Characterization of NMB, GRP and their receptors (BRS3, NMBR and GRPR) in chickens

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Abstract

The two structurally and functionally related peptides, gastrin-releasing peptide (GRP) and neuromedin B (NMB) play critical roles in many physiological/pathological processes in mammals. However, the information regarding the expression and functionality of avian NMB, GRP and their receptors is limited. Here, we characterized cNMB, cGRP and their receptors (cNMBR, cGRPR and cBRS3) in chickens. Our results showed that: (1) cNMBR and cGRPR expressed in CHO cells could be potently activated by cNMB and cGRP, respectively, as monitored by cell-based luciferase reporter assays, indicating that cNMBR and cGRPR are cNMB- and cGRP-specific receptors; strikingly, BRS3 of chickens (/spotted gars), which is orthologous to mouse bombesin receptor subtype-3 (BRS3), could be potently activated by GRP and NMB, demonstrating that both peptides are the endogenous ligands for chicken (/spotted gar) BRS3; (2) quantitative real-time PCR (qPCR) revealed that *cGRPR* is widely expressed in chicken tissues with abundant expression in the ovary, pancreas, proventriculus, spinal cord and brain, whereas *cNMB*, *cNMBR* and *cBRS3* are mainly expressed in the brain and testes; (3) interestingly, qPCR, Western blot and immunostaining revealed that cGRP is predominantly expressed in the anterior pituitary and mainly localized to LH-cells, suggesting that cGRP is likely a novel pituitary hormone in chickens. In summary, our data help to uncover the roles of GRP, NMB and their receptors in birds, and provide the first persuasive evidence from an evolutionary prospective that in vertebrates, GRP and NMB are the endogenous ligands for BRS3, an orphan receptor that has puzzled endocrinologists for more than two decades.

Key Words

- ▶ chicken
- ▶ NMB
- ▶ GRP
- ▶ BRS3
- ▶ GRPR
- ▶ NMBR
- ▶ pituitary hormone

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Introduction

Bombesin (BN) is a 14-amino acid peptide originally isolated from amphibian skin in 1971 (Erspamer *et al.* 1970, Anastasi *et al.* 1971), which has a potent stimulatory action on preparations of intestinal, uterine and urine tract smooth muscle (Erspamer *et al.* 1972). Subsequently, two structurally and functionally related bombesin-like

peptides encoded by separate genes, gastrin-releasing peptide (GRP) and neuromedin B (NMB), have also been identified in mammals (McDonald *et al.* 1979, Minamino *et al.* 1983, Spindel *et al.* 1984, Krane *et al.* 1988). Two active forms of GRP with an identical C terminus, GRP₂₇ (27 amino acids) and GRP₁₀ (10 amino

acids), have been isolated in porcine stomach and spinal cord, respectively (McDonald *et al.* 1979, Minamino *et al.* 1984). NMB is a 10-amino acid peptide originally isolated from porcine spinal cord (Minamino *et al.* 1983). Shortly after its discovery, the two N-terminally extended forms of NMB (designated as NMB₃₀ and NMB₃₂, respectively) have also been identified in porcine brain and spinal cord (Minamino *et al.* 1985).

In mammals, GRP and NMB are widely expressed in the central nervous system (CNS) and peripheral tissues (Wada *et al.* 1990, Moody & Merali 2004), including the gastrointestinal (GI) tract, urogenital and reproductive system, lung and anterior pituitary (Jones *et al.* 1992, Houben *et al.* 1993, Jensen *et al.* 2008). In accordance with their wide distribution, GRP is reported to regulate many physiological/pathological processes including food intake, circadian rhythm, itching, smooth muscle contraction, GI tract motility and cell proliferation in normal tissues and many cancerous tissues (Rozengurt 1988, Merali *et al.* 1999, McArthur *et al.* 2000, Sun & Chen 2007, Jensen *et al.* 2008, Sun *et al.* 2009). Like GRP, NMB is also involved in the control of many physiological/pathological processes, such as food intake, thermogenesis, pituitary hormone secretion and cell proliferation of both normal and cancerous tissues (Rettori *et al.* 1989, Ladenheim *et al.* 1994, Lach *et al.* 1995, Matusiak *et al.* 2005). It is clear that the biological actions of GRP and NMB are mediated by their specific receptors, namely GRP receptor (GRPR) and NMB receptor (NMBR), respectively (Spindel *et al.* 1990, Battey *et al.* 1991, Wada *et al.* 1991, Benya *et al.* 1995). Both GRPR and NMBR belong to bombesin receptor family of G protein-coupled receptor (GPCR) and are functionally coupled to Gq protein (Benya *et al.* 1995, Jensen *et al.* 2008). Upon ligand stimulation, both receptors can trigger multiple signaling pathways, such as calcium mobilization and activation of MAPK/ERK signaling cascade (Jensen *et al.* 2008). In consistence with the broad spectrum of GRP/NMB actions, GRPR and NMBR are reported to be widely expressed in the CNS and peripheral tissues in mammals (Wada *et al.* 1992, Sano *et al.* 2004, Ohki-Hamazaki *et al.* 2005, Jensen *et al.* 2008). Apart from GRPR and NMBR, a third bombesin receptor named bombesin receptor subtype-3 (BRS3), which shows high sequence identity to GRPR (~58%) and NMBR (~48%), has also been identified in mammals (Gorbulev *et al.* 1992, Fathi *et al.* 1993). Since BRS3 displays an extremely low affinity to GRP/NMB

($K_i \gg 1000$ nM) or other naturally occurring bombesin-related peptides (Gorbulev *et al.* 1992, Nagalla *et al.* 1996, Mantey *et al.* 1997, Ryan *et al.* 1998), thus, the endogenous ligand of BRS3 has remained an open question and BRS3 has been viewed as an orphan receptor. However, accumulating evidence from BRS3-deficient mice has suggested that BRS3 expressed in the CNS may regulate many vital physiological processes, such as energy homeostasis, body temperature, metabolic rates and obesity (Ohki-Hamazaki *et al.* 1997b, Guan *et al.* 2010, Metzger *et al.* 2010).

As in mammals, both GRP and NMB and their putative receptors also exist in non-mammalian vertebrates, including birds, frogs and teleosts (Vaillant *et al.* 1979, McDonald *et al.* 1980, Nagalla *et al.* 1992, Volkoff *et al.* 2000, Xu & Volkoff 2009, Yun *et al.* 2015). Moreover, there are also lines of evidence showing that GRP/NMB play important roles in many physiological processes in non-mammalian vertebrates, such as inhibition of food intake in chickens and teleosts following central or peripheral administration (Tachibana *et al.* 2010a,b, Schroeter *et al.* 2015), and regulation of gastric acid secretion, gallbladder motility, crop-emptying rate, pancreatic fluid and protein secretion in chickens or turkeys (Linari & Linari 1975, Campbell *et al.* 1991, 1994, Tachibana *et al.* 2010b, Scanes & Pierzchala-Koziec 2014). However, in contrast to the extensive studies in mammals, the identity, signaling property and expression of the receptors for GRP and NMB have not been fully characterized in non-mammalian vertebrates including birds (Iwabuchi *et al.* 2003, Ohki-Hamazaki *et al.* 2005). Therefore, using chicken as an animal model, our present study aimed to: (1) identify and functionally characterize all the receptors for GRP/NMB; (2) systematically investigate the spatial expression of GRP, NMB and their receptors. As a result, we identified three functional receptors (GRPR, NMBR and BRS3) for chicken GRP/NMB. Strikingly, we demonstrated that both GRP and NMB could potentially activate BRS3, suggesting they are both potent ligands of BRS3. Like chicken BRS3, spotted gar BRS3 could be effectively activated by GRP and NMB *in vitro*. Moreover, we found that GRP is predominantly expressed in chicken anterior pituitaries. Undoubtedly, our study presents the first convincing evidence that GRP and NMB are most likely the endogenous ligands for orphan receptor BRS3 in vertebrates from an evolutionary perspective, and puts forward a new concept that GRP is likely a novel pituitary hormone in an avian species (i.e. chicken).

Materials and methods

Chemicals, hormones, antibodies and primers

Chicken GRP₂₇ (cGRP₂₇), GRP₁₀ (cGRP₁₀) and NMB (cNMB) were synthesized using solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). Goat anti-GRP polyclonal antibody was purchased from Santa Cruz Biotechnology and monoclonal antibody for β -actin was obtained from Cell Signaling Technology. Anti-ACTH antibody was purchased from Abcam. Donkey anti-goat IgG (H+L) cross-adsorbed secondary antibody (DyLight 488 conjugate) and anti-rabbit IgG (H+L) cross-adsorbed secondary antibody (Dylight 550 conjugate) were purchased from Thermo Fisher Scientific. The polyclonal antibodies against recombinant full-length chicken GH, PRL and LH β were prepared in our laboratory (Huang *et al.* 2014, Meng *et al.* 2014, Bu *et al.* 2016). All primers were synthesized by Beijing Genome Institute (BGI, China) and listed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article).

Total RNA extraction

Adult chickens (1-year-old) of both sexes (Lohmann layer) were purchased from local commercial company. Chickens were killed, and various tissues including the different brain regions were isolated and collected for RNA extraction. Total RNA was prepared using RNeasy (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. All experiments were performed according to the guidelines provided by the Animal Ethics Committee of Sichuan University.

Reverse transcription and quantitative real-time PCR (qPCR)

Two micrograms of total RNA and 0.5 μ g of oligodeoxythymide were mixed in a total volume of 5 μ L, incubated at 70°C for 10 min and cooled at 4°C for 2 min. The first strand buffer, 0.5 mM each deoxynucleotide triphosphate and 100 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Takara) were then added into the reaction mix in a total volume of 10 μ L. Reverse transcription (RT) was performed at 42°C for 90 min.

According to our previously established method (Cai *et al.* 2015), quantitative real-time PCR was conducted on the CFX96 Real-time PCR Detection System (Bio-Rad) to examine the mRNA levels of target genes in chicken tissues.

Cloning the cDNAs of chicken GRPR, NMBR and BRS3

According to the genomic sequences of *cGRPR*, *cNMBR* and *cBRS3* (http://www.ensembl.org/Gallus_gallus), gene-specific primers were designed to amplify the cDNA containing the open reading frame (ORF) of the receptors from the chicken brain with the use of high-fidelity *Taq* DNA polymerase (TOYOBO). The amplified PCR products were cloned into pTA2 vector and sequenced by ABI3100 Genetic Analyzer (BGI). Finally, the cDNA sequence of each receptor was determined by sequencing three independent clones.

Functional characterization of cNMBR, cGRPR and chicken/spotted gar/mouse BRS3 in CHO cells

Based on the cDNA sequences of chicken *GRPR*, *NMBR* and *BRS3*-like genes, gene-specific primers were used to amplify the ORF from adult chicken brain using high-fidelity *Taq* DNA polymerase (TOYOBO, Japan). The amplified PCR products were cloned into the pcDNA3.1 (+) expression vector (Invitrogen) and sequenced. To compare the functional difference of chicken BRS3 with BRS3 from other vertebrate species, including mouse and spotted gar, we also amplified the complete coding regions of mouse and spotted gar *BRS3* from brain tissues and cloned them into the pcDNA3.1 (+) expression vector.

According to our previously established methods, the functionality and signaling property of each receptor was examined in Chinese hamster ovary (CHO) cells using pGL3-NFAT-RE-luciferase reporter system (Wang *et al.* 2012), pGL4-SRE-luciferase reporter system (Mo *et al.* 2015) and pGL3-CRE-luciferase reporter system (Wang *et al.* 2007a).

Western blot

To compare the protein level of cGRP in the anterior pituitary, brain and proventriculus of adult chickens, Western blot analysis was performed. The respective tissue lysates were prepared for the detection of cGRP level using corresponding antibodies (1:300), as described in our recent study (Mo *et al.* 2015). In parallel, β -actin level was examined in tissue lysates as a loading control.

To test whether cBRS3 activation can enhance ERK1/2 (44/42 kDa) and CREB (43 kDa) phosphorylation, HEK293 cells that transiently expressed cBRS3 were treated by cGRP₂₇ (100 nM) for 10 min, and the phosphorylated ERK1/2 and CREB levels in cellular lysates were examined by Western blot, as described in our recent study

(He *et al.* 2016, Cui *et al.* 2017). β -actin level was also examined and used as a loading control.

Detection of GRP mRNA and protein expression in chicken anterior pituitaries

To examine the spatial distribution of GRP in chicken anterior pituitaries, qPCR, Western blot and immunohistochemical staining (IHC) were used in this experiment, as described in our recent studies (Meng *et al.* 2014, Mo *et al.* 2015). In brief, anterior pituitaries collected from adult female chickens were washed with PBS to remove any contaminating blood cells. The cephalic lobe and caudal lobe were separated carefully by scalpels, and their respective tissue lysates and total RNA were prepared for Western blot detection of cGRP (or cGH/ β -actin) protein expression and qPCR assay of cGRP mRNA expression, respectively. For IHC, anterior pituitaries from adult female chickens were fixed in 4% paraformaldehyde, paraffinized and sliced into sections. IHC staining was performed in the pituitary sections using a Streptavidin-Biotin-Peroxidase Complex (SABC) kit (Boster Biological Technology Ltd) according to the manufacturer's instructions. Goat anti-GRP antibody (1:300) was used to probe the spatial distribution of cGRP in chicken pituitaries. Pituitary sections incubated with cGRP₂₇-preabsorbed GRP antibody (incubated overnight at 4°C with 100ng/mL of cGRP₂₇) was used as a negative control in parallel.

Double fluorescence staining

The dispersed anterior pituitary cells from adult female chickens were cultured in Medium 199 supplemented with 15% fetal bovine serum (Invitrogen) in a Corning Cell-BIND 96-well plates (Corning) at 37°C with 5% CO₂ at a density of 1×10^4 cells/well, as described in our previous study (Meng *et al.* 2014). After 4 h of culture, the pituitary cells were fixed in 4% paraformaldehyde and washed with PBS. Then, the cells were treated by 0.1% Triton X-100 for 10 min, washed with PBS and incubated with the blocking buffer (1% BSA in PBS) for 30 min at room temperature to minimize non-specific adsorption. After blocking, the two primary antibodies, goat anti-GRP and rabbit anti-cGH (or anti-cPRL/-cLH β /-ACTH) diluted in blocking buffer (1:500 or 1:1000), were added to each well and incubated at 4°C overnight. The cells were washed and incubated with fluorochrome-conjugated secondary antibodies (1:300) for 1 h at room temperature. Finally, the pituitary cells were counterstained with DAPI

and observed under a fluorescence microscope (Nikon ECLIPSE Ti).

Data analysis

The mRNA level of target gene was normalized by that of β -actin and then expressed as fold difference compared to chosen tissue. The luciferase activities of CHO cells expressing GRPR/NMBR/BRS3 in peptide treatment group were expressed as relative fold increase compared to the control group (without peptide treatment). The data were analyzed by one-way ANOVA followed by Dunnett's test. The dose-responsive curves were constructed using nonlinear regression models, and the corresponding half-maximal effective concentration (EC₅₀) values were evaluated with GraphPad Prism 5 (GraphPad Software). All experiments were repeated at least twice to validate our results.

Results

Cloning of NMBR cDNA and identification of BRS3 in chickens

Although *NMBR* is predicted to exist in chickens and other lower vertebrates, *NMBR* cDNA has not been reported in any non-mammalian vertebrates. In this study, we cloned the cDNA containing an ORF of *NMBR* from the chicken brain (Supplementary Fig. 1). The cloned *cNMBR* cDNA is predicted to encode a receptor of 388 amino acids (accession no.: KU887757), which shows high amino acid sequence identities with NMBR of mice (72%), *Xenopus tropicalis* (82%), Nile tilapia (68%) and spotted gars (74%). Like human NMBR, *cNMBR* contains seven transmembrane domains (TMD1–7), a DRY motif critical for G protein coupling, and two cysteines for disulfide bond formation (Fig. 1). Moreover, *cNMBR* shows high amino acid sequence identities with chicken GRPR (53%) and BRS3 (48%).

Although *BRS3* exists in mammals, it remains uncertain whether *BRS3* exists in chickens. Using synteny analysis, we found that *BRS3* exists in chickens and is located on chromosome 4, which differs from the chromosomal location of *NMBR* and *GRPR* (Fig. 2). Interestingly, we noted that chicken *BRS3* is identical to a previously reported 'bombesin receptor subtype-3.5 (*BRS3.5*)' (Iwabuchi *et al.* 2003), and thus, we designated this *BRS3.5* as *BRS3* in this study.

As in chickens, *BRS3* also exists in other non-mammalian vertebrates including western painted turtles,

Figure 1

Amino acid alignment of chicken NMBR (KU887757) with that of mice (NP_032729), *Xenopus tropicalis* (XP_012818691), Nile tilapia (XP_005452510) and spotted gars (XP_006643031), or with chicken BRS3 (NP_989737) and GRPR (NP_989738). The conserved 'DRY motif' is boxed and the seven transmembrane domains (TMD1–7) are shaded; the potential *N*-glycosylation site (NXT/S) is underlined; the pair of cysteine residues proposed for disulfide bond formation are boxed; dots indicate amino acids identical to chicken NMBR; dashes denote gaps in the alignment.

Xenopus tropicalis and spotted gars (Fig. 2). Interestingly, *BRS3* is likely lost in teleosts (e.g. Nile tilapia), as revealed by synteny analysis. In addition, some of the paralogous genes adjacent to *BRS3/NMBR/GRPR* (e.g. *ADGRG2/4/6*) could be identified in these vertebrate species examined, implicating that *BRS3*, *GRPR* and *NMBR* were likely duplicated from a common ancestral gene by the 2 rounds of genome duplication (2R) occurred during early vertebrate evolution (Fig. 2) (Yun *et al.* 2015). Using RT-PCR, we cloned the cDNA of spotted gar *BRS3* from brain tissue (KX447669) (Supplementary Figs 2 and 3). This finding further indicates the presence and expression of *BRS3* in lower vertebrate species.

Functional characterization of chicken NMBR, BRS3 and GRPR

To compare the functional difference between the three chicken bombesin receptors (cNMBR, cGRPR and cBRS3), each receptor was transiently expressed in CHO cells and treated by chicken GRP₂₇, GRP₁₀ and NMB₁₀ (Fig. 3).

Receptor activation was then examined by pGL3-NFAT-RE-luciferase reporter, pGL4-SRE-luciferase reporter and pGL3-CRE-luciferase reporter systems, which could monitor receptor-activated calcium mobilization (Wang *et al.* 2012), MAPK/ERK (Mo *et al.* 2015) and adenylate cyclase (AC)/cAMP/PKA signaling pathways (Wang *et al.* 2007a), respectively.

Using the pGL3-NFAT-RE-luciferase reporter system, we demonstrated that cNMB could potentially activate cNMBR expressed in CHO cells (EC₅₀: 8.2 nM). On the other hand, cGRP₂₇ and cGRP₁₀ are much less potent, indicating that cNMBR is a NMB-specific receptor. By contrast, cGRPR could be potentially activated by cGRP₂₇ and cGRP₁₀ (with cGRP₂₇ being slightly more potent than cGRP₁₀), while cNMB₁₀ is ineffective. These findings indicate that GRPR is a GRP-specific receptor. Strikingly, we found that cBRS3 could be effectively activated by the three peptides (EC₅₀ of cGRP₂₇: 8.3 nM; EC₅₀ of cGRP₁₀: 9.7 nM; EC₅₀ of cNMB: 28.2 nM) (Fig. 3). This finding indicates that cBRS3 can function as a receptor common for both cGRP and cNMB, and display a weak selectivity toward cGRP

Figure 2

(A, B and C) Synteny analysis showing that *NMBR* (A), *BRS3* (B) and *GRPR* (C) are located in distinct syntenic regions conserved between chickens, western painted turtles, mice, *Xenopus tropicalis*, spotted gars and Nile tilapia. The two copies of *GRPR* (*GRPRa* and *GRPRb*) identified in Nile tilapia are likely to be paralogs generated by the third round (3R) of teleost-specific genome duplication (WGD) event (Meyer & Van de Peer 2005), while *BRS3* is likely lost. Dashed lines denote the genes of interest, while dotted lines indicate the syntenic genes identified in these species. Genes were labeled based on their gene symbols in the human genome. Chr, chromosome; Chr Un, chromosome unknown. (D) Schematic diagram showing the existence of *BRS3*, *GRPR* and *NMBR* in spotted gars, teleosts (e.g. Nile tilapia, *BRS3* is lost), frogs (e.g. *Xenopus tropicalis*), reptiles (e.g. western painted turtles), birds (e.g. chickens) and mammals (e.g. mice), which were likely duplicated from an ancestral *GRPR*-like gene (*GRPR-L*) by the two rounds (2R) of genome duplication event during vertebrate evolution (Yun et al. 2015).

(Table 1). Furthermore, our findings also suggest that as in mammals, activation of all three receptors may cause calcium mobilization in chickens (Jensen et al. 2008).

Using pGL4-SRE-luciferase reporter system, we also demonstrated that cNMBR expressed in CHO cells

could be potentially activated by cNMB (EC_{50} , 5.8 nM), but not by cGRP, also supporting that cNMBR is a NMB-specific receptor (Fig. 4). By contrast, both cGRP₂₇ and cGRP₁₀ (and not cNMB₁₀) could potentially activate cGRPR expressed in CHO cells, further supporting the notion

Figure 3

(A) Amino acid sequence alignment of chicken NMB (cNMB₁₀) or GRP (cGRP₂₇ and cGRP₁₀) with NMB/GRP from mouse (mNMB₁₀/mGRP₁₀) and spotted gar (sNMB₁₀/sGRP₁₀); (B, C, D, E and F) effects of cGRP₂₇, cGRP₁₀ and cNMB₁₀ on activating chicken (c-) GRPR (B), cNMBR (C), cBRS3 (D), spotted gar BRS3 (sBRS3) (E), and mouse BRS3 (mBRS3) (F) expressed in CHO cells, monitored by pGL3-NFAT-RE-luciferase reporter system; (G) cells co-transfected with the empty pcDNA3.1 (+) vector and pGL3-NFAT-RE-luciferase reporter construct were used as internal controls, and peptide treatment did not alter the luciferase activity of CHO cells at any concentration tested. Each data point represents mean $\pm$ S.E.M. of four replicates ($N=4$).

Table 1 EC₅₀ values of cGRP and cNMB in activating different signaling pathways in CHO cells expressing chicken (c-) GRPR/ NMBR/BR53 or spotted gar (s-)/mouse (m-) BR53.

Peptide	EC ₅₀ values (nM)				
	cGRPR	cNMBR	cBR53	sBR53	mBR53
Calcium signaling pathway					
cGRP ₂₇	3.4	>200 ^a	8.3	9.2	–
cGRP ₁₀	15.7	>200 ^a	9.7	17.9	–
cNMB	–	8.2	28.2	44	–
MAPK/ERK signaling pathway					
cGRP ₂₇	6.1	~100 ^a	7.5	4.6	–
cGRP ₁₀	16.9	>100 ^a	8.4	5.9	–
cNMB	>100 ^a	5.8	28.3	30.8	–
cAMP/PKA signaling pathway					
cGRP ₂₇	66.8	>200 ^a	41.8	35.9	–
cGRP ₁₀	>100 ^a	>200 ^a	71	42.2	–
cNMB	–	23.3	>200 ^a	125.4	–

‘–’ means that the EC₅₀ values could not be calculated based on the experimental data.

^aIndicates that EC₅₀ value was roughly estimated based on the experimental data.

that GRPR is a GRP-specific receptor. In contrast, cBR53 could be effectively activated by all three peptides tested (EC₅₀ of cGRP₂₇: 7.5 nM; EC₅₀ of cGRP₁₀: 8.4 nM; EC₅₀ of cNMB: 28.3 nM) and it shows a weak selectivity toward GRP (Fig. 4) (Table 1). This finding substantiates the idea that cBR53 can function as a receptor common for both cGRP and cNMB. Meanwhile, our findings also suggest the functional coupling of the three receptors to the MAPK/ERK signaling cascade.

Using the pGL3-CRE-luciferase system, cGRPR and cNMBR were also shown to be preferentially activated by cGRP and cNMB, respectively, while cBR53 was activated by both GRP and NMB with similar potencies (Fig. 5). This finding suggests that all three receptors are also coupled to AC/cAMP/PKA pathway. However, we noted that much higher concentrations of cGRP/cNMB are required to activate the AC/cAMP/PKA signaling pathway than the other two pathways tested (Table 1).

To further verify the functional coupling of cGRPR, cNMBR and cBR53 to the calcium mobilization, MAPK/ERK

Figure 4

(A, B, C, D and E) Effects of cGRP₂₇, cGRP₁₀ and cNMB₁₀ on activating chicken (c-) GRPR (A), cNMBR (B), cBR53 (C), spotted gar BR53 (sBR53) (D) and mouse BR53 (mBR53) (E) expressed in CHO cells, monitored by pGL4-SRE-luciferase reporter system; (F) CHO cells co-transfected with empty pcDNA3.1 (+) vector and pGL4-SRE-luciferase reporter construct were used as internal controls, and peptide treatment did not alter the luciferase activity of CHO cells at any concentration tested. Each data point represents mean ± S.E.M. of four replicates (N=4).

Figure 5

(A, B, C, D and E) Effects of cGRP₂₇, cGRP₁₀ and cNMB₁₀ on activating chicken (c-) GRPR (A), cNMBR (B), cBRS3 (C), spotted gar BRS3 (sBRS3) (D) and mouse BRS3 (mBRS3) (E) expressed in CHO cells, monitored by pGL3-CRE-luciferase reporter system. CHO cells co-transfected with empty pcDNA3.1 (+) vector and pGL3-CRE-luciferase reporter construct were used as internal controls, and peptide treatment did not alter the luciferase activity of CHO cells at any concentration tested (*data not shown*). Each data point represents mean $\pm$ S.E.M. of four replicates ($N=4$). (F) Western blot showing that cGRP₂₇ treatment (100 nM, 10 min) can enhance the phosphorylation levels of ERK1/2 (pERK1/2) and CREB (pCREB) in HEK293 cells expressing chicken BRS3 (cBRS3).

and AC/cAMP/PKA signaling pathways, pharmacological drugs targeting these signaling pathways, such as 2-APB (an inhibitor of IP₃ receptor, which blocks IP₃-induced calcium mobilization from the endoplasmic reticulum), PD98059 (an inhibitor of the MEK/ERK signaling cascade), MDL12330A (an AC inhibitor) and H89 (a PKA inhibitor) were used to test whether they can inhibit receptor-activated signaling pathways. As shown in Fig. 6, all drugs tested could inhibit GRP-induced luciferase activity of CHO cells expressing cGRPR, cNMBR or cBRS3. Moreover, U73122, a phospholipase C (PLC) inhibitor, could inhibit the receptor-activated MAPK/ERK signaling pathway, suggesting that the three receptors require PLC to activate downstream MAPK/ERK signaling cascade (Fig. 6).

Using Western blot, we demonstrated that the activation of cBRS3 transiently expressed in HEK293 cells by cGRP₂₇ treatment could enhance ERK1/2 (44/42 kDa) and CREB (43 kDa) phosphorylation, further confirming the functional coupling of cBRS3 to both MAPK/ERK

and AC/cAMP/PKA/CREB pathways (Fig. 5). Similarly, cNMBR and cGRPR activation can also enhance the phosphorylation of ERK1/2 and CREB *in vitro* (*data not shown*).

Functional characterization of spotted gar and mouse BRS3

The high potency of GRP and NMB in activating chicken BRS3 led us to test whether GRP/NMB could activate BRS3 from other lower vertebrate species such as spotted gar (a primitive freshwater fish). Using the same approach, we found that like cBRS3, spotted gar BRS3 expressed in CHO cells could be effectively activated by cGRP (cGRP₂₇/cGRP₁₀) and cNMB (with cGRP being slightly more potent than cNMB), as monitored by the three luciferase reporter systems. In contrast, mouse BRS3 could neither be activated by cGRP nor by cNMB at any concentration tested (10^{-12} to 10^{-6} M) (Figs 3, 4, 5 and Table 1).

Figure 6

(A) Effects of 2-APB (100 μ M) on cGRP₂₇ (10 nM, 6 h)-induced luciferase activity of CHO cells expressing cGRPR, cNMBR or cBRS3, monitored by pGL3-NFAT-RE-luciferase reporter system; (B) effects of U73122 (U7, 20 μ M) and PD98059 (PD, 100 μ M) on cGRP₂₇ (10 nM, 6 h)-induced luciferase activity of CHO cells expressing cGRPR, cNMBR or cBRS3, monitored by pGL4-SRE-luciferase reporter system; (C) effects of MDL12330A (MDL, 20 μ M) and H89 (10 μ M) on cGRP₂₇ (100 nM, 6 h)-induced luciferase activity of CHO cells expressing cGRPR, cNMBR or cBRS3, monitored by pGL3-CRE-luciferase reporter system. Each drug was added 1 h before cGRP treatment. In each graph, 'T' represents peptide treatment and 'C' represents control without peptide treatment. Each data point represents mean $\pm$ S.E.M. of four replicates ($N=4$). * $P < 0.01$ vs control (in the absence of drug); # $P < 0.01$ vs peptide treatment (in the absence of drug).

Tissue expression of NMBR, GRPR, BRS3, GRP and NMB in adult chickens

Using qPCR, we examined the mRNA expression of *cNMB*, *cNMBR*, *cGRP*, *cGRPR* and *cBRS3* in adult chicken tissues, including the anterior pituitary, heart, kidneys, liver, lung, muscle, ovary, testes, spleen, pancreas, subcutaneous fat, skin, spinal cord, different brain regions (telencephalon, midbrain, cerebellum, hindbrain and hypothalamus) and various parts of gastrointestinal tract (crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum and colon).

As shown in Fig. 7, *cNMBR* is highly expressed in the testes and weakly expressed in the duodenum, colon, kidneys, spinal cord and various brain regions including the hypothalamus. Like *cNMBR*, *cNMB* is highly expressed

in the testes. In addition, *cNMB* is moderately expressed in the hypothalamus and ovary, and weakly in other tissues examined, except the kidneys, liver, lung and muscle.

cGRPR is widely expressed in nearly all tissues examined except kidneys and fat. It is highly expressed in the ovary, many brain regions (hypothalamus, midbrain and hindbrain), spinal cord, proventriculus and pancreas, and weakly expressed in other tissues examined.

Strikingly, unlike *cGRPR*, *cGRP* is predominantly and abundantly expressed in the anterior pituitary and weakly expressed in most of the tissues examined. The mRNA level of *GRP* in the anterior pituitary is, at least, a 100-fold higher than that in other tissues examined including the proventriculus. Within the CNS, a relatively high mRNA level of *cGRP* was detected in the spinal

Figure 7

Quantitative real-time PCR assay of *cNMB*, *cNMBR*, *cGRP*, *cGRPR* and *cBRS3* mRNA expression in adult chicken tissues, including the telencephalon (Tc), midbrain (Mb), cerebellum (Cb), hindbrain (Hb), hypothalamus (Hp), spinal cord (Sc), anterior pituitary (Pi), heart (He), crop (Cp), proventriculus (Pr), gizzards (Gi), duodenum (Du), Jejunum (Je), ileum (IL), cecum (Ce), colon (Co), kidneys (Ki), liver (Li), lung (Lu), muscle (Mu), ovary (Ov), testes (Te), spleen (Sp), pancreas (Pa), subcutaneous fat (Fat) and skin (Sk). The mRNA levels of each gene were normalized to that of β -actin and expressed as the fold difference compared with that of hypothalamus (Hp). 'nd' indicates that mRNA of target gene is undetectable. Each data point represents the mean $\pm$ S.E.M. of 6 adult chickens ($N=6$) (3 males and 3 females).

cord, telencephalon and hypothalamus, whereas *cGRP* is weakly expressed in other brain regions (Fig. 7). Within the GI tract, a comparatively high mRNA level of *cGRP* was detected in the proventriculus, whereas only a very low mRNA level was detected in other parts of the GI tract (Fig. 7). *cGRP* mRNA is also weakly expressed in the ovary, testes and spleen, while *cGRP* mRNA is undetectable in other remaining tissues.

Unlike *cGRPR*, *cBRS3* mRNA expression is restricted to several tissues including the testes, subcutaneous fat,

skin and brain. Within the CNS, *cBRS3* is highly expressed in the telencephalon and weakly in other brain regions including the hypothalamus, while it is undetectable in the spinal cord (Fig. 7).

Characterization of GRP expression in chicken anterior pituitary

cGRP is expressed in cephalic and caudal lobe of anterior pituitary The extremely abundant mRNA

expression of cGRP in chicken pituitaries led us to further examine whether cGRP protein is highly expressed in the pituitary by Western blot. As shown in Fig. 8, a strong protein band corresponding to cGRP precursor (~13 kDa) was detected in the anterior pituitary. In contrast, only a faint band was detected in the proventriculus, and no visible band detected in the whole brain.

To probe the spatial distribution of cGRP within the anterior pituitary, qPCR, Western blot and immunohistochemical staining (IHC) were conducted. qPCR and Western blot revealed that cGRP mRNA and protein are abundantly expressed in caudal and cephalic lobes of chicken pituitaries. As a control, a protein band of 23 kDa corresponding to cGH was only detected in the caudal lobe (Fig. 8). In accordance with this finding, IHC staining revealed that

cGRP-immunoreactive (cGRP-ir) cells were densely distributed in both lobes (Fig. 8).

cGRP is expressed mainly in LH-cells It is documented that the chicken anterior pituitary contains multiple hormone-producing cells, including somatotrophs (GH-cells) (Harvey *et al.* 1979), lactotrophs (PRL-cells) (Ohkubo *et al.* 2000, Liang *et al.* 2006), corticotrophs (ACTH-cells) and gonadotrophs (e.g. LH-cells) (Proudman *et al.* 1999, Scanes 2015). To determine whether cGRP is produced by these hormone-producing cells, double immunofluorescence staining was performed in cultured dispersed pituitary cells. As shown in Fig. 9, approximately 11.7% of cultured pituitary cells are GRP-ir cells, and the majority of cGRP-ir cells (~70.6%) are LH-cells. Moreover, we also noted that most of the

Figure 8

(A) Amplification plot of *cGRP* and β -actin using the same amount of adult chicken pituitary cDNA template; (B) qPCR assay of *cGRP* mRNA levels in the cephalic (Ce) and caudal (Ca) lobes of adult chicken anterior pituitaries. Each data point represents means $\pm$ S.E.M. of five female individuals ($N=5$); (C) the cGRP band (13 kDa) was detected in pituitary lysate incubated with anti-GRP, whereas cGRP band was not detected in the pituitary lysate incubated with anti-GRP pre-absorbed by 100 ng/mL cGRP₂₇, validating the specificity of antibody in recognizing GRP expressed in chicken pituitary. (D) Western blot detection of cGRP band (13 kDa) in the whole brain (Br), anterior pituitary (Pi) and proventriculus (Pr) tissue lysates; (E) Western blot detection of cGRP (13 kDa) and cGH (23 kDa) in the cephalic (Ce) and caudal (Ca) lobes of adult chicken anterior pituitaries. (F, G and H) Immuno-histochemical staining of GRP protein in the anterior pituitary of adult female chickens. Histological examination was performed under bright-field illumination with a magnification at 40 $\times$ (F) and 400 $\times$ (H). GRP-ir cells were densely distributed in both cephalic (Ce) and cephalic (Ca) lobes of anterior pituitaries (F, H), whereas no staining was observed in pituitary section incubated with anti-GRP pre-absorbed by cGRP₂₇ (G).

Figure 9

(A) Double immunofluorescence staining showing that cGRP signal (green fluorescence) is mainly localized in LH-cells (top panel, marked by white arrowheads), but not in ACTH-, GH- or PRL-cells in cultured pituitary cells of adult female chickens. (B) Graph shows the relative proportion (%) of GRP-ir cells (~11.7%), LH-cells (~9.1%), ACTH-cells (~8.6%), GH-cells (~6.2%) and PRL-cells (~21.2%) in disperse pituitary cells from adult female chickens. (C) Graph shows that the majority (~70.6%) of GRP-ir cells are LH-cells, but not other hormone-producing cells (including ACTH-, GH- and PRL-cells). 'nd' indicates no co-localization with GRP-cells. (D) Graph shows that most of LH-cells (~87.1%) are GRP-positive. Each data point in graphs (B), (C) and (D) represents mean $\pm$ S.E.M. of 5 replicates (N=5).

LH-cells (~87.1%) are cGRP-positive. In contrast, cGRP signal was not detected in other hormone-producing cells, including GH-cells, PRL-cells and ACTH-cells. These findings clearly indicate that cGRP is mainly expressed in LH-cells.

Discussion

In this study, we characterized cNMBR, cBRS3, cGRPR, cGRP and cNMB in chickens. Functional assay demonstrated that cNMBR and cGRPR can be preferentially activated by cNMB and cGRP, respectively, while chicken (/spotted gar) BRS3 can be activated by both cGRP and cNMB effectively, indicating that GRP and NMB are the native ligand for BRS3 in chickens (/spotted gars). qPCR assay revealed that *cGRPR* is widely expressed in chicken tissues, while *cBRS3*, *cNMBR* and *cNMB* are mainly expressed in the brain and testes. Furthermore, we found that cGRP is predominantly expressed in the anterior pituitary. To our knowledge, our study represents the first to present a new concept that cGRP is likely a novel pituitary hormone in birds. Meanwhile, the discovery of the endogenous ligands of BRS3 in chickens and spotted gars will facilitate our better understanding of the mysterious roles of BRS3 play in vertebrates.

cNMBR and cGRPR are functional receptors specific to cNMB and cGRP respectively

In this study, an *NMBR* orthologous to human *NMBR* was cloned in chickens. cNMBR shows a high degree of amino acid sequence identity (72–74%) with NMBR of humans and mice. Functional assays proved that cNMBR can be preferentially activated by cNMB. This finding is consistent with those observations in mammals, in which NMBR can bind to NMB with higher affinity than to GRP and be preferentially activated by NMB (Wada *et al.* 1991, Benya *et al.* 1995). Our findings indicate that, as in mammals (Jensen *et al.* 2008), cNMBR is a cNMB-specific receptor (Fig. 10).

In parallel, we found that both cGRP₂₇ and cGRP₁₀ are 100-fold more potent than cNMB₁₀ in activating cGRPR expressed in CHO cells. This finding coincides with a previous study, in which cGRPR expressed in CHO-K1 cells can bind to cGRP₂₇ with a 1000-fold higher affinity than to porcine NMB₁₀ and its activation causes the elevation of intracellular calcium concentration upon 10-nM GRP₂₇ treatment (Iwabuchi *et al.* 2003). The findings from our present and previous studies indicate that, as in mammals (Benya *et al.* 1995, Jensen *et al.* 2008), cGRPR is a functional receptor specific to cGRP.

Figure 10

(A) The bombesin receptors (NMBR, GRPR and BRS3) and their endogenous ligands identified in chickens and mammals. In chickens, GRPR and NMBR function as the receptors specific to GRP and NMB, respectively, whereas BRS3 can be potentially activated by GRP and NMB and thus act as a receptor common for both peptides. In mammals, GRPR and NMBR function as the receptors specific to GRP and NMB, respectively, while BRS3 cannot be potentially activated by GRP/NMB *in vitro* ($EC_{50} \gg 1000$ nM) (Jensen *et al.* 2008). Interestingly, chicken GRPR, NMBR and BRS3 are likely coupled to Gq and Gs proteins, and their activation triggers calcium mobilization, MAPK/ERK and cAMP/PKA signaling pathways. In contrast, mammalian GRPR, NMBR and BRS3 are coupled to Gq protein, and their activation elevates calcium concentration and stimulates MAPK/ERK signaling pathway (Jensen *et al.* 2008). (B) The proposed actions of pituitary GRP (a novel pituitary hormone) on chicken peripheral tissues. Pituitary GRP, together with GRP from other tissues (e.g. proventriculus), may contribute to the circulating GRP pool (highlighted in blue) and thus play important roles in the proventriculus, pancreas, ovary and other peripheral tissues in an endocrine (/paracrine/autocrine) manner. The localization of cGRP in pituitary LH-cells (Fig. 9), together with abundant cGRPR expression in the ovary, strongly suggests that GRP–GRPR (marked by bold arrows) may play a critical role in the pituitary–ovary axis of chickens.

It is reported that in mammals, both NMBR and GRPR are coupled to Gq protein, and their activation can lead to IP₃ accumulation and DAG production, which subsequently triggers multiple downstream signaling pathways, such as calcium mobilization and ERK signaling pathway (Fig. 10) (Benya *et al.* 1995, Lach *et al.* 1995, Jensen *et al.* 2008). As shown in our study, both cGRPR and cNMBR are also likely coupled to Gq protein, since their activation also increases intracellular calcium concentration and activates ERK signaling cascade, monitored by the cell-based reporter assays. Moreover, we noted that their activation of MAPK/ERK pathway is likely controlled by Gq-PLC. Interestingly, apart from calcium mobilization and MAPK/ERK signaling pathways, AC/cAMP/PKA signaling pathway is also likely triggered by cGRPR/cNMBR activation, suggesting that both receptors may also be coupled to Gs protein (Fig. 10). Since activation of AC/cAMP/PKA pathway requires higher concentrations of cGRP/cNMB than the other pathways, it implies that Gs-AC/cAMP/PKA pathway may represent a secondary signaling pathway for cGRPR/cNMBR. Our finding contrasts with the mammalian study, in which GRPR- or NMBR-transfected cells fail to increase cAMP levels upon ligand stimulation (Benya *et al.* 1995).

GRP and NMB are the endogenous ligands for chicken/spotted gar BRS3

There is growing evidence that BRS3 plays critical roles in the regulation of food intake, body temperature, metabolic rates, obesity, body weight and pancreatic insulin secretion in mice (Ohki-Hamazaki *et al.* 1997b, Guan *et al.* 2010, Metzger *et al.* 2010), and thus, BRS3 has been viewed as a promising therapeutic target to treat obesity and diabetes in humans (Guan *et al.* 2010, Majumdar & Weber 2012, Gonzalez *et al.* 2015). However, unlike NMBR/GRPR, human or mouse BRS3 cannot be effectively activated by GRP/NMB (EC₅₀ >> 1000 nM), or by any other naturally occurring bombesin-related peptides (Mantey *et al.* 1997, Ryan *et al.* 1998). Hence, the native ligand(s) of BRS3 has remained an open question, which has hindered our understanding of the physiological/pathological roles of BRS3 signaling (Majumdar & Weber 2012, Gonzalez *et al.* 2015).

In this study, we found that unlike mouse BRS3, chicken BRS3 can be activated effectively by cGRP and cNMB, with high potencies comparable to those of cGRP/cNMB in activating their specific receptor (cGRPR/cNMBR) (Table 1). This finding clearly indicates that both

cGRP and cNMB are native ligands for chicken BRS3. Furthermore, we proved that chicken BRS3 activation can trigger calcium mobilization, as demonstrated in mammals (Ryan *et al.* 1998). Like cGRPR/cNMBR, cBRS3 activation also stimulates MAPK/ERK and AC/cAMP/PKA/CREB signaling pathways. As in chickens, BRS3 can be effectively activated by GRP and NMB in spotted gars. This finding suggests that GRP and NMB can also act as the endogenous ligands for BRS3 in other lower vertebrate species.

The discovery of native ligands of BRS3 in chickens and spotted gars strongly suggests that GRP and NMB are likely to be the putative native ligand(s) for mammalian BRS3 from an evolutionary perspective. The little or no responsiveness of mammalian BRS3 to GRP/NMB treatment observed in our present and previous studies is likely due to the dramatic structural change of BRS3 during evolution, which may result in the loss of its high affinity to GRP/NMB *in vitro* (Gorbulev *et al.* 1992, Mantey *et al.* 1997, Ryan *et al.* 1998). Despite the extremely low affinity of mammalian BRS3 to GRP/NMB *in vitro*, we cannot exclude the possibility that other unidentified accessory protein(s) may be required to aid the effective binding of mammalian BRS3 to GRP/NMB *in vivo*. Recently, Liu *et al.* reported that GRPR can heterodimerize with μ -opioid receptor (MOR1D) in mouse spinal cord and mediate the opioid (morphine)-induced scratching behavior (Liu *et al.* 2011). This finding points out another possibility that like GRPR, BRS3 may heterodimerize with other GPCR(s) and mediate actions of the cognate ligands of its combined receptor.

It is reported that a novel bombesin receptor, named BRS3.5, exists in chickens (Iwabuchi *et al.* 2003). However, our synteny analysis clearly indicates that chicken BRS3.5 is orthologous to mouse BRS3. Moreover, we demonstrated that chicken BRS3 can be activated by cGRP and cNMB potently. Our finding contrasts with a previous study, in which cBRS3.5 (cBRS3) expressed in CHO-K1 cells binds cGRP (K_i =318 nM) or porcine NMB (K_i =678 nM) with an extremely low affinity (Iwabuchi *et al.* 2003). The discrepancy between the two studies is unclear. However, like chicken BRS3, spotted gar BRS3 can be potently activated by GRP and NMB, substantiating our hypothesis that GRP and NMB are the native ligands of BRS3 in non-mammalian vertebrates (Fig. 10).

In *Bombina orientalis*, the fourth bombesin receptor subtype (BB4) was identified in 1995 (Nagalla *et al.* 1995). Pharmacological study showed that BB4 can bind to frog [Phe¹³]-bombesin with an affinity (K_i =0.2 nM) higher

than to human GRP ($K_i=2.1$ nM) and NMB ($K_i=30$ nM). In addition, [Phe¹³]-bombesin treatment causes calcium mobilization in *Xenopus* oocytes transiently expressing BB4 (Nagalla *et al.* 1995). Interestingly, we noted that *Bombina* BB4 share a considerable amino acid sequence identical to chicken/*Xenopus tropicalis* BRS3 (77%/84%) (Supplementary Fig. 4). Moreover, *Bombina* BB4 is expressed exclusively in the forebrain and cortex (Nagalla *et al.* 1995), a tissue expression pattern nearly identical to chicken BRS3 (Fig. 7). The remarkable similarity in structure and tissue expression shared between *Bombina* BB4 and chicken BRS3 led us to hypothesize that *Bombina* BB4 is orthologous to chicken BRS3, or a BRS3-like receptor duplicated from BRS3 very recently, even though *Bombina* BB4 has evolved an unusual high affinity to frog [Phe¹³]-bombesin peptide (Nagalla *et al.* 1995), which only exists in some amphibian species (Nagalla *et al.* 1996).

Tissue expression of BRS3 in chickens

In this study, *cBRS3* is widely expressed in various brain regions with the highest expression noted in the telencephalon. Our finding is consistent with previous reports, in which *cBRS3.5* is highly expressed in the telencephalon by RT-PCR or *in situ* hybridization (Iwabuchi *et al.* 2003, Ohki-Hamazaki *et al.* 2005). These findings, together with the expression of *cGRP/cNMB* detected in these regions (Fig. 7), strongly suggest that *cBRS3* can mediate the actions of *cGRP/cNMB* in chicken CNS. Apart from the CNS, *cBRS3* and *cNMB/cGRP* are also expressed in the testes, adipose tissue and skin, suggesting a role of *cBRS3* in mediating *cGRP/cNMB* actions in these tissues.

It is of particular interest to note that the tissue distribution of *cBRS3* is similar, but non-identical, to that in mammals (Ohki-Hamazaki *et al.* 2005). For instance, BRS3 is expressed in the brain and testes of chickens (Fig. 7), humans (Sano *et al.* 2004) and rodents (Fathi *et al.* 1993, Ohki-Hamazaki *et al.* 1997a); however, within the CNS, an obvious difference in BRS3 distribution has been observed between chickens and mammals. BRS3 is mainly expressed in the hypothalamus of humans and rodents (Ohki-Hamazaki *et al.* 1997a, 2005, Sano *et al.* 2004), whereas BRS3 is highly expressed in chicken telencephalon, but not the hypothalamus (Fig. 7) (Ohki-Hamazaki *et al.* 2005). This remarkable difference hints that hypothalamic BRS3 signaling plays a critical role in mammals such as the control of body weight and metabolic rate (Ohki-Hamazaki *et al.* 1997b), while in chicken hypothalamus,

BRS3 signaling is less important and may be functionally replaced by its family member(s), such as *cGRPR*, which is highly expressed in the hypothalamus (Fig. 7).

Tissue expression of NMB and NMBR in chickens

In this study, *cNMB* and *cNMBR* were detected to be widely expressed in the CNS including the hypothalamus (Fig. 7). This finding is consistent with those reports in humans (Sano *et al.* 2004), pigs (Ma *et al.* 2016) and rats (Wada *et al.* 1992), suggesting that *cNMB* may act as a neuropeptide to regulate some physiological processes, such as food intake, as demonstrated in chickens and mammals (Jensen *et al.* 2008, Tachibana *et al.* 2010a,b).

Among the peripheral tissues, the highest mRNA levels of *cNMB* and *cNMBR* were noted in the testes (Fig. 7). As in chickens, both NMB and NMBR are highly expressed in the testes of humans (Sano *et al.* 2004), pigs (Ma *et al.* 2016) and mice (Ohki-Hamazaki *et al.* 1997a). These findings strongly suggest that NMB may act as an autocrine/paracrine factor to regulate the functions of testes in vertebrates. In addition, we noted that *cNMB* is expressed in the ovary and other peripheral tissues. However, the low or no expression of *cNMBR* in these tissues questions an active role of *cNMB* in these tissues.

In this study, *cNMBR* mRNA is undetectable in the anterior pituitary. This finding contrasts with the observation in rodents. In rats, both NMB and NMBR are expressed in the anterior pituitary, and NMB can act as an autocrine/paracrine factor to inhibit thyroid-stimulating hormone (TSH) release (Rettori *et al.* 1989, Jones *et al.* 1992). In mice, disruption of NMBR results in dysregulation of the pituitary–thyroidal axis (Oliveira *et al.* 2006). The absence of NMBR expression in chicken anterior pituitary hints that unlike mammalian NMB (Rettori *et al.* 1989), *cNMB* may not regulate pituitary functions, at least not directly.

Tissue expression of *cGRP* and *cGRPR*: evidence for *cGRP* being a novel potential pituitary hormone

In this study, we found that *cGRP* mRNA is widely expressed in the spinal cord and various brain regions (Fig. 7). Similarly, *cGRPR* is highly or moderately expressed in the spinal cord and nearly all brain regions examined including the hypothalamus. Our findings partially coincide with an early study in chickens, in which *GRPR* mRNA was reported to be widely expressed in all brain regions detected by RT-PCR (Iwabuchi *et al.* 2003).

The wide expression of *cGRP* and *cGRPR* also suggests a broad spectrum of GRP actions in chicken CNS, such as inhibition of food intake in the hypothalamus (Tachibana *et al.* 2010a,b), and itch sensation in the spinal cord, as demonstrated in mammals (Sun & Chen 2007, Sun *et al.* 2009).

Outside the CNS, *cGRPR* is highly expressed in the proventriculus, pancreas and ovary, and weakly in other tissues examined. This finding supports the previous observations in birds, in which GRP or bombesin (an agonist of GRPR) has been shown to stimulate proventriculus acid secretion and pancreatic protein secretion in chickens or turkeys (Linari & Linari 1975, Campbell *et al.* 1991, 1994). Unlike *cGRPR*, *cGRP* expression is restricted to several peripheral tissues including the GI tract. *cGRP* mRNA is moderately expressed in the proventriculus, but weakly expressed in other parts of GI tract (Fig. 7). Our data coincide with previous reports, in which GRP was reported to be mainly expressed in the endocrine cells of proventriculus (Vaillant *et al.* 1979, Buffa *et al.* 1982, Yamanaka *et al.* 1989, Scanes & Pierzchala-Koziec 2014). These findings suggest that *cGRP* may be secreted into the circulation by these endocrine cells under certain stimuli and thus exert its endocrine/paracrine/autocrine actions on chicken tissues (Fig. 10). Besides proventriculus, both *cGRP* and *cGRPR* were detected in the ovary and testes, also supporting an autocrine/paracrine action of GRP on chicken gonads.

The most striking observation of our present study is that *cGRP* mRNA is abundantly and predominantly expressed in chicken anterior pituitaries. In agreement with this finding, a strong *cGRP* protein band was detected in anterior pituitaries (Fig. 8). Moreover, qPCR assay showed that the mRNA level of *cGRP* in the pituitary seems to be even higher than that of β -actin, as estimated by their amplification plots shown in Fig. 8, in which Ct value of *cGRP* is much lower than that of β -actin when the same amount of cDNA template was used. These findings not only suggest that the anterior pituitary may be a major source of *cGRP* in chickens, but also raise the new hypothesis that *cGRP* is likely a novel pituitary hormone. Our finding contrasts with those in mammals and frogs, in which GRP is mainly produced in the brain and/or gut (Wada *et al.* 1990, Nagalla *et al.* 1992), and weakly expressed in the anterior pituitary (Houben *et al.* 1993).

Using qPCR, IHC and Western blot, we proved that both *cGRP* mRNA and protein are abundantly expressed in both cephalic and caudal lobes. In accordance with

this finding, GRP-ir cells were also shown to be densely distributed in both lobes (Fig. 8), a spatial distribution similar to that of gonadotrophs (e.g. LH-cells), which is distributed throughout both lobes (Proudman *et al.* 1999, Scanes 2015). Double fluorescence staining further elucidated that GRP-ir cells account for 11.7% of total pituitary cells, and GRP is mainly expressed in LH-cells (Fig. 9). Moreover, our preliminary study demonstrated that gonadotropin-releasing hormone 1 (GnRH1) can stimulate GRP expression and secretion in cultured chick pituitary cells (data not shown). All these findings support the notion that *cGRP* is a novel pituitary hormone, which may be released and contribute to a large portion of the circulating *cGRP* pool (Fig. 10). Considering that *cGRPR* is highly expressed in the ovary, proventriculus and pancreas, it is likely that pituitary GRP, together with GRP from other tissues (e.g. proventriculus), may act in a coordinated fashion to regulate reproduction, GI tract activity (Campbell *et al.* 1994) and pancreatic functions (Campbell *et al.* 1991). Meanwhile, the abundant expression of GRP-GRPR in the pituitary-ovary axis also hints that GRP may be a novel 'gonadotrophic factor' in chickens. Our future study on GRP-GRPR actions in chicken ovary, a dynamic organ showing spectacular granulosa cell proliferation, differentiation and apoptosis, and an extremely high incidence (30–35% at 3.5 years of age) of ovarian cancer (Wang *et al.* 2007b, Johnson & Giles 2013, Johnson & Lee 2016), will aid to define the novel facet of roles played by bombesin-like peptides in ovarian physiology/pathology, a topic which has long been neglected in vertebrates.

In summary, chicken GRP, NMB, GRPR, NMBR and BRS3 were characterized in this study. Functional assays revealed that *cGRPR* and *cNMBR* can function as the receptors specific to *cGRP* and *cNMB*, respectively, while *cBRS3* is a functional receptor common for both peptides. Like *cBRS3*, spotted gar BRS3 can also act as a receptor for GRP and NMB. Moreover, we demonstrated that *cGRP* is predominantly expressed in the anterior pituitary. Collectively, our study not only presents a new concept that GRP is likely a novel pituitary hormone in chickens, but also identifies GRP and NMB as the native ligands of BRS3 in two representative vertebrate species, an issue that has puzzled endocrinologists for more than two decades. The discovery of the native ligands for BRS3 in non-mammalian species also promotes us to re-think the physiological/pathological roles of BRS3 in mammals, including humans.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-17-0020>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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