., 2001; Denbow et al., 1988; Kuenzel et al., 1987; Tachibana et al., 2006), whereas in mammals, NPY stimulates food intake whilst PYY and PP inhibit it (Holzer et al., 2012). Moreover, unlike mammalian PYY, chicken PYY has 37 amino acids, and its N-terminally truncated form (PYY_{3-36}), a known potent appetite-suppressor in mammals, seems nonexistent in chickens (Cerdá-Reverter and Larhammar, 2000; McGowan and Bloom, 2004). These structural and functional differences between avian and mammalian NPY family peptides lead us to question whether the 6 NPY receptors could mediate the actions of NPY/PYY/PP in birds, and if so, whether their functions are analogous to that in mammals. Therefore, the present study aims to examine the gene structure, tissue expression, and signaling properties of the three NPY receptors (Y2, Y5, and Y7) in chickens. These data, together with our recent characterization of cY1, cY4, cY6 and their ligands in chickens (Gao et al., 2016), will establish a molecular basis to interpret the roles of the three NPY family peptides and their receptors in birds, and facilitate a more comprehensive understanding of the structural and functional changes of the NPY system during vertebrate evolution.

2. Materials and methods

2.1. Chemicals, peptides, primers and antibodies

The chemicals were all obtained from Sigma-Aldrich (St. Louis, MO) and restriction enzymes were purchased from Takara (Takara, Dalian, China) unless stated otherwise. Chicken NPY ($cNPY_{1-36}$), PYY ($cPYY_{1-37}$), PP (cPP_{1-36}) were synthesized with solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). The purity of synthesized chicken peptides is >95% (analyzed by HPLC), and their structures were verified by mass spectrometry. Primers used in this study were synthesized by Beijing Genome Institute (BGI, Shanghai, China) and listed in Table 1. Antibodies for β-actin and phosphorylated ERK1/2 were purchased from Cell Signaling Technology Inc. (CST, Beverly, MA).

2.2. Total RNA extraction and RT-PCR assay

Chickens (Lohmann layers) were purchased from local suppliers. Chickens were killed and different tissues including the heart, duodenum, kidneys, liver, lung, muscle, ovary, testes, pituitary, spleen, pancreas, and various brain regions including the telencephalon, cerebellum, hindbrain, midbrain and hypothalamus, were collected for total RNA extraction. All animal experiments were performed according to the guidelines provided by the Animal Ethics Committee of Sichuan University.

Total RNA was extracted from tissues with RNAzol Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and reversely transcribed using MMLV reverse transcriptase (Takara). These RT samples were then used for PCR assay of *cY2*, *cY5* and *cY7* expression in chicken tissues. For *cY2* and *cY7*, 33 PCR cycles of 30 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C were used, followed by a 5-min extension at 72 °C. For *cY5*, 33 PCR cycles of 30 s at 94 °C, 30 s at 62 °C, 60 s at 72 °C were used, followed by a 5-min extension at 72 °C. For *β-actin*, 23 PCR cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C were used followed by a 5-min extension at 72 °C. The PCR products were visualized on a UV-transilluminator (Bio-Rad Laboratories, Inc. Herculas, CA) after electrophoresis on 2% agarose gel containing ethidium bromide. The identity of PCR products, particularly those PCR bands with strong intensity, was verified by sequencing (BGI).

2.3. Rapid amplification of 5′- and 3′-cDNA ends (RACE)

To characterize the complete gene structure of chicken *Y2*, *Y5* and *Y7*, rapid amplification of 5′- or 3′-cDNA ends (RACE) was performed using SMART-RACE cDNA amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The amplified PCR products were cloned into pTA2 vector (TOYOBO, Osaka, Japan) for sequencing. Finally, the sequence and structure of 5′-untranslated region (5′UTR) and 3′-untranslated region (3′UTR) of each receptor gene were determined by comparison to the chicken genome database (www.ensembl.org/gallus_gallus).

2.4. Identification of the promoter regions of chicken Y2, Y5 and Y7

According to the genomic sequences of *cY2*, *cY5* and *cY7*, gene-specific primers were designed to amplify the 5′-flanking regions of *cY2*, *cY5* and *cY7* in various lengths with high-fidelity Taq DNA polymerase (TOYOBO) (Table 1). The amplified fragments were cloned into pGL3-Basic vector to generate the promoter-luciferase reporter constructs. In this experiment, six promoter-luciferase constructs containing different stretches of the *cY2* 5′-flanking region (−1564/−68Luc; −1564/+582Luc; −500/+582Luc; −87/+582Luc; +357/+582Luc) were prepared. Furthermore, five promoter-luciferase constructs (−1745/+149Luc; −833/+149Luc; −343/+149Luc; −91/+149Luc; −91/+57Luc) for *cY5* and 4 constructs for *cY7* (−882/+333Luc; −289/+333Luc; −73/+333Luc; −73/+48Luc) were also prepared. In this study, the first nucleotide on exon 1 of each receptor gene determined by 5′-RACE was designated as "+1", while the first nucleotide upstream of exon 1 was designed as "−1".

The promoter activity of the 5′-flanking fragment of each gene was tested in DF-1 cells by the Dual-luciferase reporter assay, as previously reported (Wang et al., 2010). In brief, DF-1 cells were cultured in a 48-well plate at a density of 1×10^5 cells per well before transfection. After 24-h incubation, a mixture containing 100 ng of promoter construct, 5 ng of pRL-TK construct and 0.5 μL of JetPRIME (Polyplus-transfection, France) was prepared in 20 μL of PBS and transfected following the manufacturer's instructions when the cells reached 70% confluence. 24 h later, the medium was removed and 100 μL 1× passive lysis buffer (Promega) was added to each well. Luciferase activities of 15 μL of cellular lysates were measured by using Dual-luciferase Assay Kit (Promega). The cells transfected with the empty pGL3-Basic vector was used as an internal control group.

2.5. Functional characterization of chicken Y2, Y5 and Y7 in cultured HEK293 cells

Based on the coding sequences of *cY2*, *cY5*, and *cY7* deposited in the GenBank (NM_001031128; NM_001031130; NM_001037824), gene-specific primers were used to amplify the open reading frame (ORF) of each receptor gene with the use of high-fidelity Taq DNA polymerase (TOYOBO). The amplified fragments were cloned into pcDNA3.1 (+) expression vector and sequenced (BGI).

Table 1
Primers used.[a]

Gene	Sense/antisense	Primer sequence (5′–3′)	Size (bp)
Primers for construction of the expression plasmids[b]			
cY2	Sense	CGG*ggtacc*AGCGCTATTGCTACTGAAAG	1158
	Antisense	CCG*gaATTC*CTTTGCCCACAACATGTG	
cY5	Sense	CGG*ggtacC*TAAGCAGGACTATAAAATGGA	1342
	Antisense	CCG*gaattc*TATATGCATTAGTATGCACGA	
cY7	Sense	CGG*ggtaCC*AGGCCAGGCATGGGACTGCT	1158
	Antisense	CCG*gaatTC*AGTCCTGGAGGCGGACAT	
Primers for rapid amplification of cDNA ends (RACE)			
cY2-rL1	Antisense	GCTTGGGCATAAGGCACCAAGTGGC	
cY2-rL2	Antisense	TCAGCCACAGCCAGGTTGGCGATGA	
cY5-rL1	Antisense	GCCGTTAAGTTGCTGGAGAGGGGAT	
cY5-rL2	Antisense	GCATTATGTGGCACATGACAGTGCC	
cY7-rL1	Antisense	GGCAGGCAGAGGGTGTCCACCATC	
cY7-rL2	Antisense	TCAGCCACAGCCAGGTTGGCGATGA	
Primers for RT-PCR assays			
cY2	Sense	TGCCTCCAAACTTTCTATGCT	223/83[c]
	Antisense	CCCATCCTTTCAGTAGCAATA	
cY5	Sense	TCAGCCCCGGGGCTATGAGCT	401/279/171[c]
	Antisense	CTTGTAGTCCTCCCAGGCAGA	
cY7	Sense	TCACCTGACCTCCTCGCTGTT	224/253[c]
	Antisense	AGCTTCTCATCTACTGCCAGCT	
β-actin	Sense	TGTGCTACGTCGCACTGGAT	401
	Antisense	GCTGATCCACATCTGCTGGA	
Primers for preparation of the promoter-luciferase constructs[b]			
cY2(-1564/-68Luc)	Sense	CGG*ggtacc*TGTTGGAAGTGCTGTAAGA	1496
	Antisense	*gctagc*ACTAGCTTCAGCCAGCACTG	
cY2(-1564/+582Luc)	Sense	CGG*ggtacc*TGTTGGAAGTGCTGTAAGA	2146
cY2(-500/+582Luc)	Sense	CGG*ggtacc*AGGGATCATTACCTTTGGCCA	1082
cY2(-87/+582Luc)	Sense	CGG*ggtacc*AGTGCTGGCTGAAGCTAGT	669
cY2(+357/+582Luc)	Sense	CGG*ggtacc*GCTGCCCCGCCGCGCAT	225
	Antisense[d]	*gctagc*ATAGAAAGTTTGGAGGC	
cY5(-1745/+149Luc)	Sense	CGG*ggtacc*ATTGCTGGTTGATGAACTCT	1894
cY5(-833/+149Luc)	Sense	CGG*ggtacc*TACAAGTCGGCTGATCCAGCT	982
cY5(-343/+149Luc)	Sense	CGG*ggtacc*TGCTCTGAAATAACGGTGGAT	492
cY5(-91/+149Luc)	Sense	CGG*ggtacc*CTGGGGTTTGTTTACAGT	240
	Antisense[d]	CCG*ctcgag*ATCTTACGCGGGACTGC	
cY5(-91/+57Luc)	Sense	CGG*ggtacc*ATTGCTGGTTGATGAACTCT	148
	Antisense	CCG*ctcgag*CTCTGCCGGCACCGAGGCT	
cY7(-882/+333Luc)	Sense	CGG*ggtacc*TCTGCCGAGCCACTCAAACTT	1215
cY7(-289/+333Luc)	Sense	CGG*ggtacc*GTGCTGTGTGAGCGCAGATAGA	622
cY7(-73/+333Luc)	Sense	CGG*ggtacc*TGGATGTGGAGGAAAAGCT	406
	Antisense[d]	CCG*ctcgag*TGTCCTTCTGGTTCGGGATC	
cY7(-73/+48Luc)	Sense	CGG*ggtacc*TCTGCCGAGCCACTCAAACTT	121
	Antisense	CCG*ctcgag*GCGTGTGTTTAACAAACAGA	

[a] All primers were synthesized by BGI (Shanghai, China).
[b] The restriction sites added are underlined.
[c] Two or three PCR bands were detected using this pair of primers.
[d] The antisense primer was used to prepare multiple promoter-luciferase constructs of *cY2*/*cY5*/*cY7*.

The signaling pathways coupled to each receptor were assessed in HEK293 cells through luciferase reporter assays. Two reporter systems (pGL3-CRE-luciferase reporter system and pGL4-SRE-luciferase reporter system) with the firefly luciferase gene expression driven by promoters containing cAMP response elements (CRE), and serum response elements (SRE) were used to monitor the receptor activated (or inhibited) cAMP/PKA and MAPK/ERK signaling pathways, respectively (Ho et al., 2012; Meng et al., 2014; Mo et al., 2015). In brief, HEK293 cells were cultured in Dulbecco minimal Eagle medium (DMEM) supplemented with 5% (vol/vol) fetal bovine serum (Thermo), 100 U/mL penicillin G, and 100 μg/mL streptomycin 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 1 day before transfection. A mixture containing 700 ng of pGL3-CRE-luciferase construct (or pGL4-SRE-luciferase construct), 200 ng of expression plasmid (or empty pcDNA3.1 vector) and 2 μL of jetPIRIME were prepared in 100 μL buffer. Transfection was performed according to the manufacturer's instructions when cells reached 70% confluence. After 24-h incubation, HEK293 cells were subcultured in a 96-well plate at 37 °C for 24 h before peptide treatment. After removal of medium from the 96-well plate, the cells were treated with 100 μL peptide-containing medium (or peptide-free medium) in the presence or absence of forskolin (an activator of adenylate cyclase, 5 μM). The cells were incubated for 6 h at 37 °C before being harvested for luciferase assays. After removal of culture medium, HEK293 cells were lysed by adding 50 μL of 1× Passive Lysis Buffer per well, and the luciferase activity of 15 μL cellular lysates was determined with luciferase assay reagent (Promega). The luciferase activities in each treatment group were expressed as relative fold increase compared with the control group.

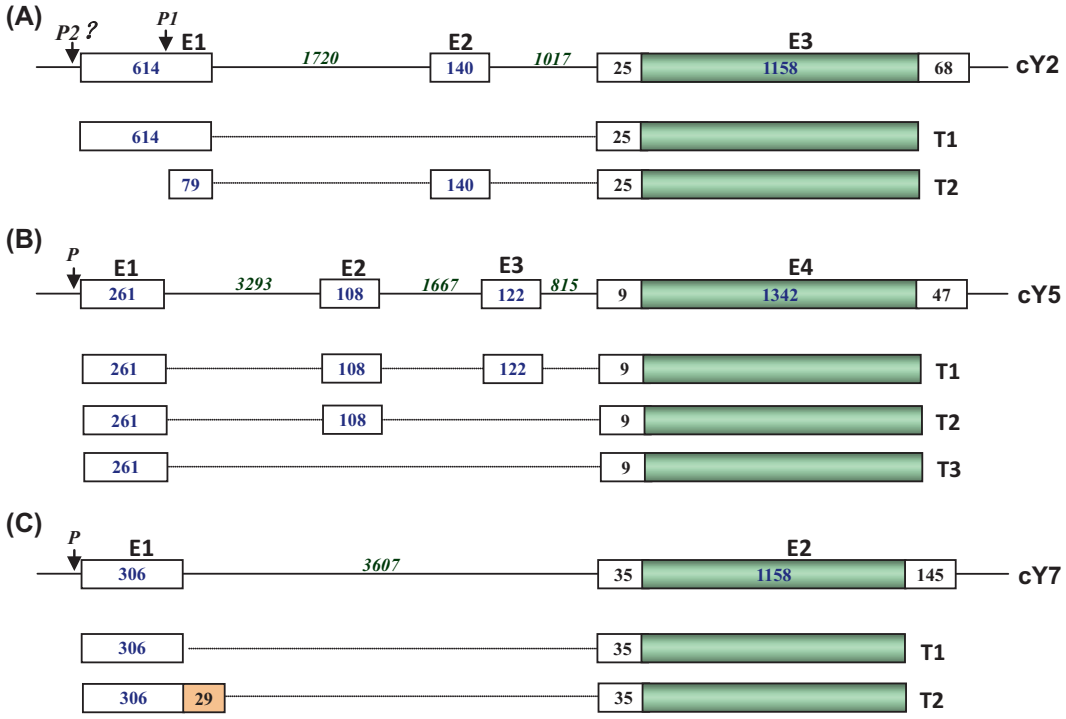

Fig. 1. Gene structure of chicken *Y2*, *Y5* and *Y7* and their mRNA splice variants. (A) Chicken *Y2* (*cY2*) consists of three exons (E1, E2 and E3) and it has two types of transcript variants (T1 and T2) generated by alternative mRNA splicing. Interestingly, one functional promoter region (named P1) was found to be located within exon 1, while an additional putative promoter (P2) may be located upstream of or distal to exon 1. (B) Chicken *Y5* (*cY5*) consists of four exons (E1, E2, E3 and E4) and three types of transcript variants (T1-T3) generated by alternative mRNA splicing were identified. (C) Chicken *Y7* (*cY7*) consists of two exons (E1 and E2) and two types of transcript variants (T1 and T2) generated by alternative usage of splice donor site at intron 1 were identified. In (A–C), the coding region of each receptor gene is shaded. Exons were represented by boxes, while introns as lines. Numbers in the boxes indicate the sizes (bp) of respective exons; while numbers in italic denote the sizes of respective introns. The promoter region (P1/P) of each receptor gene was found to be located near (or within) exon 1, which was proved to be functional in later experiments (please refer to Figs. 3–5 for details).

2.6. Western blot

To test whether the activation of cY2, cY5 and cY7 can stimulate ERK1/2 phosphorylation, HEK293 cells transfected with 500 ng of receptor expression plasmid [or empty pcDNA3.1 (+) vector] were cultured for 24 h in a 24-well plate and then treated with $cNPY_{1-36}$ (100 nM) for 10 min. 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 protein level was also examined and used as a control to monitor protein loading.

2.7. 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 as compared with the control group (promoter-less pGL3-Basic vector). The data were analyzed by one-way ANOVA followed by Dunnett's test with the use of GraphPad Prism 5 (GraphPad Software, San Diego, CA). To validate our results, all experiments were repeated at least two to four times.

3. Results

3.1. Characterization of the gene structure of chicken Y2, Y5 and Y7

The coding region of chicken *Y2*, *Y5* and *Y7* is intronless (Bromee et al., 2006; Holmberg et al., 2002; Salaneck et al., 2000); however, whether extra exon(s) exists within these receptor genes, particularly at their 5′-untranslated regions (5′UTR), has yet been determined. Therefore, 5′-RACE and 3′-RACE PCR were performed to clone the 5′-UTR and 3′-UTR of *Y2*, *Y5* and *Y7* from adult chicken brains in this experiment.

Comparison of the cloned 5′-UTR and 3′-UTR sequences of *cY2*, *cY5* and *cY7* with chicken genome (http://www.ensembl.org/Gallus_gallus) revealed that: (1) *cY2* contains three exons, including two novel non-coding exons (exon 1, 614 bp; exon 2, 140 bp) identified within its 5′UTR. Moreover, two types of 5′-UTRs generated by alternative mRNA splicing were identified and designated as T1 (accession no.: KU185985) and T2 (KU185987) transcripts, respectively (Fig. 1); 2) *cY5* contains 4 exons, including 3 novel non-coding exons (exon 1, 261 bp; exon 2, 108 bp; exon 3, 122 bp) identified in its 5′UTR. Interestingly, three types of *cY5* 5′UTRs generated by alternative mRNA splicing were also identified, and they were designated as T1 (KU185978), T2 (KU185979) and T3 (KU185980) transcripts, respectively; 3) *cY7* has two exons, including a novel non-coding exon (exon 1, 306 bp) identified in its 5′-UTR. Similarly, two types of *cY7* 5′UTR were identified and designated as T1 (KU185974) and T2 (KU185975) transcripts, respectively. T2 transcript is generated by the alternative usage of a splice donor site on intron 1 and thus causes the retention of 29-bp intronic sequence (Fig. 1); 4) no extra exon was identified in the 3′-UTRs of the three receptor genes by 3′-RACE (Fig. S1).

The complete gene structure of *cY2*, *cY5* and *cY7*, and the exon organization of their transcripts are schematically depicted in Fig. 1.

3.2. Tissue expression of Y2, Y5 and Y7 in chickens

Since multiple mRNA species of *cY2*, *cY5* and *cY7* were identified in this study (Fig. 1), therefore we designed gene-specific primers to examine their expression in adult chicken tissues, including

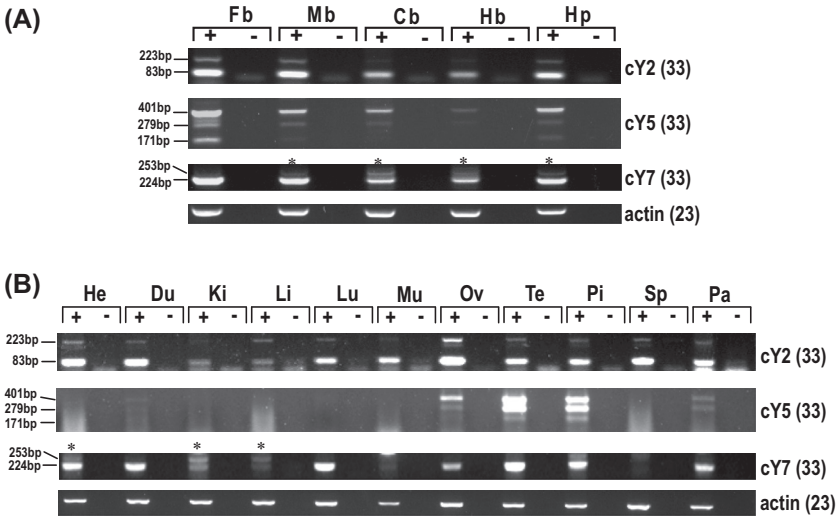

Fig. 2. Tissue expression of *Y2*, *Y5*, and *Y7* in adult chickens. (A) RT-PCR detection of multiple mRNA variants of *cY2*, *cY5*, and *cY7* expressed in various brain regions, including the telencephalon (Fb), midbrain (Mb), cerebellum (Cb), hindbrain (Hb), and hypothalamus (Hp). (B) RT-PCR detection of *Y2*, *Y5*, and *Y7* transcripts in peripheral tissues including the heart (He), duodenum (Du), kidneys (Ki), liver (Li), lung (Lu), muscle (Mu), ovary (Ov), testes (Te), pituitary (Pi), spleen (Sp), and pancreas (Pa). Asterisks in (A) and (B) indicate the weak PCR band (253 bp) corresponding to *cY7* T2 transcript expressed in these tissues. Numbers in brackets indicate the numbers of PCR cycles used. No PCR band was detected in all RT negative controls (–).

the heart, duodenum, kidneys, liver, lung, muscle, ovary, testes, pituitary, spleen, pancreas and various brain regions by RT-PCR.

As shown in Fig. 2, two PCR bands corresponding to *cY2* T1 (83 bp) and T2 (223 bp) transcripts were detected in all tissues examined including various brain regions, indicating that both transcripts are widely expressed in chicken tissues. Moreover, we noted that the 83-bp band has a much stronger intensity than the 223-bp band in all tissues examined.

Like *cY2*, *cY7* transcripts (T1 and T2) were found to be expressed in various brain regions including the hypothalamus. In peripheral tissues, *cY7* transcripts were also found to be widely distributed in nearly all tissues examined except the spleen. Although T1 and T2 transcripts were detected to be co-expressed in some chicken tissues, the signal intensity of PCR band (224 bp) corresponding to *cY7* T1 transcript is much stronger than that of the large PCR band (253 bp: T2 transcript) in most tissues examined (Fig. 2).

Similarly, multiple PCR bands corresponding to the T1 (401 bp), T2 (279 bp), and T3 (171 bp) transcripts of *cY5* were detected in various brain regions including the hypothalamus. In peripheral tissues, multiple PCR bands could be detected in the duodenum, ovary, testes, pituitary, and pancreas, and the strongest PCR signal was detected in the pituitary and testes (Fig. 2).

3.3. Promoter analyses of cY2, cY5 and cY7

The identification of the leading exon 1 of *cY2*, *cY5* and *cY7* by 5′-RACE (Fig. 1) led us to speculate that a promoter region capable of driving gene transcription may be located near exon 1 of each receptor gene. To test this possibility, we cloned the 5′-flanking regions of *cY2*, *cY5* and *cY7* near exon 1 into the pGL3-Basic vector and test their promoter activities in cultured DF-1 cells.

As shown in Fig. 3, the 5′-flanking region from −1564 to +582 of *cY2* (KU185983) displayed a strong promoter activity in DF-1 cells and caused more than 60-fold increase in luciferase activity compared to control cells transfected with the promoter-less pGL3-Basic vector. Using a deletion approach, we further noted that the removal of the 5′-end (from −1564 to −500) could enhance the promoter activity, implying that this region may contain a negative regulatory element(s). Further deletion from −500 to +356 could not reduce the promoter activity. Interestingly, the maximal promoter activity was noted within +357/+582, a region located within exon 1 (Fig. 3), whereas the construct (from −1564/−68) without this fragment displays no promoter activity, supporting the notion that a core promoter region is located within this region (+357/+582). Using the two online softwares, TFBind (http://tfbind.hgc.jp/) and JASPAR (http://jaspar.genereg.net), the putative binding sites for many transcription factors, including Sp1, MyoD, STAT3, FoxL1 and P53, were predicted to exist within or near the core promoter region (+357/+582) (Fig. 3).

Since 5′RACE assays reveal the presence of an alternative transcription start site on *cY2*, which is 356-bp upstream of this putative core promoter region (+357/+582), it suggests that an extra upstream promoter (P2) may exist in *cY2* (Fig. 1).

Like *cY2*, *cY5* 5′-flanking region from −1745/+149 near exon 1 (KU185982) also displayed a strong promoter activity in chicken DF-1 cells. Removal of its 5′-end from −1745 to −833 could not reduce its promoter activity significantly, however, further deletion of the 5′-end or 3′-end of this fragment (−833/+149) could gradually decrease its promoter activity, hinting that some *cis*-regulatory elements are necessary for its full promoter activity. Since the 5′-flanking region from −91/+149 of *cY5* still maintained a strong promoter activity in DF-1 cells, the core promoter region is hence suggested to localize within this region. Using online softwares, the putative binding sites for several transcription factors, including Sp1, NRF1, NF-kB, Pdx1, GATA, CREB, STATs, RXRa and RUNX1, were predicted to exist within or near the core promoter region (−91/+149) (Fig. 4).

Similarly, *cY7* flanking region from −882/+333 near exon 1 (KU185977) also displayed a strong promoter activity in DF-1 cells. Removal of its 5′-end from −882 to −290 could not reduce its promoter activity significantly; however, further deletion of the 5′-end, or 3′-end of this fragment (−289/+333) could diminish its promoter activity significantly. This finding suggests that the core promoter region is located within this region (−289/+333), where the putative binding sites for many transcription factors, such as GATA3, EBF1, Smad2/3, Sp1, STAT3, STAT5, MyoD, Nkx3-2 and ESR2, were also predicted (Fig. 5).

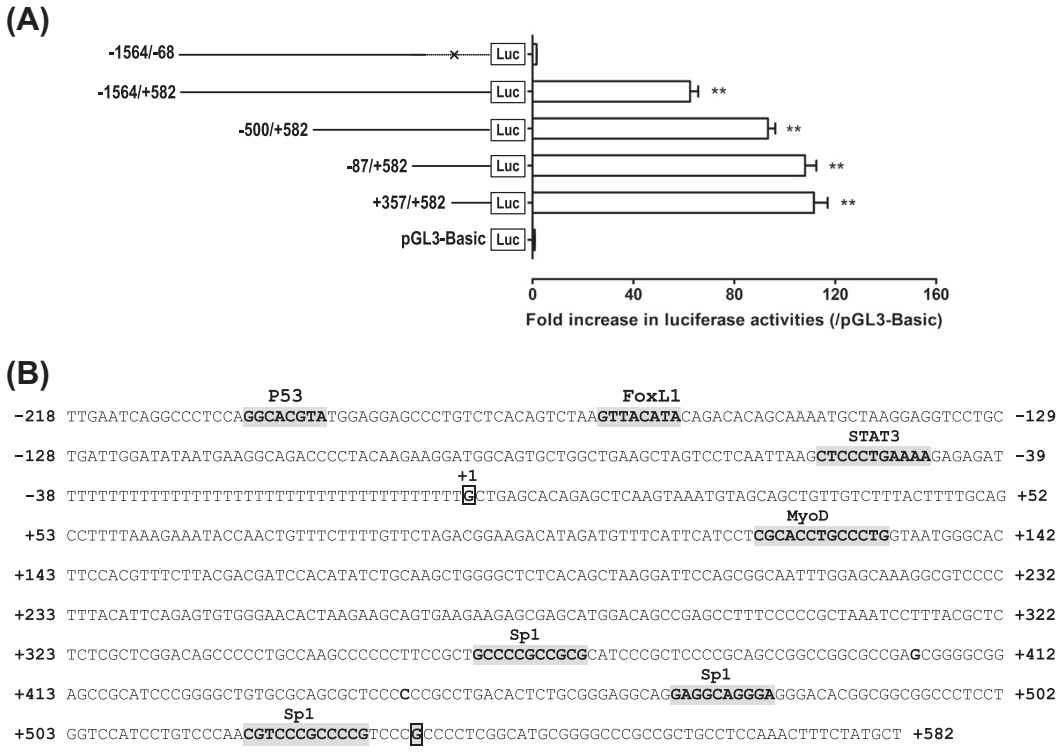

Fig. 3. Promoter analysis of chicken *Y2*. (A) Detection of the promoter activities of the 5′-flanking region of c*Y2* gene in cultured DF-1 cells. Various stretches of the 5′-flanking regions of *cY2* were cloned into pGL3-Basic vector for the generation of 6 promoter-luciferase constructs (−1564/−68Luc; −1564/+582Luc; −500/+582Luc; −87/+582Luc; +357/+582Luc). These promoter-luciferase 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 study (Wang et al., 2010). Each value represents the means ± SEM of three replicates ($N = 3$). **, $P < 0.001$ *vs* pGL3-Basic; (B) partial sequence (−218/+582) of the *cY2* promoter region. The predicted binding sites for transcriptional factors including P53, FoxL1, STAT3, MyoD, and Sp1 were shaded. The transcriptional start site 'G' identified by 5′-RACE was boxed and designated as '+1′. Interestingly, an extra transcription start site 'G' (boxed and shaded) was also identified within exon 1.

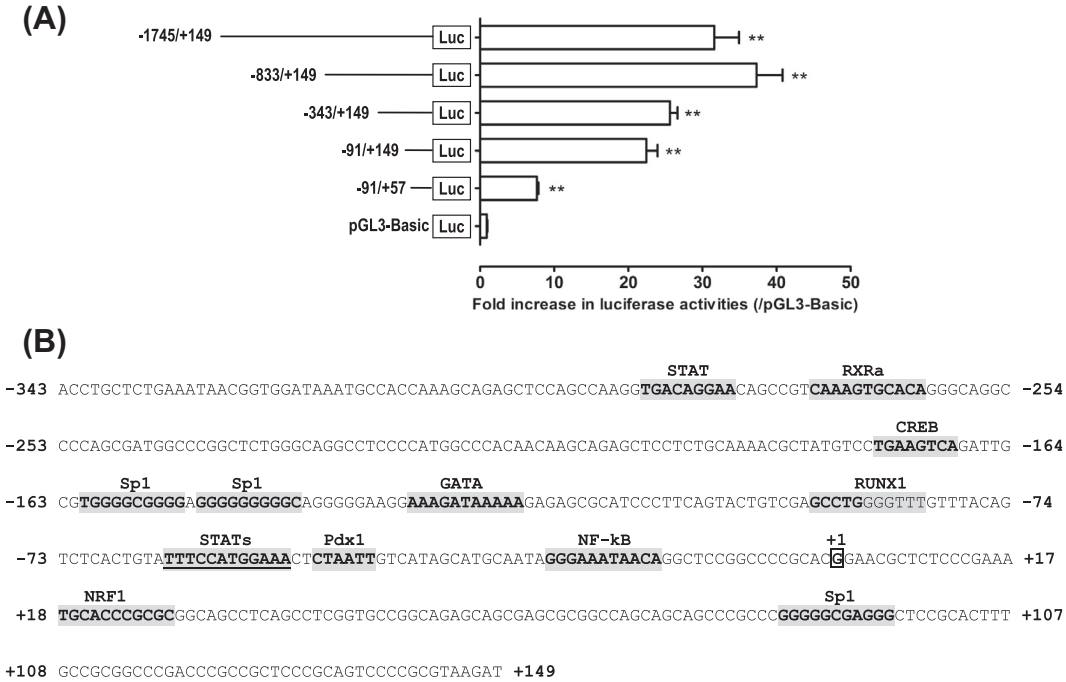

Fig. 4. Promoter analysis of chicken *Y5*. (A) Detection of the promoter activities of the 5′-flanking region of *cY5* in cultured DF-1 cells. Various stretches of the 5′-flanking regions of *cY5* gene were cloned into pGL3-Basic vector to create 5 promoter-luciferase constructs (−1745/+149Luc; −833/+149Luc; −343/+149Luc; −91/+149Luc; −91/+57Luc). The promoter activities of these constructs were 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 (−343/+149) of the *cY5* promoter region. The predicted binding sites for transcriptional factors including STATs, RXRa, CREB, Sp1, GATA, RUNX1, Pdx1, NF-kB, and NRF1 were shaded. The transcriptional start site 'G' identified by 5′-RACE was boxed and designated as +1.

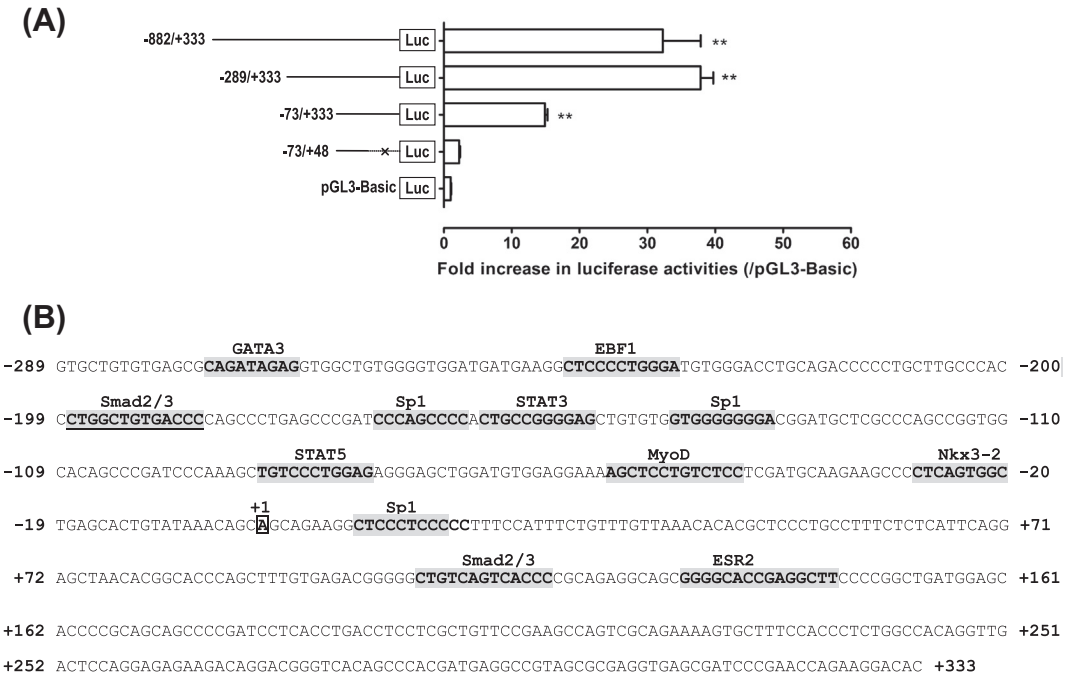

Fig. 5. Promoter analysis of chicken *Y7*. (A) Detection of the promoter activities of the 5′-flanking region of *cY7* in cultured DF-1 cells. The 5′-flanking regions of *cY7* were cloned into pGL3-Basic vector to generate 4 promoter-luciferase constructs (−882/+333Luc, −289/+333Luc, −73/+333Luc, −73/+48Luc). The promoter activities of these constructs were then measured via 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 (−289/+333) of the *cY7* promoter region. The predicted binding sites for transcriptional factors including GATA3, EBF1, Smad2/3, Sp1, STAT3, STAT5, MyoD, Nkx3-2 and ESR2 were shaded. The transcriptional start site 'A' identified by 5′-RACE was boxed and designated as +1.

3.4. Functional characterization of chicken Y2, Y5 and Y7 in cultured HEK293 cells

cY2, cY5 and cY7 have been shown to be capable of binding NPY, PYY and PP peptides from chickens or pigs *in vitro* (Bromee et al., 2006; Holmberg et al., 2002; Salaneck et al., 2000), however, their downstream signaling pathways have not been fully characterized. In this experiment, cY2, cY5 and cY7 were transiently expressed in HEK293 cells and subjected to peptide treatment ($cNPY_{1-36}$, $cPYY_{1-37}$, and cPP_{1-36}). The receptor-activated signaling pathways were then analyzed by the pGL3-CRE-luciferase and pGL4-SRE-luciferase reporter systems established in our previous studies (Ho et al., 2012; Meng et al., 2014; Mo et al., 2015).

Using the pGL3-CRE-luciferase reporter system, we found that both cNPY and cPYY treatment could inhibit forskolin-stimulated luciferase activities of HEK293 via activation of cY2 dose-dependently, with EC_{50} values at 0.69 nM and 0.56 nM, respectively. In contrast, cPP are 1000-fold less potent than cNPY and cPYY (Fig. 6). These findings not only suggest that cY2 is functionally coupled to Gi protein, and its activation can decrease the intracellular cAMP levels and CRE-mediated reporter gene expression induced by forskolin (Fig. 6), but also indicate that cY2 can function as a receptor common for both cNPY and cPYY. Similarly, cNPY (EC_{50}: 0.14 nM) and cPYY (EC_{50}: 0.46 nM), but not PP (EC_{50}: >1000 nM), could potently activate cY7 expressed in HEK293 cells, and hence caused the reduction in forskolin-induced luciferase activity. This finding indicates that like cY2, cY7 is a receptor common for cNPY and cPYY, but not for cPP. Unlike cY2 and cY7, cY5 could be activated by all three peptides, though with low potencies, and the potency of cNPY and cPYY are 6-fold and 3-fold greater than cPP in cY5 activation (EC_{50} of cNPY: 2.35 nM; EC_{50} of cPYY: 4.78 nM; EC_{50} of cPP: 15.70 nM).

Using the pGL4-SRE-luciferase reporter system, we also demonstrated that peptide treatment could stimulate the luciferase activities of HEK293 cells expressing cY2, cY5 and cY7 dose-dependently (Fig. 7 and Table 2), suggesting that cY2, cY5 and cY7 are also coupled to MAPK/ERK signaling pathway. This idea is substantiated by the fact that cNPY treatment (100 nM) could enhance ERK1/2 phosphorylation in HEK293 cells expressing cY2, cY5 or cY7 (Fig. 7).

4. Discussion

In this study, *cY2*, *cY5* and *cY7* were revealed to contain novel non-coding exon(s), and alternative mRNA splicing at their 5′UTRs result in the expression of multiple mRNA species in chicken tissues. RT-PCR clearly showed that *cY2*, *cY5* and *cY7* transcripts are widely expressed in chicken tissues including various brain regions, and their expression are likely controlled by their respective functional promoters near or upstream of exon 1. Functional studies further proved that activation of cY2, cY5 and cY7 results in both the inhibition of cAMP/PKA and activation of MAPK/ERK signaling pathways. Evidently, our findings establish a molecular basis to elucidate the physiological roles of cY2, cY5 and cY7 and their ligands in avian species.

4.1. Unique gene structure of chicken Y2, Y5 and Y7

The coding regions of *cY2*, *cY5* and *cY7* are reported to be intronless (Bromee et al., 2006; Holmberg et al., 2002; Salaneck et al., 2000) (Fig. S1); however, their detailed gene structures have not been reported in any avian species. In this study, we found that *cY2*, *cY5* and *cY7* contain one or more novel non-coding exons upstream of their coding regions (Fig. 1). Although the information regarding the detailed gene structure of *Y2*, *Y5* and *Y7* is lacking in non-mammalian vertebrates, our observation is consistent with the findings in mammals, in which *Y2* and *Y5* contain non-coding exon(s) at their 5′-UTRs (Ammar et al., 1996; Herzog et al., 1997; Nakamura et al., 1997; Parker and Xia, 1999).

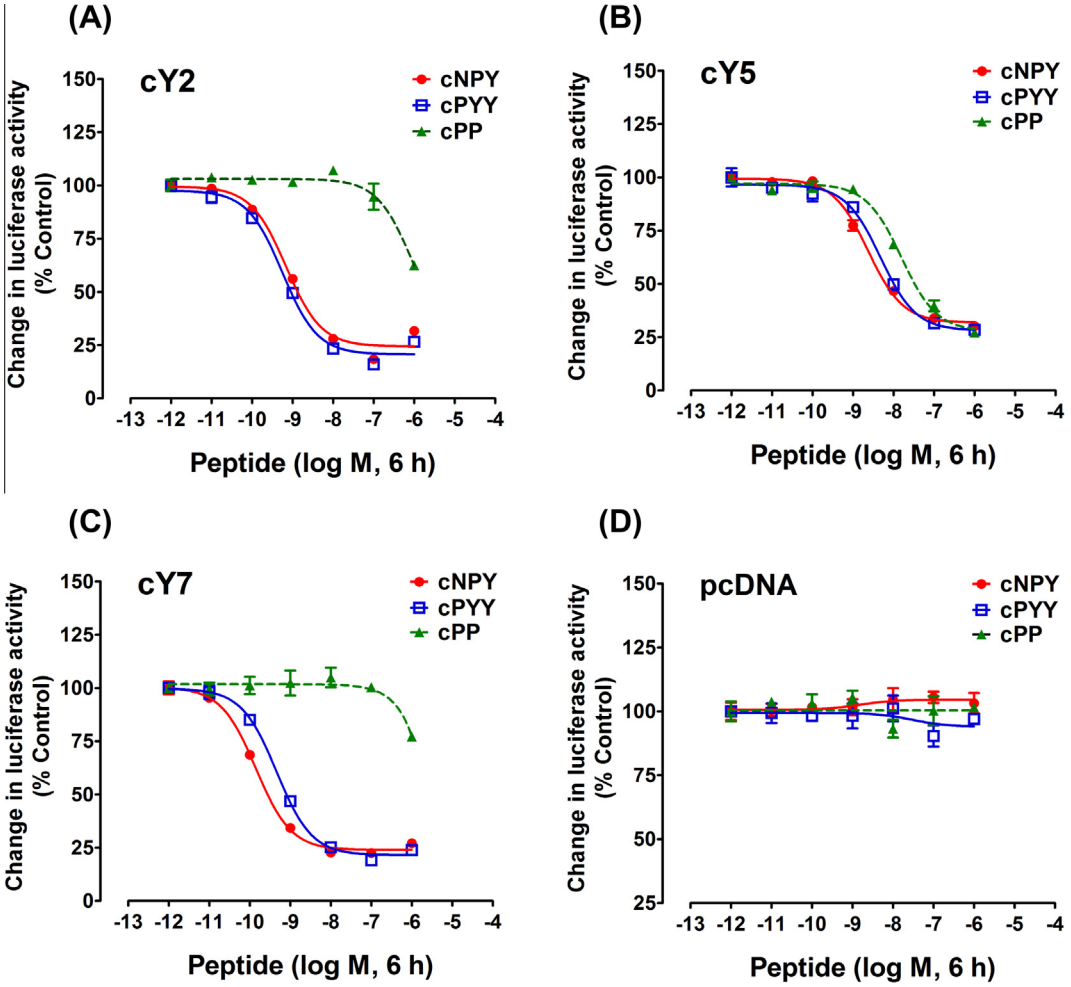

Fig. 6. Functional analyses of chicken Y2, Y5 and Y7. (A–C) Inhibition of forskolin (5 μM)-induced luciferase activities of HEK293 cells expressing cY2 (A), cY5 (B), cY7 (C) in response to various concentrations of each NPY family peptide, as monitored by the pGL3-CRE-luciferase reporter system (HEK293 cells treated by forskolin only were set as the control). (D) HEK293 cells co-transfected with the empty pcDNA3.1 (+) vector and pGL3-CRE-luciferase reporter construct were used as negative controls and peptide treatment did not alter their luciferase activities. In (A–D), each data point represents the mean ± SEM of 3 replicates ($N = 3$).

In this study, we also noted that alternative mRNA splicing in the 5′UTR results in the formation of multiple mRNA species of *Y2*, *Y5* and *Y7* in chickens (Figs. 2 and 3). Similar to our findings in chickens, multiple *Y5* splice variants, which are consisted of different combinations of exons in their 5′-UTRs, have also been identified in rats and humans, and these transcripts are likely expressed in cell/tissue-specific manners (Parker and Xia, 1999). The presence and expression of multiple 5′-UTR splice variants of c*Y2*, *cY5* and *cY7* may be closely associated with the regulation of mRNA stability and translational efficiency in different tissues, as previously reported in mammals (Chatterjee and Pal, 2009; Parker and Xia, 1999).

4.2. Tissue expression of chicken Y2, Y5 and Y7

The identification of multiple transcripts of *cY2*, *cY5* and *cY7* led us to further examine their tissue expression in chickens. Our study clearly showed that both types of *cY2* (T1 and T2) mRNA transcripts are ubiquitously expressed in all chicken tissues examined including various brain regions. This finding is slightly different from a previous report in chickens, in which *cY2* mRNA was demonstrated to be expressed only in the chicken hippocampus within the CNS by *in situ* hybridization (Salaneck et al., 2000). As in chickens, *Y2* is reported to be widely expressed in various regions of the CNS, including the hippocampus and hypothalamus in humans (Gehlert et al., 1996; Gerald et al., 1995; Rose et al., 1995). This finding implies that cY2 may play diverse roles in the chicken CNS, such as mediating the actions of NPY/PYY on food intake, learning and memory, and anxiety as proposed in mammals (Ando et al., 2001; Holzer et al., 2012; Loh et al., 2015). Interestingly, c*Y2* transcripts are also ubiquitously expressed in all chicken peripheral tissues examined. This finding is consistent with the wide expression of *Y2* in various peripheral tissues in rainbow trouts and frogs (Larsson et al., 2006; Sundström et al., 2012), but contrasts the findings in humans, in which little or no expression of *Y2* was detected (Gehlert et al., 1996; Gerald et al., 1995; Rose et al., 1995). The remarkable difference noted between non-mammalian vertebrates and mammals is highly likely due to species difference, and it may further suggest that Y2 may mediate a broader spectrum of actions of NPY/PYY in peripheral tissues of non-mammalian species, such as GI tract activity and lipid accumulation in chickens (Pedrazzini et al., 2003; Zhang et al., 2015). Furthermore, a functional promoter (−1564/+582), which contains multiple putative transcriptional factor binding sites, has been identified in this study. Since this promoter displays a strong promoter activity in DF-1 cells, it is highly likely responsible in driving *cY2* expression in chicken tissues (Figs. 1 and 3). Interestingly, an alternative transcriptional start site is noted at exon 1, implying

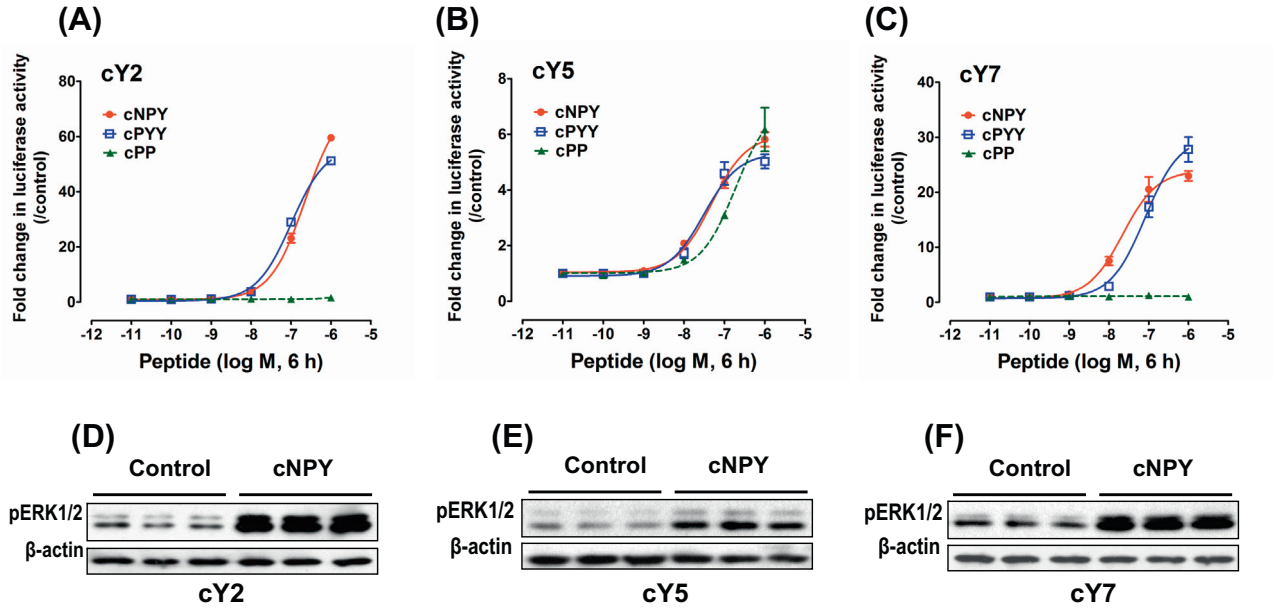

Fig. 7. Functional coupling of chicken Y2, Y5 and Y7 to the intracellular MAPK/ERK signaling pathway. A–C, Activation of the MAPK/ERK signaling pathway of HEK293 cells expressing cY2 (A), cY5 (B), and cY7 (C) in response to NPY family peptide treatment (10^{-11}–10^{-6} M, 6 h), as monitored by the pGL4-SRE-luciferase reporter system (HEK293 cells without peptide treatment were set as the control). In contrast, peptide treatment (10^{-11}–10^{-6} M, 6 h) did not alter the luciferase activities of the negative control HEK293 cells co-transfected with empty pcDNA3.1 vector and pGL4-SRE-luciferase construct (*data not shown*). (D and E) Western blot showed that cNPY treatment (100 nM, 10 min) could enhance the phosphorylation of ERK1/2 (pERK1/2: 44 kDa/42 kDa) of HEK293 cells expressing cY2 (D) or cY5 (E) or cY7 (F). As a control, $cNPY_{1-36}$ treatment causes no change in ERK1/2 phosphorylation of HEK293 cells transfected with empty pcDNA3.1 (+) vector (*data not shown*). Only one representative set of 3 independent experiments is shown here.

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

EC_{50} (nM)						
Inhibition of cAMP/PKA signaling pathway						
	cY2	cY5	cY7	cY1[a]	cY4[a]	cY6[a]
$cNPY_{1-36}$	0.69	2.35	0.14	0.90	~687.5	–
$cPYY_{1-37}$	0.56	4.78	0.46	4.67	48.01	–
cPP_{1-36}	>896.1	15.70	>1000	>230.2	13.8	–
Activation of MAPK/ERK signaling pathway						
	cY2	cY5	cY7	cY1[a]	cY4[a]	cY6[a]
$cNPY_{1-36}$	223.2	48.1	22.04	114.6	–	–
$cPYY_{1-37}$	100.7	30.7	81.38	497.5	–	–
cPP_{1-36}	–	189.9	–	–	–	–

Note: 'a' indicates that the corresponding EC_{50} values are from Gao's work (Gao et al., 2016).
'–' denotes that EC_{50} values cannot be estimated.

that an additional promoter (designated herein as P2), either upstream of or distal to exon 1, may exist in *cY2* (Fig. 1). Further identification of the alternative promoter(s) of *cY2* will provide clue to investigate the complex transcriptional regulation behind *cY2* expression.

Like *cY2*, *cY7* transcripts are widely expressed in nearly all chicken tissues examined including the hypothalamus, of which its expression is also likely controlled by a functional promoter near exon 1 (Fig. 2). Our finding contrasts a previous report, in which *cY7* mRNA was only detected in the adrenal gland, but not in the CNS and other peripheral tissues by RT-PCR (Bromee et al., 2006). The discrepancy between the present and previous studies is unclear. However, similar to our findings in chickens, *Y7* is reported to be widely expressed in other non-mammalian species including elephant sharks (Larsson et al., 2009), rainbow trouts (Larsson et al., 2006) and frogs (Sundström et al., 2012). The wide tissue expression of *cY2* and *cY7* (Fig. 2), together with their high similarity in structure (58% amino acid identity) (Bromee et al., 2006) (Fig. S2), ligand binding (Salaneck et al., 2000), and signaling property demonstrated in the present study (Fig. 6), not only supports the notion that both *Y2* and *Y7* were generated by a recent genome duplication event during vertebrate evolution (Larhammar and Bergqvist, 2013), but also hints that both receptors may serve similar, or overlapping, roles in mediating the actions of NPY/PYY, such as regulation of food intake (Denbow and Cline, 2015), in the CNS and peripheral tissues of chickens.

Like *cY2* and *cY7*, *cY5* transcripts are widely expressed in various brain regions including the hypothalamus, and its expression is controlled by a functional promoter near exon 1. As in chickens, multiple *Y5* transcripts are co-expressed in various human brain regions (Parker and Xia, 1999). Our finding partially coincides with a previous report in chickens, in which a strong hybridization signal of *cY5* is detected in chicken infundibular nucleus, the brain region structurally equivalent to the mammalian hypothalamic arcuate nucleus (Holmberg et al., 2002). The similar expression pattern of *Y5* noted in chickens and mammals also suggest that cY5, as in mammals, may play an important role in mediating the orexigenic action of NPY (/PYY) in the chicken hypothalamus (Denbow and Cline, 2015; Kuenzel et al., 1987; Tachibana et al., 2006). Since all NPY receptors seem to be expressed in the chicken hypothalamus (Fig. 2) (Yi et al., 2015), future studies on the anatomic distribution of each receptor in the chicken hypothalamus will help to define the precise role of each receptor in mediating the central actions of NPY/PYY/PP in birds. Besides the hypothalamus, *cY5* is also expressed in other brain regions, hinting it has regulatory roles in other parts of the brain (Holzer et al., 2012). Interestingly, *cY5* was also found to be expressed in several peripheral tissues including the pituitary and testes. The peripheral expression of *Y5* has also been reported in frogs, elephant sharks and humans (Larsson et al., 2009; Rodriguez et al., 2003; Statnick et al., 1998; Sundström et al., 2012), further supporting the role of Y5 in mediating the actions of NPY/PYY/PP in peripheral tissues of vertebrates.

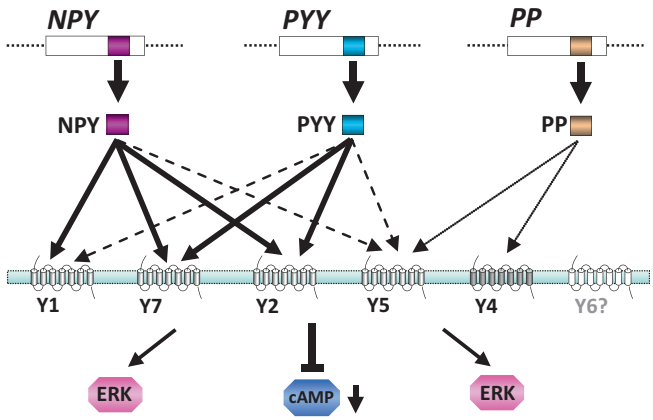

Fig. 8. A proposed model for the interaction between multiple ligands and receptors in the chicken NPY system. Six NPY receptors (Y1, Y2, Y4, Y5, Y6, and Y7) and three NPY family peptides (NPY_{1-36}, PYY_{1-37}, and cPP_{1-36}) encoded by separate genes have been identified in chickens (Bromee et al., 2006; Holmberg et al., 2002; Lundell et al., 2002; Salaneck et al., 2000; Gao et al., 2016). $cNPY_{1-36}$ can activate cY1, cY7, cY2 and cY5 expressed in HEK293 cells with EC_{50} values ranging from 0.14 nM to 2.35 nM (Gao et al., 2016), indicating that $cNPY_{1-36}$ can act as an endogenous ligand for the four Y receptors; Similarly, $cPYY_{1-37}$ can activate cY1, cY7, cY2 and cY5 effectively (Gao et al., 2016), indicating that $cPYY_{1-37}$ can function as an endogenous ligand for the four Y receptors *in vivo*. In contrast, cPP_{1-36} could only activate cY4 and cY5 with EC_{50} values higher than 10 nM, implying that cPP_{1-36} may function as an endogenous ligand for cY4 and cY5. Since $cPYY_{1-37}$ activates cY4 with a much low potency (EC_{50}: 48.01 nM), thus, it remains to be clarified whether cY4 can mediate $cPYY_{1-37}$ actions *in vivo* (Gao et al., 2016). Activation of cY1, cY7, cY2, cY5 and cY4 can cause the inhibition of cAMP signaling pathway and the activation of MAPK/ERK signaling cascade, as reported in mammals. However, the little or undetectable response of cY6 to treatment of NPY family peptides at physiological concentrations *in vitro* questions whether cY6 is functional in chickens. *Note*: The bold arrows indicate that cNPY and cPYY have low EC_{50} values (<1.0 nM) in activating Ys; the dashed arrows indicate that cNPY and cPYY have moderate EC_{50} values (2.0 ~ 5.0 nM) in activating Ys; dotted arrows denote that cPP has high EC_{50} values (>10 nM) in activating cY4 and cY5.

4.3. Functional characterization of chicken Y2, Y5 and Y7: finding the potential endogenous ligands for chicken Y receptors

In this study, we proved that cY2, cY5, and cY7 expressed in HEK293 cells are functionally coupled to G_i protein, and thus their activation results in the inhibition of the cAMP/PKA signaling pathway (Fig. 6). Furthermore, we proved that the activation of the three receptors also triggers the intracellular MAPK/ERK signaling cascade (Fig. 7). These findings are consistent with the findings in mammals, in which all NPY receptors except *y6* are coupled to G_i-cAMP pathway and ERK signaling pathway (Michel et al., 1998; Persaud and Bewick, 2014). Moreover, our findings also partially coincide with an early study on cY2, in which activation of cY2 expressed in HEK293-EBNA cells by pig NPY_{1-36} and chicken PYY_{1-37} can lower forskolin-induced cAMP levels (Salaneck et al., 2000).

The activation of cY2 by both $cNPY_{1-36}$ and $cPYY_{1-37}$ with equally high potencies (EC_{50}: <1.0 nM) clearly indicates that cY2 is a functional receptor common for both peptides (Fig. 8). Likewise, cY7 is potently activated by both NPY_{1-36} and $cPYY_{1-37}$ (EC_{50}: <1.0 nM), but not by cPP_{1-36}, as monitored by pGL3-CRE-luciferase reporter system, proving that cY7 is another functional receptor common for both $cNPY_{1-36}$ and $cPYY_{1-37}$ in chickens (Fig. 8). Our finding is different from the previous study describing that cY7 expressed in HEK293 cells binds $cPYY_{1-37}$ (Ki: 41 nM) and pig NPY_{1-36} (Ki: 10 nM) with much lower affinities (Bromee et al., 2006). We repeated this experiment four times, either in HEK293 (Fig. 6) or CHO cells (*data not shown*), with similarly high potencies noted. In addition, consistent with our finding in chickens, Y7 was also shown to bind PYY_{1-36} and NPY_{1-36} with high affinities in zebrafish and rainbow trouts (Fredriksson et al., 2004; Larsson et al., 2006).

Unlike cY2 and cY7, cY5 is activated by all three peptides with EC_{50} values ranging from 2.35 nM to 15.7 nM, as monitored by the pGL3-CRE-luciferase reporter system, indicating that cY5 may function as the common receptor for all three peptides (Fig. 8). Moreover, we noted that cY5 displays a weak selectivity for $cNPY_{1-36}$ and $cPYY_{1-37}$. This finding is consistent with the previous report in chickens, in which cY5 can bind $pNPY_{1-36}$ and $cPYY_{1-37}$ with affinities approximately 10-fold higher than that for cPP_{1-36} (Holmberg et al., 2002). In rats, Y5 can bind to NPY and PYY with higher affinities than to PP (Gerald et al., 1996). Interestingly, in frogs *Silurana tropicalis*, Y5 binds PYY with much higher affinity than NPY and PP (Sundström et al., 2012). The difference in pharmacological profile of Y5 among various classes of vertebrates may hint of change in its functions during vertebrate evolution.

It should be noted that unlike cY2 and cY7, cY5 activation requires relatively higher concentrations of each peptide (EC_{50}: 2.35–15.7 nM) *in vitro* (Table 2). This finding also raises a fundamental question whether cY5 alone could effectively mediate the NPY/PYY/PP actions *in vivo*, such as mediating NPY/PYY-stimulated food intake in chickens (Denbow and Cline, 2015), or Y5 could form hetero-dimers with other Y receptor(s) (*e.g.* Y1), thus increasing Y5 signaling following ligand stimulation (Gehlert et al., 2007). Future studies on receptor hetero-dimerization will help to solve this interesting puzzle.

Given the functional analysis of cY2, cY5 and cY7 (Fig. 6), together with the functional assay of cY1, cY4 and cY6 (Table 2) performed in our recent study (Gao et al., 2016), we proposed here that: 1) $cNPY_{1-36}$ can function as the endogenous ligand for cY7, cY2, cY1 and cY5 and its actions are most likely mediated by these receptors; 2) $cPYY_{1-37}$ can function as an endogenous ligand for cY7, cY2, cY1 and cY5 (Gao et al., 2016), and its actions are mediated by these receptors; 3) cPP_{1-36} may function as an endogenous ligand for cY4 and cY5, and its actions are likely mediated by the two receptors in chickens; however, the low potencies of cPP in activating cY4 (EC_{50}: 13.8 nM) and cY5 (EC_{50}: 15.7 nM), together with the restricted expression of cPP in the pancreas (Gao et al., 2016), cast doubt on the question whether cPP can exert endocrine actions in extra-pancreatic tissues despite the wide expression of *cY4* and *cY5* in many non-pancreatic tissues; 4) cY6 alone cannot be activated by cNPY, cPYY and cPP *in vitro* (Gao et al., 2016), thus, it remains unclear whether it is functional *in vivo*. Since our hypothesis is based on data obtained *in vitro*, more studies conducted *in vivo* are required to substantiate these ideas, such as the anatomic distribution of each Y receptor in the chicken CNS and peripheral tissues. These data would undoubtedly help to uncover the roles of each Y receptor in birds.

In summary, cY2, cY5 and cY7 were characterized in this study. RACE PCR assay revealed that *cY2*, *cY5* and *cY7* contain novel non-coding exons in their 5′-UTR. RT-PCR showed that *cY2*, *cY5* and *cY7* transcripts are widely expressed in chicken tissues, and their expression are likely controlled by their respective promoters near or upstream of exon 1. Functional studies demonstrated that cY2 and cY7 can function as the receptor for cNPY and cPYY, but not for cPP, while cY5 may function as the receptor for all three peptides with a weak selectivity for cNPY and cPYY. These findings, together with our recent study on the other three NPY receptors (cY1, cY4 and cY6) (Gao et al., 2016) will greatly extend the previous findings on NPY system and depict a fuller picture regarding the expression and signaling of these ligand-receptor pairs in an avian species. Undoubtedly, these findings will not only facilitate the comprehensive understanding on the physiological roles of NPY system in birds, but also help to uncover the structural and functional changes of these ligand-receptor pairs in different classes of vertebrates, such as their actions on food intake.

Acknowledgments

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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.2016.04.019.

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