Characterization of Neuropeptide B (NPB), Neuropeptide W (NPW), and Their Receptors in Chickens: Evidence for NPW Being a Novel Inhibitor of Pituitary GH and Prolactin Secretion

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The 2 structurally and functionally related peptides, neuropeptide B (NPB) and neuropeptide W (NPW), together with their receptor(s) (NPBWR1/NPBWR2) constitute the NPB/NPW system, which acts mainly on the central nervous system to regulate many physiological processes in mammals. However, little is known about this NPB/NPW system in nonmammalian vertebrates. In this study, the functionality and expression of this NPB/NPW system and its actions on the pituitary were investigated in chickens. The results showed that: 1) chicken NPB/NPW system comprises an NPB peptide of 28 amino acids (cNPB28), an NPW peptide of 23 or 30 amino acids (cNPW23/cNPW30), and their 2 receptors (cNPBWR1 and cNPBWR2), which are highly homologous to their human counterparts. 2) Using a pGL3-CRE-luciferase reporter system, we demonstrated that cNPBWR2 expressed in Chinese hamster ovary cells can be potently activated by cNPW23 (not cNPB28), and its activation inhibits the intracellular cAMP signaling pathway, whereas cNPBWR1 shows no response to peptide treatment, suggesting a crucial role of cNPBWR2 in mediating cNPW/cNPB actions. 3) Quantitative real-time PCR revealed that *cNPW* and *cNPB* are widely expressed in chicken tissues, including hypothalamus, whereas *cNPBWR1* and *cNPBWR2* are mainly expressed in brain or pituitary. 4) In accordance with abundant *cNPBWR2* expression in pituitary, cNPW23 could dose dependently inhibit GH and prolactin secretion induced by GHRH and vasoactive intestinal polypeptide, respectively, in cultured chick pituitary cells, as monitored by Western blotting. Collectively, our data reveal a functional NPB/NPW system in birds and offer the first proof that NPW can act directly on pituitary to inhibit GH/prolactin secretion in vertebrates. **(*Endocrinology* 157: 3562–3576, 2016)**

In mammals, neuropeptide B (NPB) and neuropeptide W (NPW) are the 2 structurally and functionally related peptides encoded by *NPB* and *NPW* genes, respectively (1). Two forms of NPW peptides, NPW23 and NPW30 (23 and 30 amino acids in length, respectively), have been identified from the porcine hypothalamus. NPW23 is identical to the N-terminal portion of NPW30 (2). Similarly, 2 forms of NPB peptides, NPB23 and NPB29 (23 and 29 amino acids in length, respectively), are predicted to exist in humans (3), whereas only NPB29 exist in other mammalian species (4, 5). The 2 receptors for NPB and NPW were cloned and originally named as GPR7 and G protein-coupled receptor (GPR)8 in humans, both of which share high structural similarity with somatostatin receptor (SSTR)3 and opioid receptors (6). Later studies revealed that these 2 receptors can bind NPB and NPW peptides with high affinities, thus they have been renamed as NPBWR1 and NPBWR2, respectively (1). Interestingly,

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Abbreviations: BGI, Beijing Genome Institute; c, chicken; CHO, Chinese hamster ovary; CNS, central nervous system; go, goose; GPR, G protein-coupled receptor; h, human; IHC, immunohistochemical; NPB, neuropeptide B; NPBWR, NPB/NPW receptor; NPW, neuropeptide W; OPR, opioid receptor; ORF, open reading frame; PRL, prolactin; pVIP, pig VIP; qPCR, quantitative real-time RT-PCR; SSTR, somatostatin receptor; TM, transmembrane domain; VIP, vasoactive intestinal polypeptide.

in rodents, only *NPBWR1* (*GPR7*) exists, whereas *NPBWR2* (*GPR8*) is absent, probably lost during evolution (7). It is clear that both receptors are functionally coupled to Gi proteins, and their activation decreases intracellular cAMP level, which is crucial for mediating diverse actions of NPB/NPW in vivo (1).

In mammals, NPB/NPW have been reported to regulate many physiological processes such as food intake (8, 9), energy homeostasis (9, 10), pain perception, locomotor activity, obesity (5, 11, 12), stress (13, 14), sleep (15), neuroendocrine activity (16), and social behavior (17) via its action on the central nervous system (CNS), although reports on some of these roles remain controversial. In agreement with their actions on the CNS, the expression of NPB, NPW and their receptor(s) have been reported in many brain regions including the hypothalamus (4, 5, 8). In addition to the CNS, NPB/NPW and/or their receptor(s) are also expressed in the peripheral tissues of rodents or humans, such as the pancreas (18, 19), stomach (20, 21), adrenal cortex (22), anterior pituitary (6, 23), and adipocytes (24). Furthermore, there is also a handful of studies showing that NPB/NPW may be involved in the control of adrenal corticosterone secretion (22), pancreatic insulin secretion (18), and lipolysis (24), even though their actions on peripheral tissues remains largely obscure.

Unlike that in mammals, the information regarding the functionality and roles of NPB/NPW and their receptor(s) in nonmammalian vertebrates is rather scarce. There are only a few studies showing the expression and actions of NPB in teleosts. In Nile tilapia, *NPB* mRNA is mainly expressed in the CNS region involved in the regulation of food intake and pituitary gene expression (25). In medaka, *NPB* mRNA is predominantly expressed in the brain and its expression in the telencephalic and preoptic nuclei displays sexual dimorphism (26). The extremely limited information from nonmammalian vertebrates has undoubtedly impeded the progress on understanding the conserved roles of this NPB/NPW system in vertebrates (27). Therefore, using chicken as a model organism, the present study aims to examine the functionality of the NPB/NPW system and investigate their actions on the pituitary. Our data establish a clear concept that a functional NPB/NPW system exists in chickens, and report a novel inhibitory action of NPW on GHRH-induced pituitary GH and vasoactive intestinal polypeptide (VIP)-induced prolactin (PRL) secretion (28–34), which has not been reported in any vertebrate species before. Undoubtedly, our findings provide novel insights into the roles of the NPB/NPW system in vertebrates, including their direct actions on pituitary hormone secretion.

Materials and Methods

Chemicals, primers, peptides, and antibodies

All chemicals were purchased from Sigma-Aldrich and restriction enzymes were obtained from Takara unless stated otherwise. All primers used in this study were synthesized by Beijing Genome Institute (BGI) and listed in Supplemental Table 1. Chicken (c)NPB28, cNPW23, and cGHRH were synthesized by GL Biochem Ltd. The purity of synthesized peptides is more than 95% (analyzed by HPLC), and their structures were verified by mass spectrometry. Pig VIP (pVIP), which is potent in stimulating chicken PRL secretion, was also synthesized by GL Biochem Ltd. Monoclonal antibody against β-actin was purchased from Cell Signaling Technology, Inc, and polyclonal antibodies against recombinant cGH or cPRL were prepared in our laboratory (32, 35, 36).

Animals and tissues

One-week-old (or 3-wk-old) chicks and adult chickens (8 mo old) of both sexes (Lohmann layer) were purchased from a local commercial company. Chickens were killed, and tissues were collected either for total RNA extraction or for cell culture. All the experiments were performed under the guidelines of the Animal Ethics Committee of Sichuan University.

Total RNA extraction, RT-PCR, and quantitative real-time PCR assays

Total RNA was extracted from chicken tissues using RNAzol (Molecular Research Center) and dissolved in diethylpyrocarbonate-treated H_2O. These RNA samples were then reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Takara) and were either subjected to PCR amplification of target genes from chicken pituitaries, or quantitative real-time PCR assay of gene expression, as described in our previous study (37).

Cloning of full-length chicken NPB, NPW, NPBWR1, and NPBWR2 cDNAs

According to the predicted cDNA sequences of chicken NPB (*cNPB*, XM_001231799) and *NPBWR1* (*cNPBWR1*, XM_419201) deposited in GenBank, gene-specific primers were designed to amplify the cDNAs of *cNPB* and *cNPBWR1* from the chicken pituitary. The amplified PCR product of *cNPB* was cloned into pTA2 vector (Toyobo), whereas the amplified cDNA of *cNPBWR1* was cloned into pcDNA3.1(+) expression vector (Invitrogen).

Using the predicted partial sequence of ground tit *NPW* as a reference (XM_005523213), we blasted the chicken EST Database (www.chick.manchester.ac.uk) and identified an EST sequence (ID, ChEST454a4) homologous to tit *NPW*. According to this EST sequence, gene-specific primers were designed to amplify the full-length cDNA of *NPW* (*cNPW*) from the chicken brain using SMART-RACE cDNA amplification kit (Clontech). The amplified PCR product was then cloned into pTA2 vector.

Using human (h)*NPBWR2* as a reference, we blasted the chicken genome database (www.ensembl.org/gallus_gallus) and identified a putative chicken *NPBWR2* (*cNPBWR2*) on chromosome 20. *cNPBWR2* cDNA containing the complete open reading frame (ORF) was then amplified from the chicken pituitary by high-fidelity KOD *Taq* DNA polymerase (Toyobo) and cloned into the pcDNA3.1(+) expression vector. In this

study, all PCR products inserted into pTA2 [or pcDNA3.1(+)] vector were sequenced by BGI.

Cloning of human and goose *NPBWR1* and *NPBWR2* genes

Using human genomic DNA (extracted from HEK293 cells) or goose brain cDNAs as templates, the coding regions of human *NPBWR*s (*hNPBWR1* and *hNPBWR2*) and goose (go)*NPBWR*s (*goNPBWR1* and *goNPBWR2*) were amplified by PCR and cloned into pcDNA3.1 (+) vector and sequenced (BGI).

The cDNA sequences of chicken *NPB*, *NPW*, *NPBWR1*, and *NPBWR2*, and goose *NPBWR1* and *NPBWR2*, have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers: KU059545, KU059546, KU059547, KU059548, KU059549, and KU059550.

Functional characterization of chicken/goose/human NPBWR1 and NPBWR2 in Chinese hamster ovary (CHO) cells

CHO cells (with a passage number <10) were cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum (HyClone), 100-U/mL penicillin G, and 100-g/mL streptomycin (Thermo Fisher Scientific, Inc) in a 90-cm culture dish (NUNC) and incubated at 37°C with 5% CO_2.

According to our previously established methods (32, 34), the functionality of receptors (chicken NPBWR1/NPBWR2, goose NPBWR1/NPBWR2, and human NPBWR1/NPBWR2) was examined in cultured CHO cells by a pGL3-CRE-Luciferase reporter system, which can monitor receptor-mediated inhibition of cAMP signaling pathway.

Because cNPBWR1 and cNPBWR2 are structurally related to chicken SSTR1–SSTR5 (32), and opioid receptors (OPRD1, OPRM1, OPRK1, OPRL1), we also examined whether cNPB28 and cNPW23 treatment can activate chicken SSTR1–SSTR5 (or 4 opioid receptors) using this reporter system.

Immunohistochemical (IHC) staining

Anterior pituitaries of 3-week-old chicks were fixed in 4% paraformaldehyde, and embedded in paraffin wax for IHC staining, as described in our previous study (36). In brief, IHC staining was performed in chick pituitary sections using a streptavidin-biotin-peroxidase complex kit (Boster) according to the manufacturer's instructions. Polyclonal antibodies against cGH (1:500) or cPRL (1:200) were used to probe the spatial distribution of GH and PRL in chick anterior pituitaries. Sections incubated with rabbit preimmune serum were used as negative controls.

Effects of cNPB28 and cNPW23 on GH and PRL secretion in cultured chick pituitary cells

As described in our recent studies (32, 36), anterior pituitaries were collected from 1-week-old male chicks under sterile condition and digested by 0.25% trypsin at 37°C for 20 minutes. The dispersed pituitary cells were then cultured at a density of 5×10^5 cells/well on 48-well plates with Medium 199 containing 15% FBS at 37°C with 5% CO_2. After 18 hours of culture, the culture medium was removed and cells were treated with 100-μL Medium 199 containing various concentration of peptide (cNPB28 or cNPW23, 1nM–100nM) in the presence of $cGHRH_{1-27NH2}$ (1nM) (32), pVIP (1nM), or forskolin (2μM) for 6 hours. At the end of treatment, the culture medium was collected for measurement of GH/PRL secretion, and the pituitary cells were lysed by 1× passive lysis buffer (Promega) to examine intracellular GH/PRL and β-actin levels. In this experiment, Western blotting was used to detect protein level using antibodies against cGH, cPRL, or β-actin, as previously described (36).

Data analysis

The protein bands of Western blotting were quantitated by densitometric analyses (ImageJ software, NIH). Relative GH/PRL levels were calculated as the ratio to that of intracellular β-actin and then expressed as the percentage of control or GHRH (or VIP/forskolin) treatment group. The data were analyzed by one-way ANOVA followed by Dunnett's test using GraphPad prism 5 (GraphPad Software). All experiments were repeated at least 3 times to validate our results.

Results

Cloning of chicken NPB and NPW cDNAs

According to the predicted sequence of chicken *NPB* (*cNPB*) deposited in the GenBank, c*NPB* cDNA (399 bp) was cloned from the chicken pituitary by RT-PCR. It encodes a precursor of 119 amino acids (accession number KU059545), which shares high amino acid sequence identity (36%–40%) with NPB precursors of other vertebrate species including humans, turtles, *Xenopus tropicalis*, and zebrafish (Figure 1 and Supplemental Figure 1). Like mammalian NPB, cNPB precursor contains a signal peptide at its N terminus and a dibasic residue ($R^{51}R^{52}$) critical for proteolytic processing. cNPB precursor is thus predicted to generate a mature NPB peptide of 28 amino acids (named cNPB28), after the removal of its signal peptide and long C terminus. The length of mature chicken NPB differs from its counterparts in other species including humans, turkeys, woodpeckers, *X. tropicalis*, zebrafish, tilapia, and fugu, in which NPB has a length of 29 (or 23) amino acids (Figure 1). However, cNPB28 still shares 50%–97% amino acid sequence identity with NPB29 (or NPB23) from other species (Supplemental Figure 2). In addition, we also noted that avian NPB has a 'WYR' motif, instead of the 'WYK' motif conserved in NPB of mammals, frogs, and teleosts.

Besides c*NPB*, c*NPW* was also cloned from the chicken brain in this study (KU059546). The cloned c*NPW* cDNA is 568 bp long. It encodes a 139-amino acid precursor with a signal peptide at its N terminus (Supplemental Figure 1). As in mammals, chicken NPW precursor was also predicted to generate 2 forms of NPW peptide, cNPW23 (23 amino acids) and cNPW30 (30 amino acids), after proteolytic processing at the 2 dibasic amino acid residues ($R^{52}R^{53}$) and ($R^{59}R^{60}$) (Figure 1) (2). Unlike cNPB, cNPW23/cNPW30 show a remarkable degree of amino

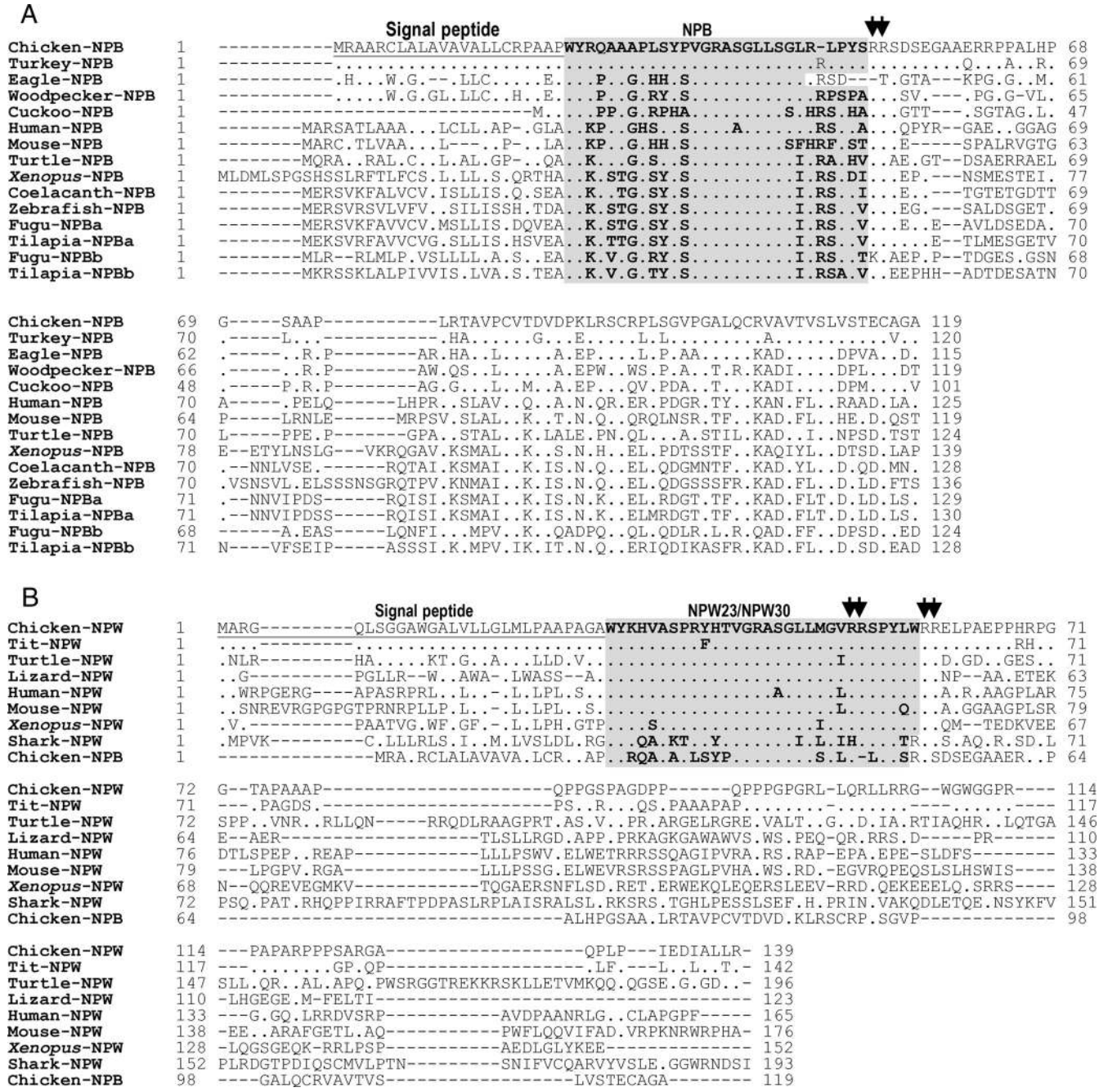

Figure 1. A, Amino acid alignment of chicken NPB precursor (KU059545) with that of turkeys (XP_010719651), bald eagles (XP_010566435), woodpeckers (XP_009896314), common cuckoos (XP_009557310.1), humans (NP_683694), mice (NP_695020), Western painted turtles (XP_005283205), *X. tropicalis* (XP_002937351), coelacanths (XP_005989170), zebrafish (NP_001120841), takifugu (NPBa, XP_003972081; NPBb, XP_003979086), and Nile tilapia (NPBa, XP_003455810; NPBb, XP_003450170). B, Amino acid alignment of chicken NPW precursor (KU059546) with that of Tibetan ground tits (XP_005523270), green sea turtles (XP_007058654), anole lizards (XP_008120357), humans (NP_001092926), mice (NP_001093134), *X. tropicalis*, (XP_004918111), elephant sharks (XP_007908641), or with chicken NPB precursor. The mature NPB/NPW peptide is shaded. Arrows indicate the putative cleavage sites. All sequences were retrieved from the GenBank. Dots indicate amino acids identical to chicken NPB/NPW precursor; dashes denote gaps in the alignment.

acid sequence identity (87%–100%) with NPW of mammals, reptiles, *X. tropicalis*, and other birds, with only 1- to 4-amino acid substitution noted between them (Supplemental Figure 2). Moreover, a characteristic 'WYK' motif was identified at the N termini of cNPW23/cNPW30.

Cloning of NPBWR1 and NPBWR2 in chickens

According to the predicted cDNA sequence (XM_419201) in the GenBank, c*NPBWR1* cDNA was cloned from the chicken pituitary. It encodes a 331-amino acid protein (accession number KU059547), which shares high amino acid sequence identities with NPBWR1

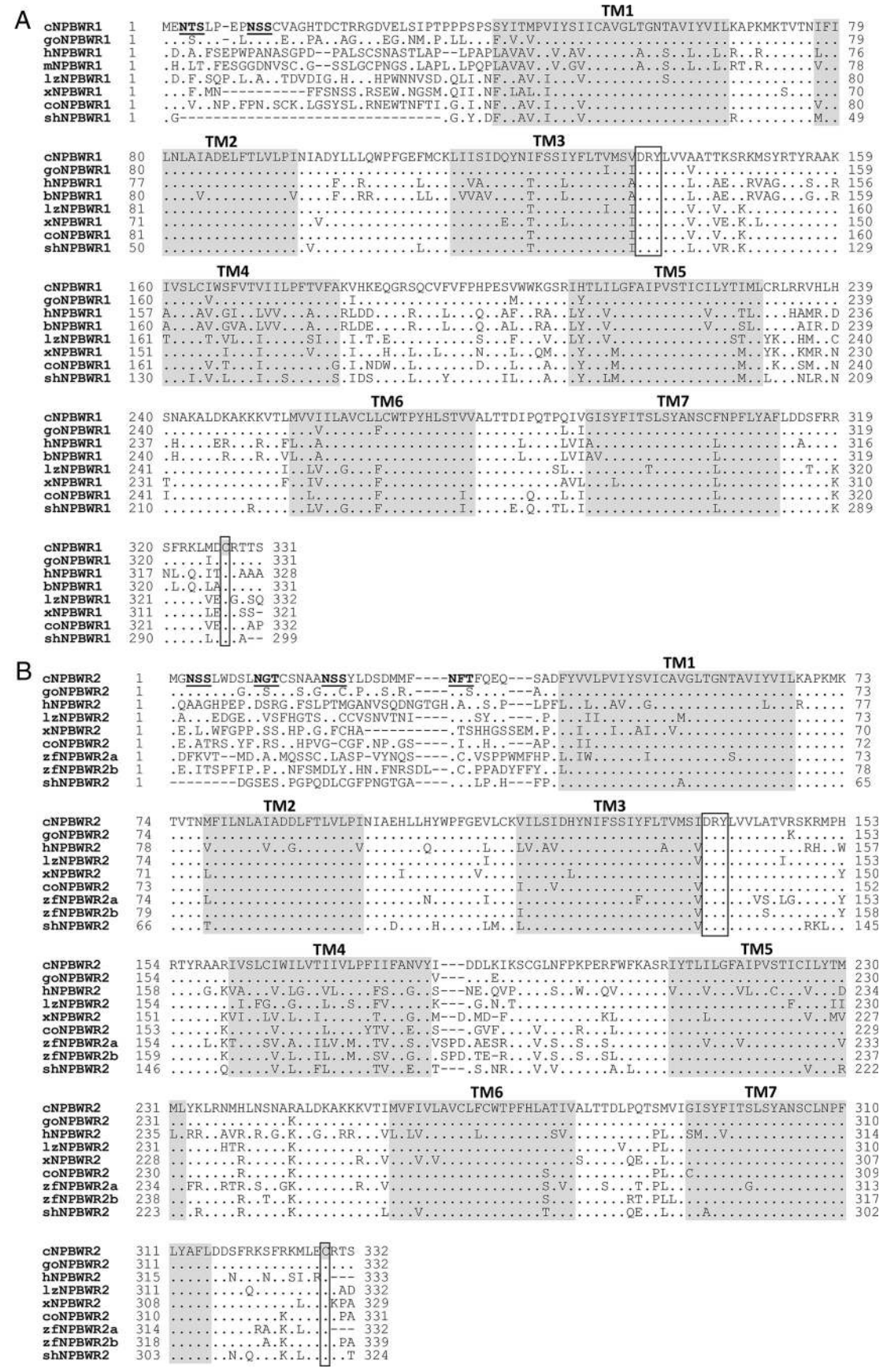

Figure 2. A, Alignment of chicken NPBWR1 (cNPBWR1, KU059547) with that of geese (goNPBWR1, KU059549), humans (hNPBWR1,NP_005276), mice (mNPBWR1, NP_034472), anole lizards (lzNPBWR1, XP_003224368), *X. tropicalis* (xNPBWR1, XP_002934151), coelacanths (coNPBWR1, XP_006011643),

(GPR7) of humans (66%), mice (67%), anole lizards (77%), *X. tropicalis* (74%), coelacanths (79%), and elephant sharks (81%). Like mammalian NPBWR1, chicken NPBWR1 contains 7 transmembrane domains (TM1–7), a 'DRY' motif at the C-terminal end of the third transmembrane domain and a cysteine residue at the C terminus, which may function as a palmitoylation site. Moreover, 2 potential *N*-glycosylation sites (NXT/Ss) were also noted at the N terminus of cNPBWR1.

NPBWR2 (*GPR8*) was originally reported to be lost in chickens during evolution (27); however, we searched the chicken genome database using human *NPBWR2* as a reference and identified the ORF encoding *NPBWR2* on chicken chromosome 20. Using RT-PCR, we cloned *cNPBWR2* from the chicken pituitary. The cloned *cNPBWR2* cDNA encodes a protein of 332 amino acids (KU059548), which shares high amino acid sequence identities with NPBWR2 of humans (65%), green anoles (83%), *X. tropicalis* (78%), coelacanths (84%), zebrafish (NPBWR2a, 64%; NPBWR2b, 78%), and elephant sharks (79%). Interestingly, cNPBWR2 also shares 68% identity with cNPBWR1. Like human NPBWR2, cNPBWR2 contains 7 transmembrane domains, a 'DRY' motif, a cysteine residue at its C-terminal tail proposed for palmitoylation. In addition, 4 potential N-glycosylation sites were found at the N terminus of cNPBWR2.

As in chickens, *NPBWR1* (KU059549) and *NPBWR2* (KU059550) were also identified and cloned in geese (Figure 2 and Supplemental Figures 3 and 4). This finding indicates the presence of 2 receptor genes in other avian species.

Synteny analysis of NPB, NPW, NPBWR1, and NPBWR2 in vertebrates

To verify whether the cloned chicken *NPB*, *NPW*, *NPBWR1*, and *NPBWR2* are orthologous to genes identified in humans and other vertebrates, synteny analysis was performed by searching their neighboring genes in the genomes of zebrafish, elephant sharks, coelacanths, *X. tropicalis*, anole lizards, mice, humans, and chickens. As shown in Figure 3, c*NPB*, *cNPW*, *cNPBWR1*, and *cNPBWR2* are orthologous to their human counterparts and located in 4 separate syntenic regions, which are conserved in all (or most) vertebrate species examined (Figure 3). With the exception of mouse *NPBWR2* (*GPR8*) which was highly likely lost in the lineage (7), *NPBWR2* is identified in all other vertebrate species examined (Figure 3).

Interestingly, our synteny analysis further indicates that a *NPB* and 2 *NPBWR2* (*NPBWR2a* and *NPBWR2b*) genes exist in the zebrafish genome, whereas *NPW* and *NPBWR1* were likely lost in zebrafish (Figure 3). In addition, 2 *NPB* (*NPBa* and *NPBb*) genes exist in tilapia and fugu genomes (Figure 1).

Functional characterization of cNPB28, cNPW23, cNPBWR1, cNPBWR2, and goose NPBWRs in cultured CHO cells

To determine whether chicken NPB/NPW are bioactive and capable of activating cNPBWR1 or cNPBWR2, each receptor was transiently expressed in CHO cells and treated with synthetic cNPB28 or cNPW23 in the presence of 2μM forskolin. The receptor-mediated inhibition of cAMP signaling pathway was subsequently monitored by a pGL3-CRE-Luciferase reporter system established in our previous studies (32, 34).

As shown in Figure 4, both cNPB28 and cNPW23 could inhibit forskolin-stimulated luciferase activity via activation of cNPBWR2. However, cNPW23 (EC_{50} = 7.63nM) is 100-fold more potent than cNPB28 (Table 1), indicating that cNPBWR2 is a receptor specific to cNPW and functionally coupled to Gi protein. Strikingly, cNPBWR1 expressed in CHO cells could neither be activated by cNPB28 nor cNPW23 at any concentration tested (10^{-11}M–10^{-6}M, 6 h).

To examine whether NPBWR1 and NPBWR2 are functional in other avian species, the functionalities of goose NPBWR1 and NPBWR2 were also examined in vitro using the same approach. As shown in Figure 4, goose NPBWR2 could be potently activated by cNPW23 (EC_{50} = 5.71nM), but not by cNPB28 ($EC_{50} > 1\mu$M), supporting the concept that NPBWR2 is a receptor specific to NPW in geese. As in chickens, goose NPBWR1 could not be activated by cNPB28 at any concentration tested (10^{-11}M–10^{-6}M, 6 h). However, it could be activated by cNPW23 at high concentrations (EC_{50} = 77.0nM), indicating that goose NPBWR1 is still functional.

Although cNPB28 failed to activate chicken or goose NPBWR1, it could activate human NPBWR1 expressed in CHO cells with a higher potency than activating human NPBWR2, indicating that cNPB28 is biologically active. In addition, we noted that cNPW23 could effectively activate both human NPBWR1 (EC_{50} = 2.31nM) and

Figure 2. (Continued). and elephant sharks (shNPBWR1, XP_007904116). B, Alignment of chicken NPBWR2 (cNPBWR2, KU059548) with that of geese (goNPBWR2, KU059550), humans (hNPBWR2, NP_005277), anole lizards (lzNPBWR2, XP_003220762), *X. tropicalis* (xNPBWR2, XP_012810330), coelacanths (coNPBWR2, XP_006006269), zebrafish (zfNPBWR2a, AAR88325; zfNPBWR2b; AAR88324), and elephant sharks (shNPBWR2, XP_007903406). The conserved 'DRY' motif and cysteine (Cys) residue are boxed and the 7 transmembrane domains (TM1–7) shaded; the potential N-glycosylation site (NXT/S, X represents any residue except proline) is underlined; dots indicate amino acids identical to chicken NPBWR1/2; dashes denote gaps in the alignment.

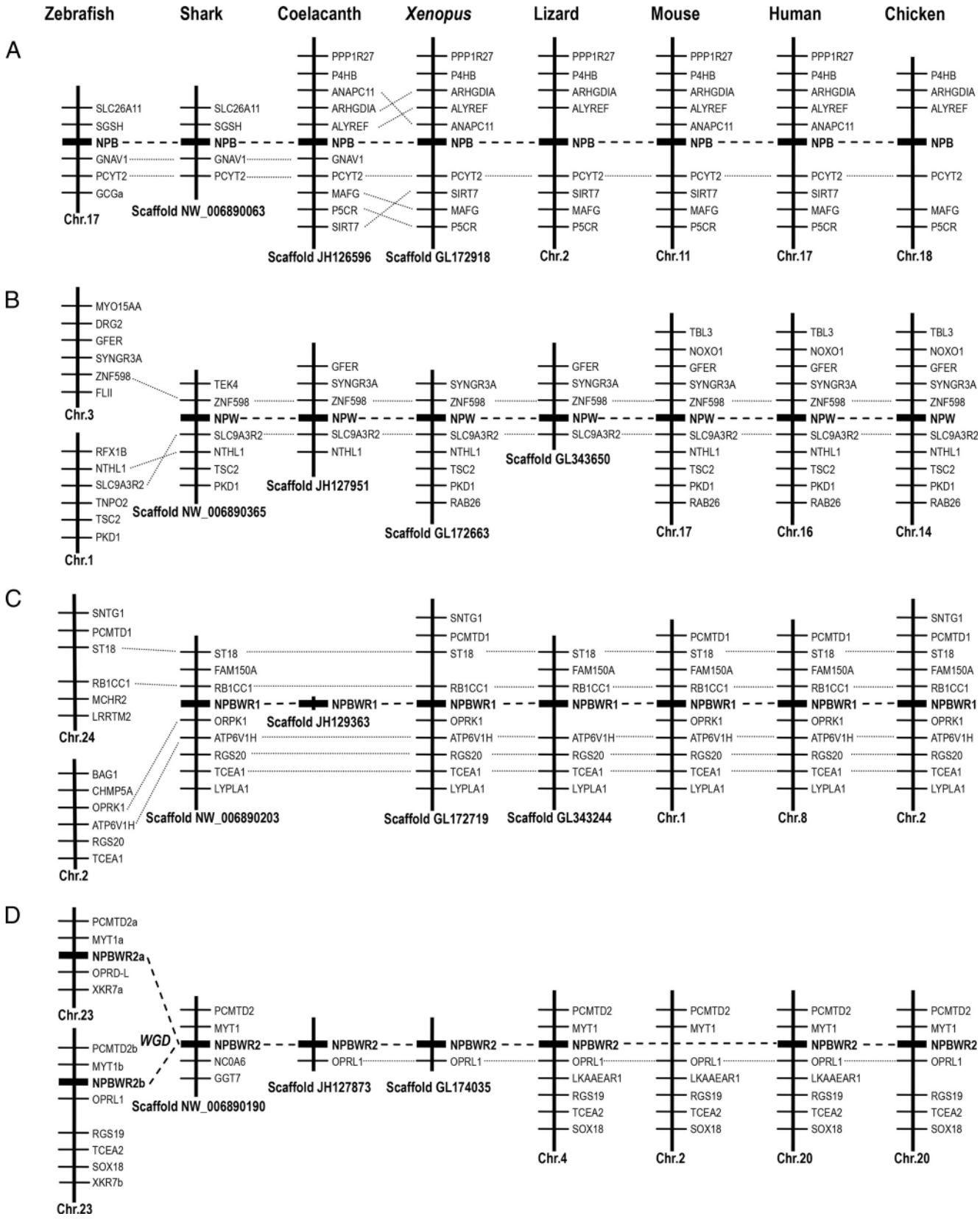

Figure 3. Synteny analyses show that (A) *NPB*, (B) *NPW*, (C) *NPBWR1*, and (D) *NPBWR2* are located in 4 separate syntenic regions conserved in all (or most) vertebrate species examined (including zebrafish, elephant sharks, coelacanths, *X. tropicalis*, anole lizards, mice, humans, and chickens). Dotted lines indicate the syntenic genes; dashed lines denote genes of interest. Note: only *NPB* and *NPBWR2s* exist in the zebrafish genome, whereas *NPW* and *NPBWR1* were seemingly lost during evolution. Interestingly, the 2 *NPBWR2* (*NPBWR2a* and *NPBWR2b*) genes identified in zebrafish were likely generated by a whole-genome duplication event (WGD) occurred in the teleost lineage (41).

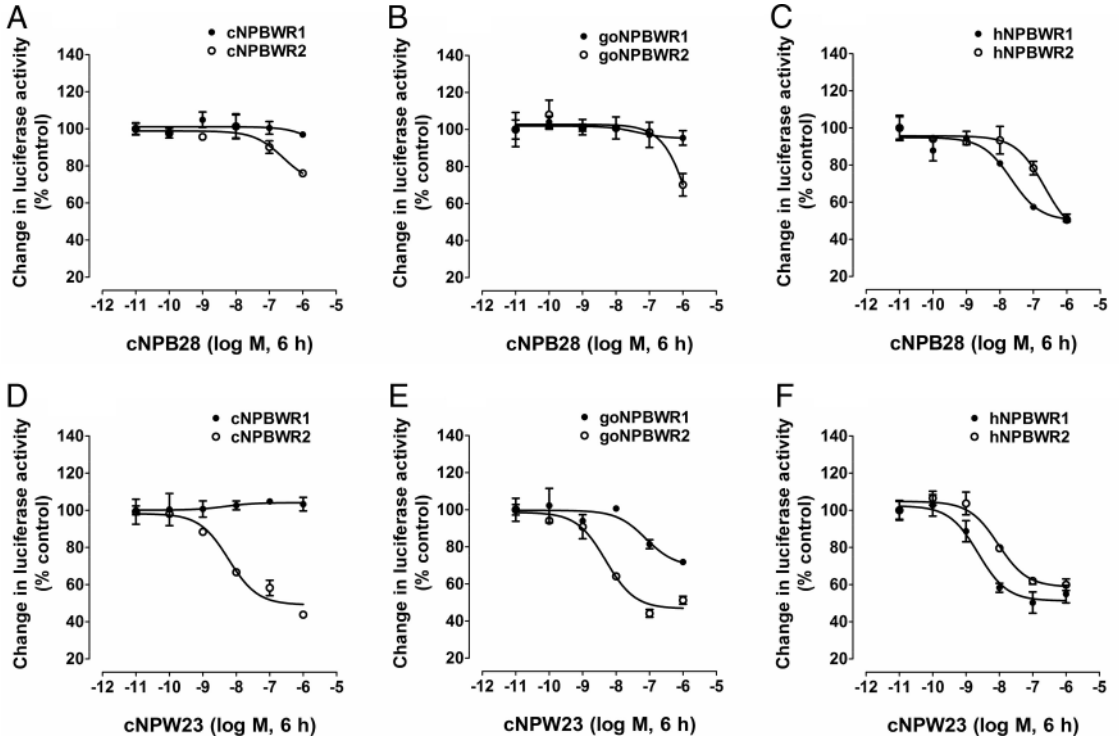

Figure 4. Effects of cNPB28 (10^{-11}M–10^{-6} M, 6 h) (A–C) or cNPW23 (10^{-11}M–10^{-6}M, 6 h) (D–F) on forskolin (2μM)-stimulated luciferase activity of CHO cells expressing NPBWR1 or NPBWR2 of chicken (cNPBWR1/cNPBWR2), goose (goNPBWR1/goNPBWR2), or human (hNPBWR1/hNPBWR2), monitored by a pGL3-CRE-luciferase reporter system. As a negative control, cNPB28/cNPW23 treatment did not inhibit forskolin-stimulated luciferase activity of CHO cells transfected with the empty pcDNA3.1(+) vector (data not shown). Each datapoint represents mean ± SEM of 3 replicates (n = 3).

NPBWR2 (EC_{50} = 9.17nM) expressed in CHO cells (Figure 4).

Despite the high amino acid sequence identity (~40%) shared between chicken NPBWRs and chicken SSTR1–SSTR5 (or opioid receptors), our preliminary study showed that cNPB28/cNPW23 treatment (10^{-11}M–10^{-6}M, 6 h) could not inhibit forskolin-stimulated luciferase activities of CHO cells expressing chicken SSTR1–SSTR5 or opioid receptors (data not shown), suggesting the inability of cNPW/cNPB in activating these receptors.

Tissue distribution of NPB, NPW, NPBWR1, and NPBWR2 in chickens

To elucidate the physiological roles of NPB/NPW in chickens, quantitative real-time RT-PCR (qPCR) was performed to examine the mRNA expression of *NPB*, *NPW*, *NPBWR1*, and *NPBWR2* in adult chicken tissues, including the heart, duodenum, kidneys, liver, lung, muscle, ovary, testes, spleen, pancreas, visceral fat, skin, pituitary, and various brain regions (telencephalon, midbrain, cerebellum, hindbrain, and hypothalamus). As shown in Figure 5, *cNPW* is abundantly expressed in the hypothalamus, moderately expressed in the lung, and weakly expressed in other tissues except liver. Interestingly, *cNPB* mRNA is widely expressed in all tissues examined including various brain regions. Unlike the wide tissue distribution of *cNPB* and *cNPW*, the mRNA expression of *cNPBWR1* and *cNPBWR2* is restricted to several tissues. c*NPBWR2* is predominantly and abundantly expressed in the anterior pituitary and weakly expressed in the hypothalamus, telencephalon, midbrain, spinal cord, spleen, and pancreas. Similarly, *cNPBWR1* is highly expressed in several brain regions (hypothalamus, telencephalon, and

Table 1. EC_{50} Values of cNPB28 and cNPW23 in Activating Chicken, Human, and Goose NPBWR1/NPBWR2 Expressed in CHO Cells

	EC_{50} (nM)					
Ligands	**cNPBWR1**	**cNPBWR2**	**hNPBWR1**	**hNPBWR2**	**goNPBWR1**	**goNPBWR2**
cNPB28	–	–	21.7	216.0	–	–
cNPW23	–	7.63	2.31	9.17	77.0	5.71

–, EC_{50} values could not be calculated based on the experimental data.

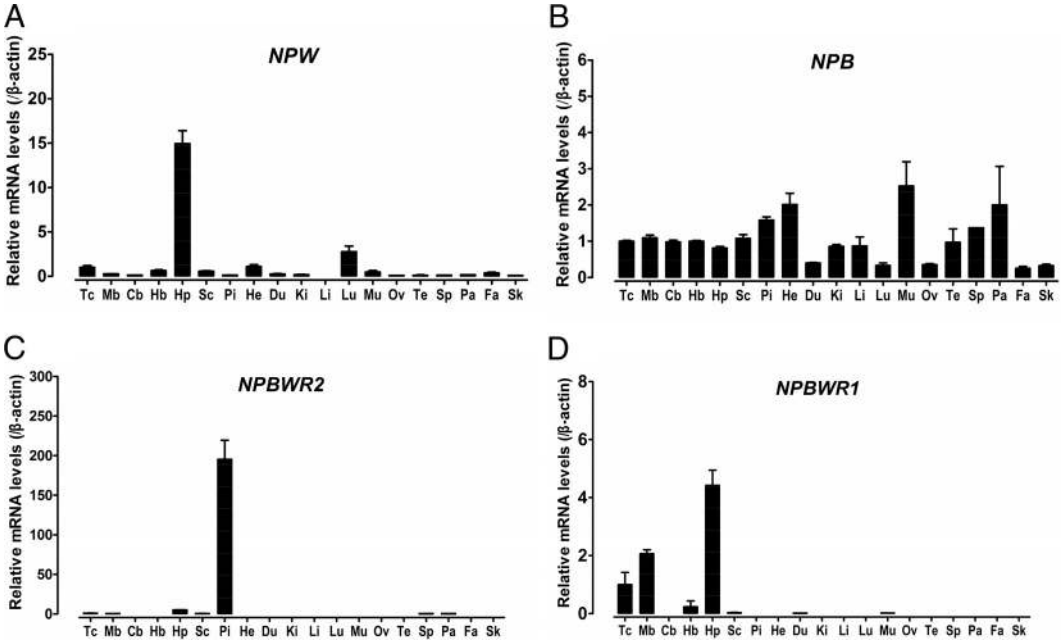

Figure 5. qPCR assay of *NPW* (A), *NPB* (B), *NPBWR2* (C), and *NPBWR1* (D) mRNA levels in different chicken tissues, including the telencephalon (Tc), midbrain (Mb), cerebellum (Cb), hindbrain (Hb), hypothalamus (Hp), spinal cord (Sc), pituitary (Pi), heart (He), duodenum (Du), kidneys (Ki), liver (Li), lung (Lu), muscle (Mu), ovary (Ov), testes (Te), spleen (Sp), pancreas (Pa), fat (Fa), and skin (Sk). The mRNA levels of target gene were normalized to that of *β-actin* and expressed as the fold difference compared with that of the telencephalon (Tc). Each data point represents the mean ± SEM of 4 adult chickens (n = 4).

midbrain) and weakly expressed in the hindbrain, spinal cord, duodenum, and muscle.

cNPW23 inhibits GHRH-induced GH and VIP-induced PRL secretion in cultured chick pituitary cells

The predominant expression of *cNPBWR2* in the chicken anterior pituitary (Figure 5) strongly suggests that NPW/NPB may affect pituitary functions. Because RT-PCR assay further revealed that *cNPBWR2* mRNA is expressed in both cephalic lobe (Ce) and caudal lobe (Ca) of anterior pituitaries (from 1- or 3-wk-old chicks and adult chickens), where PRL and GH cells reside, respectively (Figure 6) (38), it led us to hypothesize that NPW may control pituitary hormone secretion, such as GH/PRL secretion in chickens. To test this hypothesis, the effects of cNPW23 and cNPB28 on cGHRH (1nM)-induced GH secretion or pVIP (1nM)-induced PRL secretion were examined in cultured chick pituitary cells by Western blotting. As shown in Figure 6, cNPW23 (1nM–100nM, 6 h) could significantly inhibit GHRH-stimulated GH secretion in cultured pituitary cells dose dependently. Likewise, cNPW23 (1nM–100nM, 6 h) could dose dependently inhibit VIP-stimulated cPRL secretion in cultured chick pituitary cells. Moreover, the inhibitory action of cNPW23 on VIP-induced cPRL secretion was further supported by the observation that cNPW23 treatment caused an obvious, dose-dependent retention of cPRL protein within the cells treated by VIP (Figure 6). In sharp contrast, cNPB28 (1nM–100nM) failed to inhibit GHRH-induced GH secretion, or block VIP-induced PRL secretion.

To clarify whether cAMP signaling pathway is involved in NPW inhibition of pituitary GH/PRL secretion, we examined the effect of cNPW23 on hormone secretion in the presence of forskolin (2μM), which is an adenylyl cyclase activator that increases intracellular cAMP levels. Expectedly, forskolin treatment (6 h) could stimulate cGH and cPRL secretion in cultured chick pituitary cells, as previously reported (39, 40). However, this stimulatory effect was significantly inhibited by cNPW23 (100nM) treatment (Figure 7), suggesting that Gi-cAMP signaling pathway coupled to cNPBWR2 may mediate its inhibitory action on pituitary GH/PRL secretion (Figure 8).

Discussion

In this study, *NPB*, *NPW*, *NPBWR1*, and *NPBWR2* have been cloned from the chicken brain or pituitary. RT-PCR assay revealed that *NPB* and *NPW* mRNA are widely expressed in chicken tissues, whereas *NPBWR1* and *NPBWR2* are mainly expressed in the brain or pituitary. Functional study demonstrated that cNPBWR2 is a receptor specific to NPW, whereas cNPBWR1 cannot be activated by cNPB/cNPW. Moreover, cNPW has been shown to be capable of inhibiting GH and PRL secretion induced

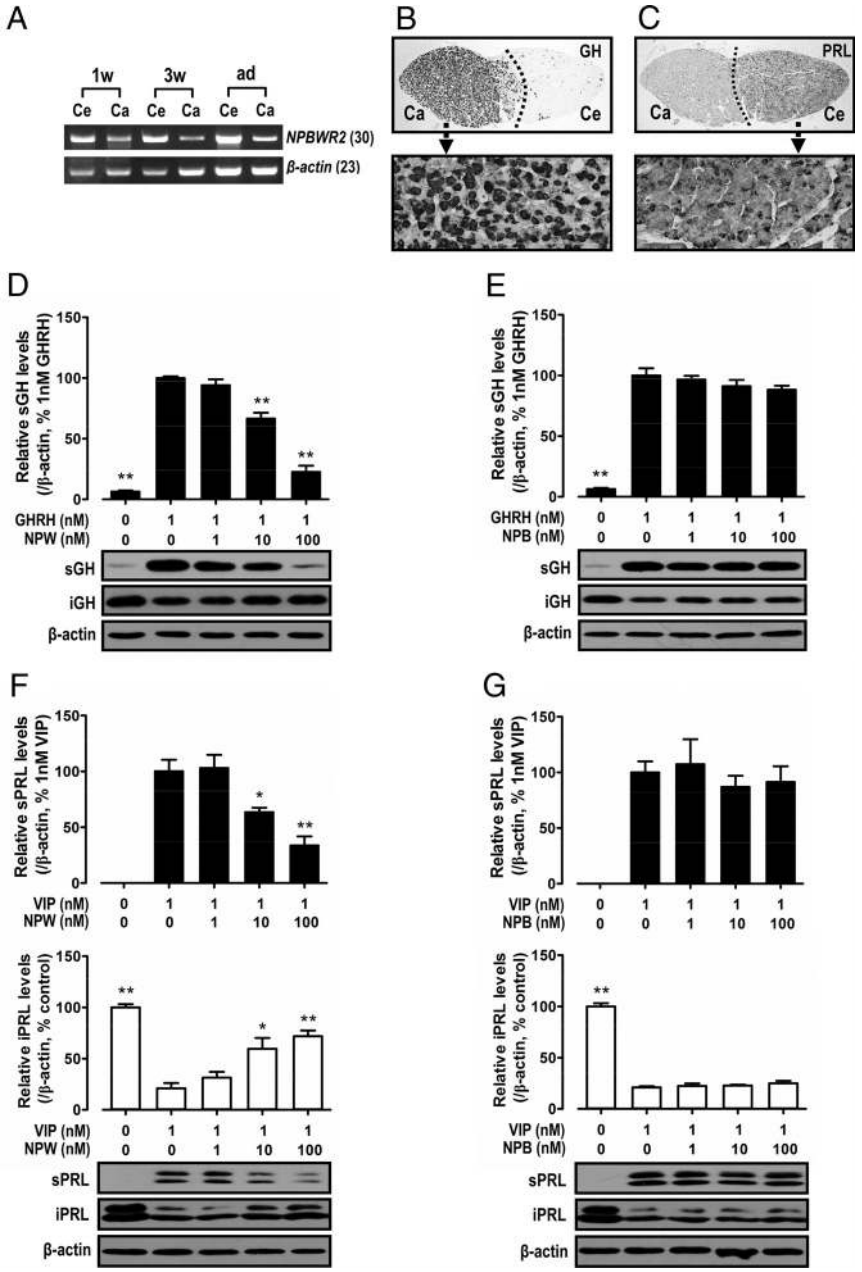

Figure 6. A, RT-PCR detection of *NPBWR2* mRNA expression in the caudal lobe (Ca) and cephalic lobe (Ce) of anterior pituitaries from 1-week-old (1w) and 3-week-old (3w) chicks and adult (ad) chickens. Numbers in brackets indicate the number of PCR cycles used. All RT negative controls show no PCR signal (data not shown). B and C, cGH and cPRL immunoreactive cells (dark color) are localized in the caudal lobe (Ca) and cephalic lobe (Ce) of 3-week-old chick pituitaries, respectively (upper panel, 4×; lower panel, 40×). D–G, Western blot analyses show that cNPW23 (1nM–100nM, 6 h), but not cNPB28, dose dependently inhibits cGHRH (1nM)-stimulated cGH secretion (D and E) and pVIP (VIP, 1nM)-stimulated PRL secretion (F and G, upper graphs) in cultured chick pituitary cells. Interestingly, cNPW, but not cNPB, treatment also caused an obvious retention of intracellular PRL protein (iPRL bands) within the cells treated by VIP (F and G, bottom graphs). The relative GH/PRL levels in culture medium (sGH/sPRL bands), or PRL levels in cell lysates (iPRL bands), were quantified by densitometry, normalized by that of β-actin in pituitary cell lysate, and then expressed as percentage of the 1nM-GHRH (1nM-VIP) treatment group, or the control. Each data point represents mean ± SEM of 3 replicates (n = 3). *, $P < .05$; **, $P < .001$, vs 1nM-GHRH (1nM-VIP) treatment group. Representative set of Western blotting is shown at the bottom of graphs (sGH/sPRL represent the secreted GH/PRL protein detected in the culture medium, whereas iGH/iPRL denote the intracellular GH/PRL). Note: 2 cPRL bands (24 and 27 kDa) were detected in the culture medium and pituitary cell lysates by Western blotting (both bands were used in densitometric analysis), whereas only a single cGH band of 23 kDa was detected, as reported in our previous studies (32, 36).

by GHRH and VIP, respectively. To our knowledge, this study represents the first to prove that an NPB/NPW system functions in birds and reveal a novel inhibitory action of NPW on pituitary hormone secretion in a vertebrate species.

NPW is highly conserved between chicken and other vertebrate species, but NPB is not

NPW/NPB peptides have been reported to play important roles on the mammalian CNS (1); however, the information regarding their structure and functions remains rather scarce in nonmammalian vertebrates. In this study, we cloned c*NPW* and *cNPB* cDNA from chicken brain or pituitary. Sequence analysis revealed that *cNPW* contains 2 coding exons (Supplemental Figure 1). As in mammals, chicken NPW precursor may generate 2 forms of NPW, cNPW23 and cNPW30. Moreover, chicken NPW shows a remarkable structural conservation with NPW of mammals, reptiles, and frogs.

Like c*NPW*, c*NPB* also contains 2 coding exons. Interestingly, chicken NPB precursor may produce a mature peptide of 28 amino acids (cNPB28), different from the 29- or 23-amino acid NPB of teleosts, frogs, and mammals (3). Moreover, we noted that cNPB28 shares only 50%–78% amino acid sequence identity with NPB29 of mammals, reptiles, frogs, and teleosts. Strikingly, chicken and other avian NPB peptide also differs in length. In turkeys and woodpeckers, NPB precursor is predicted to generate 2 forms of NPB of 23 (NPB23) and 29 (NPB29) amino acids after proteolytic processing at the 2 dibasic residues (Figure 1 and Supplemental Figure 2). In eagles and cuckoos, only 1 form of NPB (NPB23 or NPB29) is predicted. Besides the

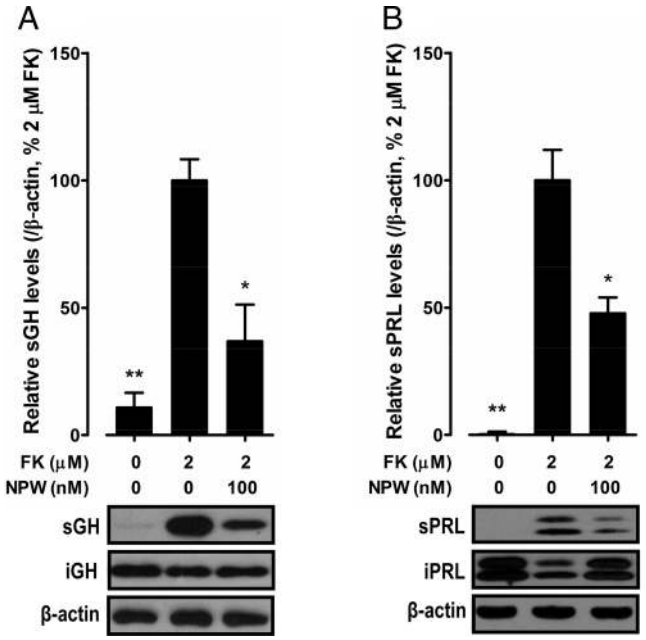

Figure 7. A and B, Western blot analyses show that cNPW23 (100nM, 6 h) inhibits forskolin (FK) (2μM)-stimulated GH and PRL secretion in cultured chick pituitary cells. The relative GH or PRL levels in culture medium (sGH/sPRL) were quantified by densitometry, normalized by that of β-actin band in pituitary cell lysate, and then expressed as percentage of the FK treatment group. Each data point represents mean ± SEM of 3 replicates (n = 3). *, $P < .05$; **, $P < .01$ vs FK treatment group. The representative set of Western blotting is shown at the bottom of each graph (sGH/sPRL represent the secreted GH/PRL protein detected in the culture medium, whereas iGH/iPRL denote the intracellular GH/PRL).

variation in length, NPB demonstrates considerable diversity in amino acid sequence between avian species. For instance, cNPB28 shares only 57% amino acid identity with cuckoo NPB29. Furthermore, all avian NPB has a 'WYR' motif at their N termini, instead of the typical 'WYK' present in NPB or NPW of mammals, frogs, and teleosts. The remarkably low degree of sequence conservation between chickens and other vertebrates and between avian species may imply a less important role of NPB in chickens, and possibly in other birds as well.

NPB/NPW systems in chickens and other vertebrates

The presence of NPW and NPB in chickens led us to further examine whether their 2 receptors, NPBWR1 and NPBWR2, exists in chickens (1). Our data clearly showed that both receptors orthologous to human NPBWR1 and NPBWR2 exist in chickens (Figure 3). As in chickens, both receptor genes could also be identified in other avian species including geese and turkeys. Our finding contrasts a previous report, which proposed *NPBWR2* was lost in chickens during evolution (27). Moreover, both receptor genes can be identified in the genome databases of anole lizards, *X. tropicalis*, coelacanths, and elephant sharks, indicating that *NPBWR1* and *NPBWR2* have emerged since the time the common ancestor of these species lived. And these 2 genes were possibly originated by the second round of genome duplication event during vertebrate evolution (27).

NPB, *NPW*, *NPBWR1*, and *NPBWR2* exist in the genomes of chickens, frogs, coelacanths, and elephant sharks (Figure 3); however, absence of particular system member(s) was observed in other species, probably due to lineage-specific gene loss. In particular, as revealed by synteny analysis, only *NPB* and *NPBWR2* remain in the zebrafish genome, whereas *NPW* and *NPBWR1* seem to be lost in zebrafish and other teleosts (eg, Nile tilapia) (Figures 3 and 8). Interestingly, 2 copies of *NPB* genes, named *NPBa* and *NPBb*, were identified in the genomes of Nile tilapia and fugu in this study. Similarly, 2 copies of *NPBWR2*, named *NPBWR2a* and *NPBWR2b*, were also identified in the zebrafish genome. The 2 *NPB* and *NPBWR2* genes were likely originated by the third round of genome duplication event occurred in the teleost lineage (41). These findings not only imply that the NPB-NPBWR2 ligand-receptor pair exist in teleosts but also indicate the fundamental difference in the member constitution of the vertebrate NPB/NPW system. These findings also provide support to the similar, but nonidentical, roles of NPB/NPW system in different vertebrate groups (Figure 8).

Chicken NPBWR2 is a receptor specific to cNPW

In this study, we proved that only cNPBWR2 can be effectively activated by NPW23, and its activation lead to inhibition of the intracellular cAMP signaling pathway. Similarly, goose NPBWR2 is also preferentially activated by cNPW23 (Figure 4). These findings indicate that NPBWR2 is a receptor specific to NPW23, and functionally coupled to Gi-cAMP signaling pathway in birds, as its mammalian ortholog (1).

Unlike cNPBWR2, chicken NPBWR1 cannot be activated by cNPB28, or by cNPW23. Similarly, goose NPBWR1 also shows no response to NPB treatment, and it can only be activated by NPW23 at concentrations far above the physiological ranges. In addition, we noted that zebra finch and budgerigar *NPBWR1* is a pseudogene that lacks a complete ORF (Supplemental Figures 5 and 6). All these findings tend to support the idea that NPBWR1 (GPR7) may play a lesser or insignificant role in mediating NPB/NPW actions in birds. The inability of NPBWR1 in signal transduction in chickens also highlights the dramatic functional switch of NPBWR1 and NPBWR2 between chickens and mammals. In chickens, only NPBWR2 can be potently activated by NPW, hinting the importance of NPBWR2 in mediating NPW (or NPB) actions. In mice,

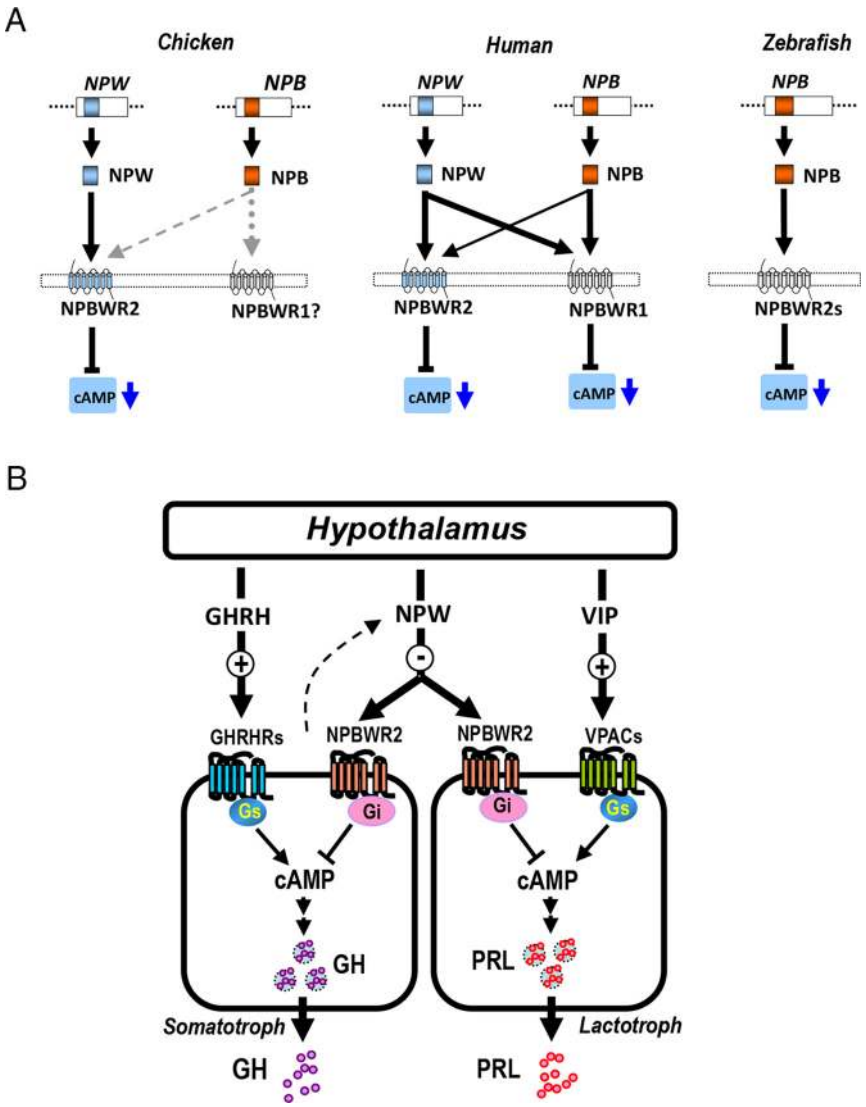

Figure 8. A, The ligand-receptor pair(s) in chickens, humans, and zebrafish. In chickens, only cNPBWR2 can be activated by cNPW effectively, suggesting that NPW-NPBWR2 is the major ligand-receptor pair functioning in chickens. The low potency of cNPB28 in activating cNPBWR2 implies that cNPB28 may be a low-affinity ligand for cNPBWR2. The question whether cNPBWR1 is functional remains unclear. In humans, both NPBWR1 and NPBWR2 can be activated by NPB and NPW effectively, hinting their importance in mediating NPB/NPW actions. In zebrafish, only 1 *NPB* and 2 *NPBWR2s* (*NPBWR2a* and *NPBWR*2b) were identified, implying that unlike that in chickens and humans, only NPB-NPBWR2 functions in zebrafish. B, Proposed model for NPW action on chicken pituitary. In somatotrophs (GH cells), hypothalamic GHRH strongly stimulates GH secretion via activation of GHRH receptors (GHRHRs) coupled to cAMP/PKA signaling pathway (34, 51, 52), whereas in lactotrophs (PRL cells), hypothalamic VIP strongly induces PRL secretion via activation of VIP receptors (VPACs) coupled to cAMP/PKA signaling pathway (52). As a novel inhibiting factor, hypothalamic NPW can inhibit GHRH-induced GH secretion or VIP-stimulated PRL secretion. And this inhibitory action is likely mediated by cNPBWR2 expressed in GH and PRL cells, which activation can decrease intracellular cAMP levels induced by GHRH or VIP, and subsequently inhibit GH/PRL secretion. In addition, the NPW signal (dashed arrow) from the pituitary or other peripheral tissues may inhibit GH/PRL secretion in an autocrine/paracrine/endocrine manner.

only NPBWR1 can be potently activated by NPB/NPW, whereas NPBWR2 is lost during speciation, indicating a pivotal role of NPBWR1 in mediating NPB/NPW actions (7). In humans, both NPBWR1 and NPBWR2 can be activated by NPB/NPW, thus both receptors can mediate the actions of NPB/NPW (Figure 8) (1).

It is of particular interest to note that cNPB28 cannot activate chicken and goose NPBWR1 at any concentration tested. This finding contrasts the finding in humans, in which NPB is a selective agonist for NPBWR1 and capable of preferentially activating human NPBWR1, but not NPBWR2 (4, 5). Despite its inability in activating chicken or goose NPBWR1, cNPB28 can still preferentially activate human NPBWR1 with an EC_{50} value of 21.7nM. In addition, cNPB28 can also activate cNPBWR2 at a high concentration (1μM). These findings suggest that cNPB28 is bioactive and may act as a low-affinity ligand for cNPBWR2. The low potency of cNPB28 in activating cNPBWR2 may be due to its remarkable structural change during speciation.

Tissue expression of NPB/NPW system: Implications for its potential roles in chickens

In this study, the expression of *NPB/NPW*, *NPBWR1*, and *NPBWR2* in chicken tissues was examined. *cNPB* is widely expressed in chicken tissues examined including peripheral tissues and various brain regions. This finding is consistent with the findings in rats (4). However, it should be reminded that cNPB28 can only activate cNPBWR2 at high concentrations (1μM) (Figure 4). Thus, it remains to be clarified whether cNPB28 derived from brain and peripheral tissues can play an active role via activation of cNPBWR2 in vivo, such as regulation of food intake and stress, as demonstrated in mammals (1).

Like *cNPB*, *cNPW* mRNA is widely expressed in chicken tissues except liver. Interestingly, the highest mRNA level of *NPW* was noted in the hypothalamus, indicating that the hypothalamus is a major source of NPW peptide in chick-

ens. Our finding coincides with the reports in rats, in which NPW is expressed in the hypothalamic paraventricular nucleus, ventromedial nucleus, arcuate nucleus and lateral hypothalamus (8, 23, 42). c*NPBWR2* is predominantly expressed in the anterior pituitary and weakly expressed in several brain regions including the hypothalamus. This spatial expression pattern of cNPW-NPBWR2 not only implies that cNPW may act on the CNS to regulate physiological processes, such as food intake and stress in the hypothalamus similar to mammalian NPB/NPW system (2, 8, 43, 44), but also strongly suggests that hypothalamic NPW may control pituitary functions directly. Although *cNPW* mRNA is moderately expressed in the lung and weakly expressed in most peripheral tissues examined, its receptor (c*NPBWR2*) mRNA is weakly expressed in several peripheral tissues including the spleen and pancreas. This finding questions whether cNPW can play autocrine/paracrine roles in these tissues.

Although cNPBWR1 cannot be activated by cNPB/cNPW, *cNPBWR1* mRNA is still abundantly expressed in several brain regions including the hypothalamus (Figure 5). This finding partially agrees with the observation in rodents, in which NPBWR1 (*GPR7*) mRNA is abundantly expressed in several brain regions including the hypothalamus (7). However, the physiological relevance of this interesting finding awaits further clarification.

NPW is a novel inhibitor of pituitary GH and PRL secretion in chickens

It is reported that the central administration of NPW or NPB can increase plasma PRL level and lower GH levels in rats (2, 13, 16); however, NPW/NPB have no effects on the basal or releasing factor-stimulated GH and PRL secretion in cultured pituitary cells (13, 16). These findings are rather confounding, considering that their receptors are expressed in the pituitary (4, 6). It is hence hypothesized that in mammals, NPB/NPW act on the hypothalamus nuclei to modulate neuroendocrine signals released to the anterior pituitary, but do not act as releasing or inhibiting factors on their own in the pituitary. In contrast to the mammalian findings, we demonstrated that cNPW23 not only inhibits GH secretion induced by chicken GHRH (32, 34), a major GH-releasing factor identified by our and other groups (29, 32, 34, 45), but also effectively suppresses PRL secretion induced by VIP, a well-known PRL-releasing factor in birds (30, 33, 46–48) and mammals (49). Undoubtedly, our findings provide the first persuasive evidence that NPW23 can act directly on the pituitary to inhibit GH/PRL secretion induced by their releasing factors.

It is reported that cAMP signaling pathway coupled to GHRH receptors or VIP receptors is involved in GHRH-induced GH secretion or VIP-induced PRL release in chickens (Figure 8) (34, 50–52). In this study, we demonstrated that cNPW23 can significantly suppress GH and PRL secretion stimulated by forskolin, an adenylyl cyclase activator which can elevate intracellular cAMP levels and stimulate pituitary GH/PRL secretion in chickens (39, 40, 53). Considering that cNPBWR2 activation can decrease intracellular cAMP levels, it is likely that cNPBWR2 activation by NPW treatment may reduce cAMP levels of pituitary cells elevated by GHRH or VIP treatment, and thus suppress GH/PRL secretion induced by GHRH or VIP.

It is well documented that hypothalamic SST can strongly inhibit pituitary hormone secretion (54–56), such as GH and TSH secretion via activation of SSTRs in chickens and mammals (32, 54, 57, 58). The structural similarity (~40% identity) between cNPBWR2 and cSSTR1–cSSTR5 casts doubt on whether cNPW23 can activate cSSTRs to modulate pituitary hormone secretion. However, our in vitro functional assay demonstrated that cNPW23 cannot activate cSSTR1–cSSTR5 at any concentration tested, ruling out such a possibility. Given that *cNPW* is highly expressed in the hypothalamus and *cNPBWR2* in the anterior pituitary, it is likely that hypothalamic NPW is a novel inhibitory factor that acts on the pituitary directly in chickens. Future studies on the distribution of cNPW within the hypothalamic nuclei and its concentration in the hypophyseal portal blood are needed to substantiate this hypothesis. Interestingly, besides the hypothalamus, *cNPW* mRNA is also expressed in the pituitary and many peripheral tissues (Figures 5), implying that NPW signals from the pituitary or peripheral tissues may also inhibit pituitary GH/PRL secretion in an autocrine/paracrine/endocrine manner (Figure 8).

In chickens, *NPBWR2* mRNA was detected to be expressed in both cephalic lobe and caudal lobe of the anterior pituitary where multiple hormone-producing cells reside (38), suggesting that NPW may affect other pituitary hormone secretion, such as TSH and ACTH secretion. Future studies on this topic will aid in defining the precise actions of this novel factor, which may also act on the pituitary of other vertebrates, given the expression of its receptor(s) in the pituitary of all vertebrate species examined so far (4, 6).

In summary, *NPW*, *NPB*, *NPBWR1*, and *NPBWR2* have been cloned in chickens. Functional studies revealed that cNPBWR2 is a receptor specific to cNPW, whereas cNPBWR1 shows no response to cNPB/cNPW treatment, suggesting that NPW-NPBWR2 is the functional ligand-receptor pair in chickens. qPCR assay showed that *cNPW* is highly expressed in the hypothalamus, whereas its receptor (*NPBWR2*) is predominantly expressed in the pi-

tuitary, strongly suggesting that NPW may control pituitary functions directly. In support of this idea, cNPW23 can significantly inhibit chicken pituitary GH and PRL secretion induced by GHRH and VIP, respectively. Collectively, our study represents the first to characterize the NPB/NPW system in birds, and provide the foremost piece of persuasive evidence that NPW can act directly on the pituitary to inhibit GH/PRL secretion in a vertebrate species (Figure 8). Undoubtedly, these findings will facilitate the better understanding of the physiological roles played by the NPB/NPW system in birds, and help to uncover the structural and functional changes of the NPB/NPW system during vertebrate evolution (Figure 8).

Acknowledgments

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