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# Endothelins (EDN1, EDN2, EDN3) and their receptors (EDNRA, EDNRB, EDNRB2) in chickens: Functional analysis and tissue distribution

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ABSTRACT

Endothelins (EDNs) and their receptors (EDNRs) are reported to be involved in the regulation of many physiological/pathological processes, such as cardiovascular development and functions, pulmonary hypertension, neural crest cell proliferation, differentiation and migration, pigmentation, and plumage in chickens. However, the functionality, signaling, and tissue expression of avian EDN-EDNRs have not been fully characterized, thus impeding our comprehensive understanding of their roles in this model vertebrate species. Here, we reported the cDNAs of three *EDN* genes (*EDN1*, *EDN2*, *EDN3*) and examined the functionality and expression of the three EDNs and their receptors (EDNRA, EDNRB and EDNRB2) in chickens. The results showed that: 1) chicken (c-) *EDN1*, *EDN2*, and *EDN3* cDNAs were predicted to encode bioactive EDN peptides of 21 amino acids, which show remarkable degree of amino acid sequence identities (91–95%) to their respective mammalian orthologs; 2) chicken (c-) EDNRA expressed in HEK293 cells could be preferentially activated by chicken EDN1 and EDN2, monitored by the three cell-based luciferase reporter assays, indicating that cEDNRA is a functional receptor common for both cEDN1 and cEDN2. In contrast, both cEDNRB and cEDNRB2 could be activated by all three EDN peptides with similar potencies, indicating that both receptors can function as common receptors for the three EDNs and share functional similarity. Moreover, activation of three EDNRs could stimulate intracellular calcium, MAPK/ERK, and cAMP/PKA signaling pathways. 3) qPCR assay revealed that *cEDNs* and *cEDNRs* are widely, but differentially, expressed in adult chicken tissues. Taken together, our data establishes a clear molecular basis to uncover the physiological/pathological roles of EDN-EDNR system in birds and helps to reveal the conserved actions of EDN-EDNR signaling across vertebrates.

## 1. Introduction

Endothelin (EDN; alias: ET) was initially isolated from cultured porcine aortic endothelial cells and identified as the most potent vasoconstrictor of coronary artery strips (Yanagisawa et al., 1988). Subsequently, three endothelin peptides with vasoconstrictive activity, named endothelin-1 (EDN1; ET-1), endothelin-2 (EDN2; ET-2) and endothelin-3 (EDN3; ET-3), have been identified in humans and other mammalian species (Inoue et al., 1989). The three EDNs are bioactive peptides of 21 amino acids and characterized by the presence of a single α-helix and 2 intrachain disulfide bonds at $Cys^3$-$Cys^{11}$ and $Cys^1$-$Cys^{15}$, which are encoded by separate genes (Davenport et al., 2016). Each EDN peptide is derived from a large precursor (prepro-EDN) of ~200 amino acids. This large precursor is first processed by furin endopeptidase to yield an intermediate product of ~38 amino acids (known as Big-EDN, which is biologically inactive), which is in turn cleaved by endothelin converting enzymes (ECEs) to give bioactive EDN peptide (Davenport et al., 2016).

In mammals, EDN biological actions are reported to be mediated by two endothelin receptors (EDNR), EDNRA and EDNRB. Both receptors belong to G protein-coupled receptor (GPCR) superfamily and share ~60% amino acid identity with each other (Davenport et al., 2016). EDNRA binds EDN1 and EDN2 with > 100-fold higher affinity than EDN3 (Arai et al., 1990), whereas EDNRB binds EDN1, EDN2 and EDN3 with equally high affinities (Sakurai et al., 1990). It is believed that both EDNRA and EDNRB are coupled to Gq (and Gs/Gi) proteins, thus their activation can trigger multiple downstream signaling pathways, including calcium mobilization, MAPK/ERK, and cAMP/protein-kinase A (cAMP/PKA) pathways (Aquilla et al., 1996; Aramori and Nakanishi, 1992; Davenport et al., 2016).

EDN-EDNR system is widely expressed in mammalian vascular and non-vascular tissues and involved in the regulation of many physiological processes, such as cardiovascular development and function, craniofacial development, blood pressure regulation, renal water and

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sodium excretion, neurotransmission, ovulation, and proliferation, migration and differentiation of cranial/cardiac/trunk/sacral neural crest cells (NCCs) (Bondurand et al., 2018; Clouthier et al., 1998; Davenport et al., 2016; Kohan et al., 2011). For instance, in blood vessels, EDN1 is mainly expressed in endothelial cells, where it can not only cause vasoconstriction via EDNRA expressed on vascular smooth muscle cells, but also limit the constriction response via EDNRB expressed on the endothelial cells to release vasodilators, such as nitric oxide (NO). In the ovary, EDN2 is mainly expressed in granulosa cells and may induce ovulation which is mediated by EDNRA/B (Ko et al., 2006; Palanisamy et al., 2006). During embryogenesis, EDN3 is reported to be expressed in the surface ectoderm and gut mesenchyme and may induce the proliferation, migration and migratory pathway-finding (dorso-ventral or dorso-lateral pathway), and differentiation of trunk NCCs and their derivatives (*e.g.* melanoblasts), thus may represent one of the key signals for pigmentation and ganglionosis (Baynash et al., 1994; Bondurand et al., 2018). Dysfunction of EDN-EDNR system is associated with various diseases, such as pulmonary arterial hypertension, heart failure, chronic kidney disease, abnormal pigmentation, growth retardation, aganglionic megacolon, and growth and progression of many cancers such as prostate, ovarian, colon, breast, bladder, cervical, hepatocellular and lung cancers (Bondurand et al., 2018; Chang et al., 2013; Clouthier et al., 1998; Hosoda et al., 1994; Kurihara et al., 1994; Puffenberger et al., 1994; Rosano et al., 2013).

As in mammals, *EDN* and *EDNR* genes have also been predicted or identified in birds and other non-mammalian vertebrate species (Watanabe et al., 1989; Braasch and Schartl, 2014). In birds, there are lines of evidence to suggest that EDN-EDNR signaling plays critical roles in many physiological/pathological processes, such as cardiovascular development (Gourdie et al., 1998; Groenendijk et al., 2008; Kanzawa et al., 2002), angiogenesis (Cruz et al., 2001), pulmonary arterial hypertension (Gomez et al., 2008; Gomez et al., 2007), morphogenesis of the face and heart (Kempf et al., 1998; Nataf et al., 1998), trunk NCCs' proliferation, migration and their final differentiation into the enteric nervous system (Nagy and Goldstein, 2006), or melanocytes (Harris et al., 2008; Lahav et al., 1996; Pla and Larue, 2003), which are likely controlled by EDN3-EDNRB2 signaling essential for pigmentation and plumage patterning in quails (Lecoin et al., 1998; Miwa et al., 2007; Miwa et al., 2006), ducks (Li et al., 2015; Wu et al., 2017), and chickens (Dorshorst et al., 2011; Han et al., 2014; Kinoshita et al., 2014; Shinomiya et al., 2012). In zebrafish and *Xenopus*, EDN-EDNR signaling has also been reported to play important roles in pigmentation and cranial NCC migration and development (Kawasaki-Nishihara et al., 2011; Nair et al., 2007; Spiewak et al., 2018). All the findings highlight the ancient and conserved roles of EDN-EDNR system across vertebrates.

Despite the fact that the three *EDN* (*EDN1*, *EDN2*, *EDN3*) and three EDN receptor (*EDNRA*, *EDNRB,* and *EDNRB2*) genes including a novel *EDNRB2* (lost in mammals) (Braasch and Schartl, 2014; Kempf et al., 1998; Lecoin et al., 1998) have been predicted or identified in birds (Braasch and Schartl, 2014), the information regarding the functionality and signaling property of avian EDN-EDNR is limited (Kempf et al., 1998; Lecoin et al., 1998), which has hindered our comprehensive understanding of the conserved roles of END-EDNR system in birds. Therefore, using chicken as the animal model, our present study aims to: 1) clone *EDN1*, *EDN2*, and *EDN3* genes, which have not been cloned in chickens; 2) investigate on the functional similarity and difference of chicken EDNs/EDNRs *in vitro*; 3) characterize the tissue expression pattern of *EDN-EDNR*s. Our data will establish a molecular basis to decipher the physiological roles of EDN-EDNR system in birds and help to reveal the conserved actions of EDN-EDNR signaling across vertebrates.

## 2. Materials and methods

### 2.1. Chemicals, primers, peptides and antibodies

All primers used in this study were synthesized by Tsingke (Beijing, China) and listed in Supplemental Table 1. Chicken EDN1, EDN2 and EDN3 were synthesized using solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). The purity of synthesized peptides is higher than 95% (analyzed by HPLC) and their structures were verified by mass spectrometry. Antibodies for β-actin, phosphorylated CREB and ERK1/2 were purchased from Cell Signaling Technology, Inc. (CST, Bevely, MA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and restriction enzymes were obtained from Takara Biotechnology Co. Ltd. (Dalian, China).

### 2.2. Tissue preparation and total RNA extraction

Adult chickens (1-year-old) of Lohmann Layer strain were purchased from local commercial companies. Six adult chickens (3 males and 3 females) were decapitated and various tissues (including the telencephalon, midbrain, cerebellum, hindbrain, hypothalamus, spinal cord, anterior pituitary, heart, crop, proventriculus, gizzards, duodenum, jejunum, ileum, cecum, colon, kidneys, liver, lung, muscle, ovary, testes, spleen, pancreas, subcutaneous fat, skin, and adrenal gland) were frozen in liquid nitrogen and stored at −80 °C before use. Total RNA was extracted with RNAzol reagent (Molecular Research Center, Cincinnati, OH) and re-suspended in $H_2O$ treated with diethylpyrocarbonate (DEPC) following the manufacturer's instructions. The animal experiments were performed in accordance with the Guidelines for Experimental Animals issued by the Ministry of Science and Technology of People's Republic of China. The experimental protocol used in this study was approved by the Animal Ethics Committee of College of Life Sciences, Sichuan University (Chengdu, China).

### 2.3. Reverse transcription and quantitative real-time PCR

Two μg of total RNA and 0.5 μg of oligo-deoxythymide were mixed in a total volume of 5 μL, incubated at 70 °C for 10 min, and cooled at 4 °C for 2 min. Then, the first strand buffer, 0.5 mM each deoxynucleotide triphosphate (dNTP), and 100 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Takara) were added into the reaction mix in a total volume of 10 μL. Reverse transcription (RT) was performed at 42 °C for 90 min. RT negative controls were performed without addition of reverse transcriptase into the reaction mix.

According to our previously established method (Cai et al., 2015), quantitative real-time PCR was performed to examine the mRNA levels of target genes. In brief, before quantitative real-time PCR assay, PCR products of target genes or *β-actin* (used as a reference gene) were quantified by a Spectrophotometer (Eppendorf, Hamburg, Germany), and the copy numbers of DNA molecules were calculated. Then, the serially diluted PCR products with known copy numbers were used to set up the standard curves in each quantitative real-time PCR assay. The real-time PCR was conducted on the CFX96 Real-time PCR Detection System (Bio-Rad) in a volume of 20 μL containing 0.5 μL RT product, 1 × PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.2 mM each primer, 0.5 U high-fidelity Taq DNA polymerase (TOYOBO, Japan), and 1 μL EvaGreen (Biotium Inc., Hayward, CA). The PCR profile consisted of 40 cycles of 94 °C for 20 s, 60–65 °C (the annealing temperature for each gene was listed in the Supplemental Table 1) for 15 s, 72 °C for 30 s. To assess the specificity of PCR amplification, melting curve analysis and agarose gel (2%) electrophoresis were performed with the PCR product. In addition, the identity of PCR products for all genes was confirmed by sequencing. Finally, according to the standard curve included in each quantitative real-time PCR assay, the copy numbers of target gene transcripts in RT samples were calculated (note: in each qPCR assay, we also set up a negative control using $H_2O$, instead of RT

sample, to exclude any possible contamination from the environment or reagents involved).

### 2.4. Cloning the cDNAs of chicken endothelin (cEDN1, cEDN2, and cEDN3)

Based on the predicted cDNA sequences of chicken *EDN1* (XM_418943.4) and *EDN3* (XM_015296553.1) deposited in the GenBank or genomic sequence of *cEDN2* (http//www.ensembl.org/Gallus_gallus), gene-specific primers were designed to amplify the complete ORFs of *cEDN1*, *cEDN2*, and *cEDN3* from adult chicken heart (*cEDN1, cEDN2*) or whole brain (*cEDN3*) tissue. The amplified PCR products were cloned into pTA2 vector (TOYOBO, Japan) and sequenced (BGI).

According to the reported DNA sequence of chicken *EDNRA* (NM_204119) (Kempf et al., 1998) and *EDNRB2* or the predicted cDNA sequences of c*EDNRB* (XM_417001.2) deposited in GenBank, gene-specific primers were designed to amplify to the complete ORF of *cEDNRA*, *cEDNRB*, and *cEDNRB2* from chicken heart. The amplified PCR products were then cloned into pcDNA3.1 (+) expression vector (Invitrogen) and sequenced (BGI).

### 2.5. Functional characterization of chicken endothelin receptors (cEDNRA, cEDNRB and cEDNRB2)

According to our previously established methods (Mo et al., 2017; Wang et al., 2012), cEDNRA, cEDNRB or cEDNRB2 was transiently expressed in human embryonic kidney 293 (HEK293) cells [purchased form American Type Culture Collection (ATCC, Manassas, VA, USA)] and treated by synthetic cEDN1, cEDN2 and cEDN3 ($10^{-12}$ to $10^{-6}$ M, 6 h). The receptor-activated signaling pathways were then examined by pGL3-NFAT-RE-luciferase, pGL4-SRE-luciferase and pGL3-CRE-luciferase reporter systems, which were capable of monitoring intracellular calcium, MAPK/ERK, and cAMP/PKA signaling pathways, respectively. In brief, HEK293 cells were cultured in Dulbecco minimal Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (Thermo Fisher Scientific Inc, Waltham, MA), 100 U/mL penicillin G, and 100 μg/mL streptomycin (Life Technologies Inc., Grand Island, NY) in a 90-mm culture dish (Nunc, Rochester, NY) and incubated at 37 °C with 5% $CO_2$. Cells were then plated in a 6-well plate at a density of $3 \times 10^5$ cells per well 18 h before transfection. A mixture containing 1000 ng of pGL3-NFAT-RE-luciferase/pGL4-SRE-lucfierase/pGL3-CRE-luciferase reporter construct, 200 ng of receptor expression plasmid/empty pcDNA3.1 (+) vector, and 2 μL of jetPRIME (Polyplus-transfection SA, Illkirch, France) were prepared in 200 μL of jetPRIME buffer solution. Transfection was performed according to the manufacturer's instruction when cells reached 70% confluence. After 24-h incubation, HEK293 cells were subcultured onto a 96-well plate at 37 °C for 24 h before treatment. After removal of medium from 96-well plate, 100 μL of EDN containing medium or peptide-free medium (used as the control) was added. The cells were incubated for an additional 6 h at 37 °C before being harvested for luciferase assay. After removal of culture medium, HEK293 cells were lysed by adding 50 μL of 1 × Passive Lysis Buffer (Promega) per well, and the luciferase activity of 15 μL cellular lysates was determined with the luciferase assay kit (Promega). The luciferase activities in peptide treatment groups were expressed as the relative fold increase compared with the control group (without peptide treatment).

Using the same luciferase reporter assays and experimental procedures, the inhibitors of signaling pathways, including MDL12330A (20 μM), H89 (10 μM), U73122 (20 μM), PD98059 (100 μM) and 2-APB (100 μM) were added 1 h before EDN treatment (10 nM, 6 h) to test whether cEDNR-activated signaling pathways could be blocked by these inhibitors.

### 2.6. Western blot

As described in our previous studies (Gao et al., 2017; He et al., 2016), HEK293 cells transfected with the expression plasmid of cEDNRA, cEDNRB or cEDNRB2 (150 ng/well) were cultured on a 24-well plate at 37 °C for 24 h and treated by cEDN1 (100 nM) for 10 min. Then, cells were lysed by adding 200 μL RIPA lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.25% Na deoxycholate) containing a final concentration of 1 × the protease/phosphatase inhibitor cocktail (Roche Diagnostics, CA, USA) per well and used for Western blot detection of intracellular β-actin level and phosphorylated ERK1/2 (pERK1/2) (44/42 kDa) and CREB (pCREB) (43 kDa).

### 2.7. Data analysis

The relative mRNA levels of *cEDN* and *cEDNR* were first calculated as the ratios to that of *β-actin* and then expressed as the fold difference compared to the chosen tissue. The luciferase activities of HEK293 cells expressing EDNRs in peptide treatment group were expressed as relative fold increase compared to the control group (without peptide treatment). The data was analyzed by Student *t*-test (between two groups), or by one-way ANOVA followed by the Dunnett's test in GraphPad Prism 5 (GraphPad Software, San Diego CA). To validate our results, three or four replicates were set up for every sample in each experiment, and each experiment was repeated twice or thrice.

## 3. Results

### 3.1. Cloning of chicken EDN1, EDN2, and EDN3

Based on the predicted cDNA sequence or genomic sequence of chicken *EDN1* (*cEDN1,* XM_418943.4), c*EDN2*, *cEDN3* (*cEDN3*, XM_015296553.1), we cloned the cDNAs containing complete open reading frame (ORF) of these genes from adult chicken heart or brain tissue by RT-PCR. The cloned *cEDN1* cDNA is 618 bp in length (Accession no. MK139007) and predicted to encode a prepro-EDN1 of 205 amino acids (a.a.), which shows 33%-52% identity with that of humans (41%), mice (52%), cow (49%), zebrafish (33%), and *Xenopus tropicalis* (42%). The cloned *cEDN2* cDNA is 540 bp in length (Accession no.: MK139008) and predicted to encode a prepro-EDN2 of 179 amino acids, which shows 37–42% amino acid identity with those from other vertebrate species including humans. The cloned *cEDN3* cDNA is 522 bp in length (Accession no.: MK139009) and encodes a prepro-EDN3 of 173 amino acids (a.a.), which shows 33%–52% identity with prepro-EDN-3 from other vertebrate species (Fig. 1).

As in mammals, the predicted bioactive EDN1, EDN2, and EDN3 are 21 amino acids in length in chickens after the removal of their signal peptides at the N-termini and cleavage at the putative proteolytic sites by furin protease (RR/KR) and ECEs (Fig. 1). Sequence alignment revealed that chicken EDN1, EDN2 and EDN3 show remarkable amino acid sequence identity with their respective mammalian counterparts (91%; 91%; 95%). Moreover, we found that cEDN1 shares a relatively high degree of amino acid sequence identity to cEDN2 (81%) than to cEDN3 (67%) (Fig. 2).

### 3.2. Functional analysis of chicken EDN1, EDN2, EDN3, EDNRA, EDNRB, and EDNRB2

To investigate the functional similarity and difference between the three chicken EDNRs (cEDNRA, cEDNRB, cEDNRB2), we first cloned the three receptor genes from heart tissue including the *cEDNRB* cDNA (accession no.: MK359292), which has not been reported in birds previously (Fig. 1). We then tested whether cEDNRA, cEDNRB and cEDNRB2 could be activated by synthetic chicken EDNs (cEDN1, cEDN2, cEDN3) *in vitro* (Fig. 2) using the three cell-based luciferase

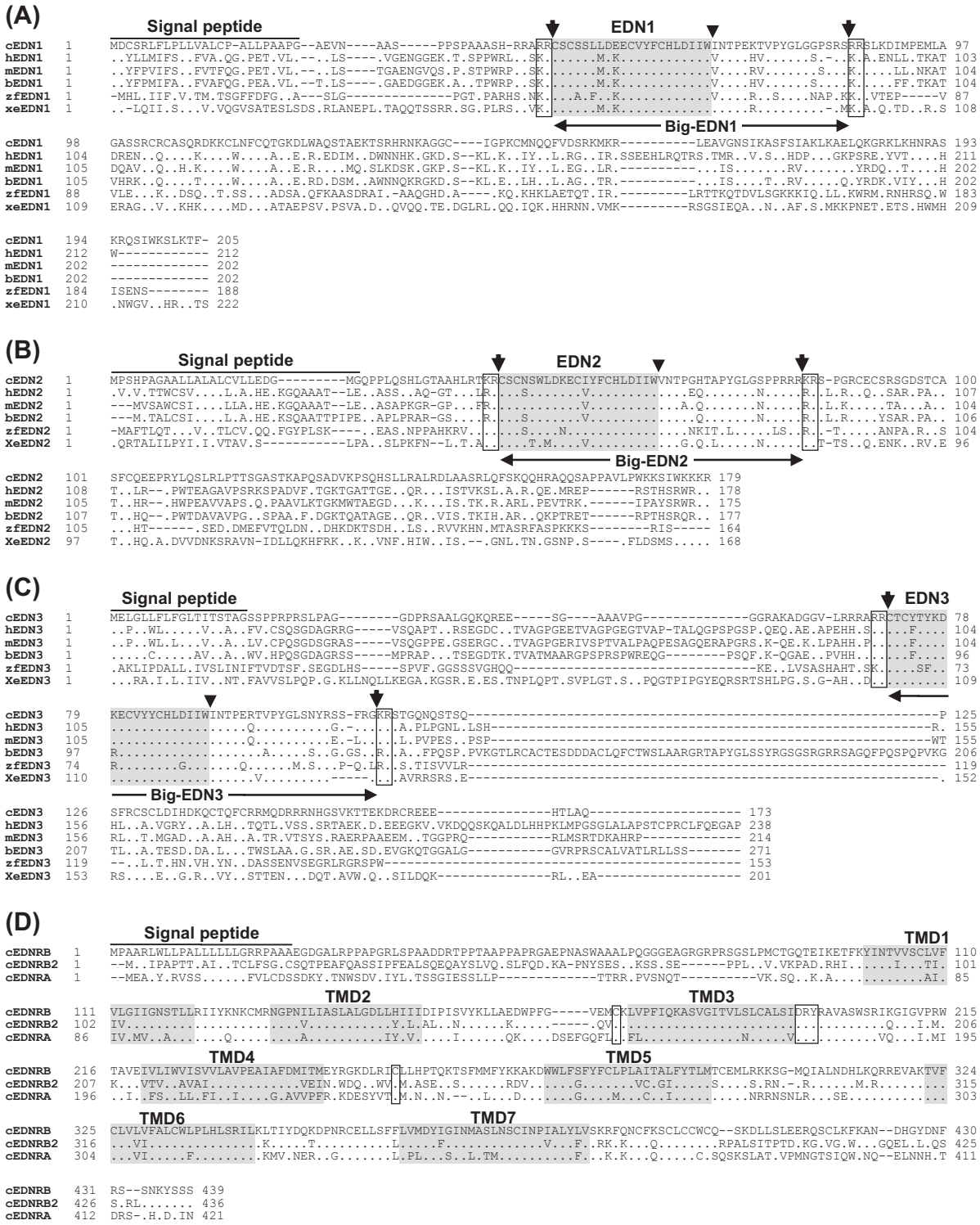


**Fig. 1.** (A-C) Amino acid alignment of chicken (c-)EDN1 (A), cEDN2 (B) and cEDN3 (C) precursors with EDN1, EDN2 and EDN3 precursors from humans (h), mice (m), cows (b), zebrafish (zf) and *Xenopus tropicalis* (Xe). The signal peptide is underlined. The putative mature EDN peptide of 21 amino acid is shaded. Arrows indicate the cleavage sites for furin-like protease, while arrowheads denote the cleavage sites for ECEs. (D) Amino acid alignment of chicken (c-)EDNRB (cEDNRB, MK359292), cEDNRB2 and cEDNRA. cEDNRB shows a relatively high amino acid sequence identity with cEDNRB2 (62%) and a comparatively low identity with cEDNRA (57%). The conserved 'DRY' motif for G protein-coupling and cysteine (C) residues for disulfide bond formation are boxed and the 7 transmembrane domains (TMD1-7) shaded. In (A-D), dots indicate the amino acids identical to cEDNs/cEDNRB, and dashes represent gaps in the sequence.

reporter assays established in our laboratory [pGL3-NFAT-RE-luciferase reporter (Wang et al., 2012), pGL4-SRE-luciferease reporter (Mo et al., 2015), and pGL3-CRE-luciferease reporter assays (Mo et al., 2017; Wang et al., 2012)].

Using pGL3-NFAT-RE-luciferase reporter system, we demonstrated that cEDNRA expressed in HEK293 cells could be potently activated by cEDN1 ($EC_{50}$, 4.5 nM) and cEDN2 ($EC_{50}$, 3.5 nM) and strongly increase luciferase activities of HEK293 cells in a dose-dependent manner. In

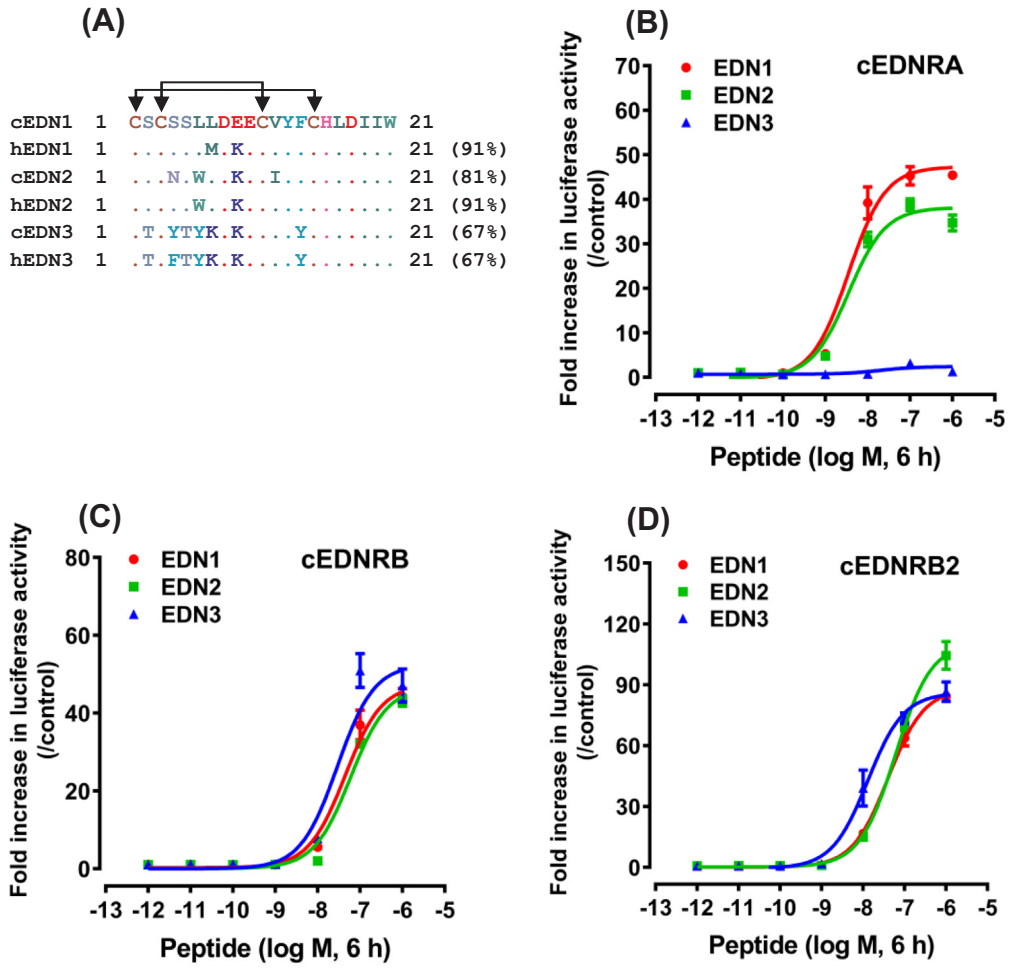


**Fig. 2.** (A) Amino acid sequence alignment of chicken (c-)EDN1, cEDN2 and cEDN3 with human (h-)EDN1, hEDN2 and hEDN3. Dots indicate identical amino acid residues. Lines linking cysteines indicate the two conserved disulfide bonds. The numbers in the brackets indicate the identity shared between cEDN1 and cEDN2/3 (hEDN1/2/3). (B-D) Effects of cEDN1, cEDN2, and cEDN3 on activating chicken (c-)EDNRA (B), cEDNRB (C), and cEDNRB2 (D) expressed in HEK293 cells, monitored by pGL3-NFAT-RE-luciferase reporter system; HEK293 cells co-transfected with 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 HEK293 cells at any concentration tested (*Data not shown*). Each data point represents mean ± SEM of three replicates ($N = 3$).

**Table 1**
$EC_{50}$ values of cEDN1, cEDN2 and cEDN3 in activating different signaling pathways of HEK293 cells expressing cEDNRA, cEDNRB and cEDNRB2.

| Peptide | $EC_{50}$ values (nM) | | |
|---|---|---|---|
| | cEDNRA | cEDNRB | cEDNRB2 |
| | Calcium signaling pathway | | |
| cEDN1 | 4.5 | 44.3 | 40.4 |
| cEDN2 | 3.5 | 59.8 | 60.7 |
| cEDN3 | – | 29.3 | 9.7 |
| | MAPK/ERK signaling pathway | | |
| cEDN1 | 2.5 | 63.4 | 42.6 |
| cEDN2 | 2.3 | 35.0 | 49.4 |
| cEDN3 | > 200 | 37.4 | 23.0 |
| | cAMP/PKA signaling pathway | | |
| cEDN1 | 3.2 | 47.3 | 83.3 |
| cEDN2 | 3.9 | 34.2 | 32.2 |
| cEDN3 | > 200 | 40.7 | 26.2 |

Note: '–' means that the $EC_{50}$ value could not be calculated based on the experimental data.

contrast, cEDN3 is > 100-fold less potent than cEDN1/cEDN2 (Fig. 2). This finding clearly indicates that as in mammals (Arai et al., 1990), cEDNRA is a functional receptor common for both EDN1 and EDN2, but not for EDN3 in chickens. Unlike cEDNRA, both cEDNRB and cEDNRB2 could be activated by all three peptides with similarly high potencies (Table 1), indicating that both receptors may act as common receptors for the three EDNs. Meanwhile, our findings indicate that cEDN1, cEDN2, and cEDN3 are all biologically active, which are likely to exert their actions via cEDNRs. Interestingly, we also noted that cEDN3 is slightly more potent than cEDN1 or cEDN2 in activating EDNRB2 ($EC_{50}$ of cEDN3: 9.7 nM, Table 1). Furthermore, our findings suggest that like mammalian EDNRs, activation of chicken EDNRA, EDNRB, and EDNRB2 may trigger calcium mobilization (Davenport et al., 2016).

Using a pGL4-SRE-luciferase reporter system, we also demonstrated that cEDN1 ($EC_{50}$: 2.5 nM) and cEDN2 ($EC_{50}$: 2.3 nM), and not cEDN3, could potently activate cEDNRA expressed in HEK293 cells. By contrast, cEDNRB and cEDNRB2 could be potently activated by all three cEDNs (Fig. 3, Table 1). The similar trend is observed with cEDNRA, cEDNRB, and cEDNRB2 expressed in HEK293 cells treated by these peptides monitored by the pGL3-CRE-luciferase reporter system (Fig. 4, Table 1). These findings lend support to the notion that cEDNRA is a common receptor for cEND1 and cEDN2 and not for cEDN3, while cEDNRB and cEDNRB2 can function as common receptor for all three cEDNs. It also suggests that cEDNRs are likely coupled to intracellular MAPK/ERK and cAMP/PKA signaling pathways.

To verify the functional coupling of cEDNRA, cEDNRB, and cEDNRB2 to the calcium, MAPK/ERK, and cAMP/PKA signaling pathways, pharmacological drugs targeting these signaling pathways including MDL12330A [an adenylyl cyclase (AC) inhibitor], H89 [a protein kinase A (PKA) inhibitor], U73122 [a phospholipase C (PLC) inhibitor], PD98059 (an inhibitor of the MEK/ERK signaling cascade) and 2-APB (an inhibitor of IP3-induced calcium mobilization) were used. As shown in Fig. 5, all drugs tested could significantly inhibit cEDN1 (10 nM, 6 h)-induced luciferase activity of HEK293 cells expressing cEDNRA, cEDNRB or cEDNRB2. These findings elucidate that activation of the three cEDNRs is coupled to the calcium mobilization, MAPK/ERK and AC/cAMP/PKA signaling pathways, similar to

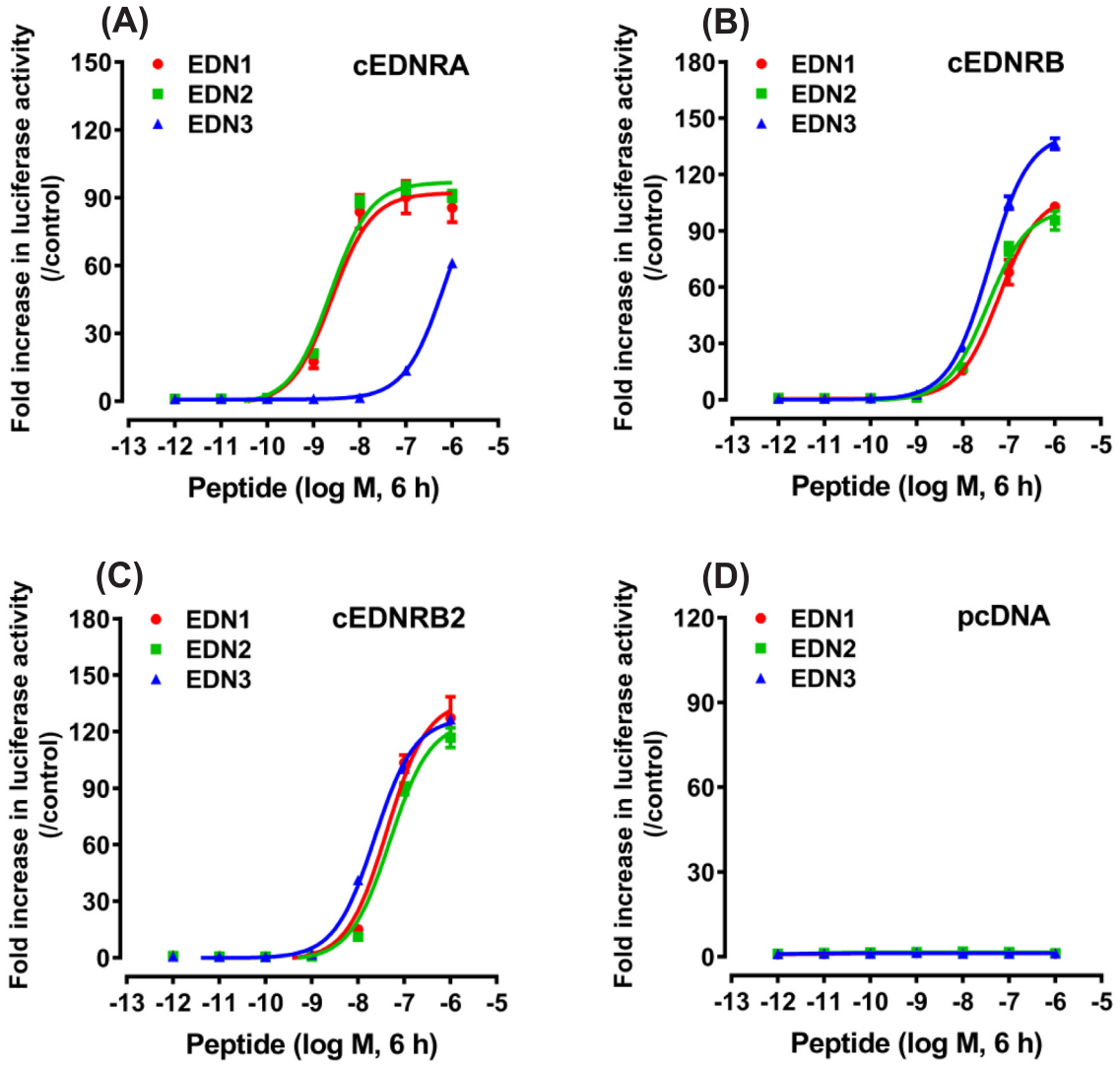


**Fig. 3.** Effects of cEDN1, cEDN2, and cEDN3 on activating cEDNRA (A), cEDNRB (B), and cEDNRB2 (C) expressed in HEK293 cells, monitored by pGL4-SRE-luciferase reporter system; (D) HEK293 cells co-transfected with empty pcDNA3.1 (+) vector and pGL4-SRE-luciferase reporter construct were used as internal controls, and peptide treatment did not alter the luciferase activity of HEK293 cells. Each data point represents mean ± SEM of three replicates ($N = 3$).

mammalian studies (Fig. 5).

Using Western blot, we cross-checked that cEDN1 treatment (100 nM, 10 min) could enhance phosphorylation of ERK1/2 (44/42 kDa) and CREB (43 kDa) in HEK293 cells expressing cEDNRA, cEDNRB or cEDNRB2. This finding substantiates the functional coupling of chicken EDNRs to the MAPK/ERK and AC/cAMP/PKA/CREB signaling pathways (Fig. 6).

### 3.3. Tissue distribution of EDN1, EDN2, EDN3, EDNRA, EDNRB, and EDNRB2 in chickens

To determine the tissue distribution patterns of *EDN-EDNRs* mRNAs in chickens, using the quantitative real-time PCR assay, we examined the mRNA expression of *EDN-EDNRs* in 27 adult chicken tissues including the heart, anterior pituitary, spinal cord, liver, kidneys, lungs, muscle, ovary, testes, spleen, pancreas, subcutaneous fat, skin, adrenal gland, various brain regions (telencephalon, midbrain, cerebellum, hindbrain, hypothalamus) and gastrointestinal tract (abbrev: GI tract; includes the crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, colon).

As shown in Fig. 7, three *EDNRs* are widely, but differentially, expressed in all chicken tissues examined. *cEDNRA* mRNA is highly expressed in the heart, and moderately or weakly expressed in other tissues examined. *cEDNRB* mRNA is highly expressed in the heart and lungs, moderately expressed in the liver, adrenal gland, kidneys, ovary, testes, spleen, pituitary and various parts of the GI tract except the gizzard, and weakly expressed in other tissues. Interestingly, we found that *cEDNRB2* mRNA is predominantly expressed in the kidneys, liver and spleen and weakly expressed in other tissues.

Like c*EDNRs*, the three *cEDNs* were also detected to be widely, but differentially, expressed in all chicken tissues examined (Fig. 7). *cEDN1* mRNA is highly expressed in the heart, ovary, testes, muscle, pancreas, fat, lungs, and proventriculus, and moderately or weakly expressed in other tissues. Unlike *cEDN1*, *cEDN2* is highly expressed in the lungs and GI tract (including the proventriculus, jejunum, ileum, cecum and colon) and moderately or weakly expressed in other tissues (Fig. 7E). *cEDN3* mRNA is highly expressed in the telencephalon and anterior pituitary, moderately expressed in the midbrain, hypothalamus, heart, duodenum, jejunum, ileum, colon, kidney, lungs, muscle, ovary, testes, spleen, pancreas, fat, skin and adrenal gland, and weakly expressed in other tissues examined.

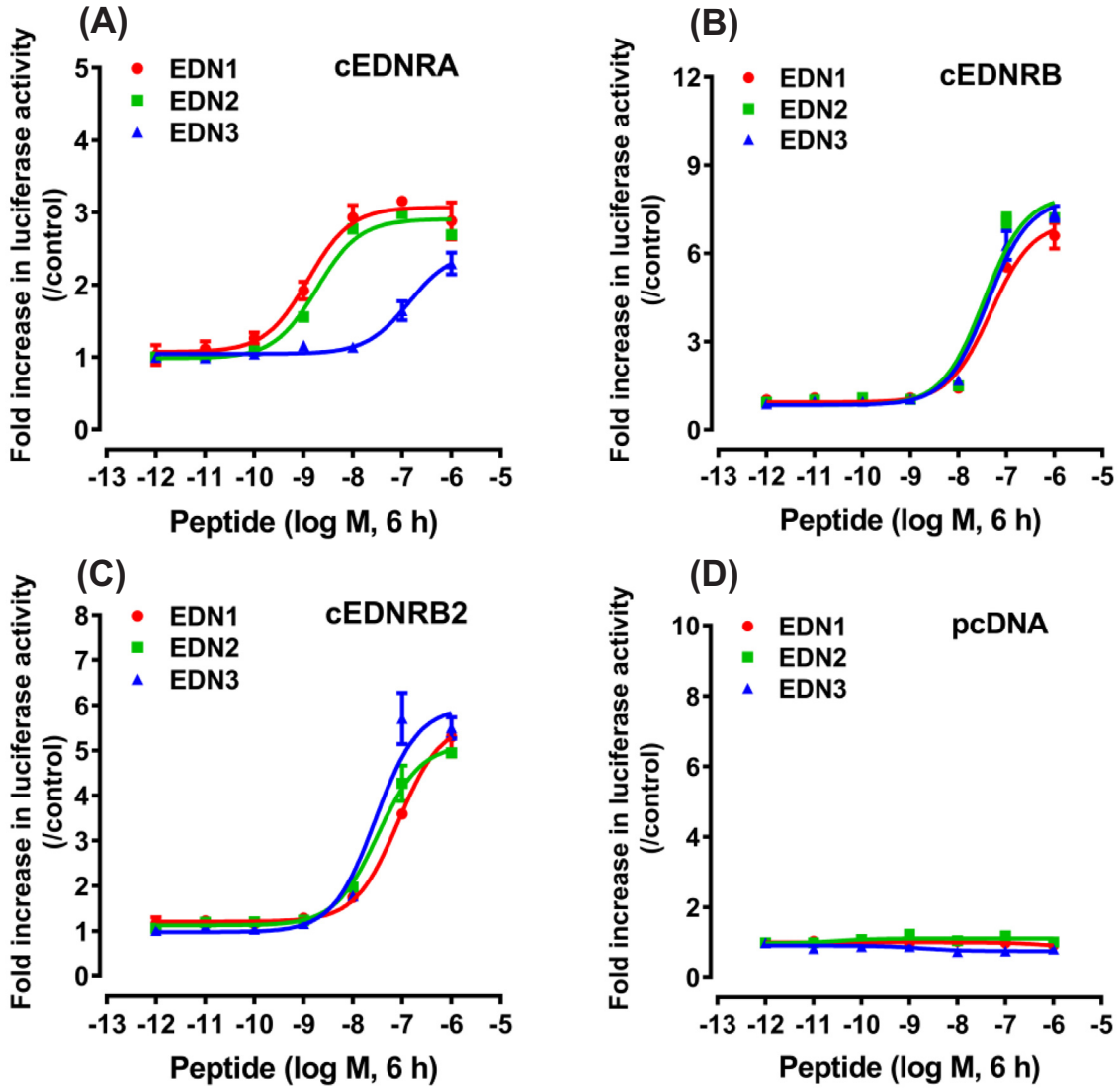


**Fig. 4.** Effects of cEDN1, cEDN2 and cEDN3 on activating cEDNRA (A), cEDNRB (B), and cEDNRB2 (C) expressed in HEK293 cells, monitored by pGL3-CRE-luciferase reporter system; (D) HEK293 cells co-transfected with empty pcDNA3.1 (+) vector and pGL3-CRE-luciferase reporter construct were used as internal controls, and peptide treatment did not alter the luciferase activity of HEK293 cells at any concentration tested. Each data point represents mean ± SEM of three replicates ($N = 3$).

## 4. Discussion

In this study, we cloned the cDNAs of chicken *EDN1*, *EDN2*, and *EDN3*, and analyzed the functionality of chicken EDN1, EDN2, EDN3, EDNRA, EDNRB, and EDNRB2 *in vitro*. Our study demonstrated that cEDNRA could be potently activated by cEDN1 and cEDN2, while both chicken EDNRB and EDNRB2 could be potently activated by all three cEDNs, clearly indicating that both receptors share functional similarity. Moreover, we found that the three *EDN*s and *EDNR*s are widely, but differentially, distributed in chicken tissues. To our knowledge, our study represents the first to reveal the functionality and expression pattern of each component of the EDN-EDNR system in an avian species.

### 4.1. Structure of chicken EDN1, EDN2 and EDN3

In chickens, EDN1, EDN2 and EDN3 have been reported to play important roles in cardiovascular development and proliferation, migration and differentiation of NCCs (Kempf et al., 1998; Pla and Larue, 2003), however, their cDNA sequences have yet been identified. In this study, we cloned cDNAs of chicken *EDN1*, *END2* and *EDN3* genes from heart/brain tissue. These cDNAs are predicted to encode the large prepro-EDNs of 205 aa, 179 aa and 173 aa respectively. Although the overall amino acid identities between chicken and mammalian prepro-EDNs are not high (41–52%), the putative bioactive cEDN1, cEDN2, and cEDN3 of 21 amino acids share a remarkable degree of amino acid sequence identities with human EDN1, EDN2, and EDN3, respectively (91–95%) (Fig. 2A) (Inoue et al., 1989; Itoh et al., 1988). And the mature peptides also contain the four conserved cysteines essential for disulfide bond formation. In addition, since the putative cleavage sites for furin endopeptidase (KR/RR) and membrane-bound metalloprotease ECEs are present in chicken prepro-EDNs and fully conserved among species (Fig. 1), it strongly suggests that like mammalian EDNs, chicken EDN1, EDN2 and EDN3 could be released from the large prepro-EDNs after two consecutive steps of proteolytic processing. The remarkable structural conservation of EDN1, EDN2 and EDN3 noted between chicken and other vertebrate species, also suggests that all chicken EDNs are biologically active, which is in line with our findings *in vitro* that they can activate chicken EDNRs efficiently (Table 1).

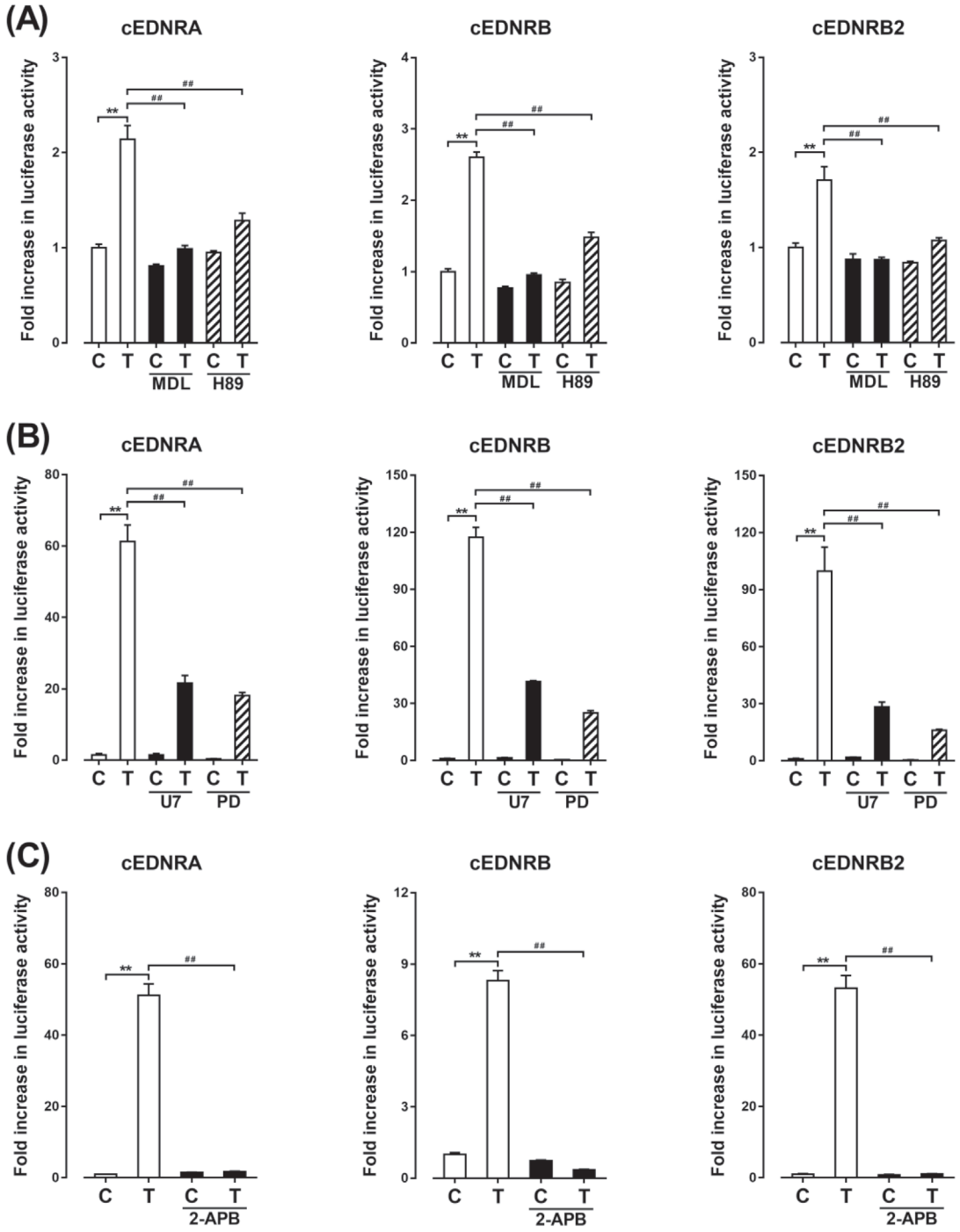


**Fig. 5.** (A) Effects of MDL12330A (MDL, 20 μM) and H89 (10 μM) on cEDN1 (10 nM, 6 h)-induced luciferase activity of HEK293 cells expressing cEDNRA, cEDNRB and cEDNRB2, monitored by pGL3-CRE-luciferase reporter system; (B) Effects of U73122 (U7, 20 μM) and PD98059 (PD, 100 μM) on cEDN1 (10 nM, 6 h)-induced luciferase activity of HEK293 cells expressing cEDNRA, cEDNRB and cEDNRB2, monitored by pGL4-SRE-luciferase reporter system; (C) Effects of 2-APB (100 μM) on cEDN1 (10 nM, 6 h)-induced luciferase activity of HEK293 cells expressing cEDNRA, cEDNRB and cEDNRB2, monitored by pGL3-NFAT-RE-luciferase reporter system. Each drug was added 1 h before cEDN1 treatment. In each graph, 'T' represents EDN1 peptide treatment and 'C' represents control without peptide treatment. Each data point represents mean ± SEM 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).

### *4.2. Functional characterization of chicken EDNRA, EDNRB and EDNRB2.*

In mammals, the functionality of EDN-EDNR system has been studied intensively *in vitro*, (Davenport et al., 2016), however, our knowledge regarding its functionality in non-mammalian vertebrates is limited. Moreover, the number of genes encoding EDNs and EDNRs, varies considerably among different vertebrate groups (Braasch and Schartl, 2014). For instance, in birds, three *EDNs* (*EDN*1, 2 and 3) and three *EDNRs* (*EDNRA*, *EDNRB* and *EDNRB2*) have been identified in their genomes, including a novel *EDNRB2*, which is absent in humans and rodents (Braasch and Schartl, 2014). In teleosts, 4 *EDNRs* (*EDNRAa*, *EDNRAb*, *EDNRBa* and *EDNRBb*) and 6 *EDNs* (*EDN1*, *EDN2a*, *EDN2b*,

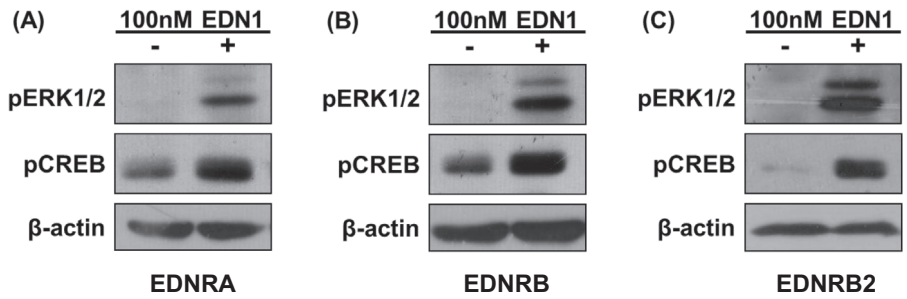


**Fig. 6.** (A-C) Western blot showing that cEDN1 treatment (100 nM, 10 min) could enhance the phosphorylation levels of ERK1/2 (pERK1/2) and CREB (pCREB) in HEK293 cells expressing chicken EDNRA (cEDNRA) (A), EDNRB (cEDNRB) (B) and EDNRB2 (cEDNRB2) (C). Each experiment was repeated thrice.

*EDN3a*, *EDN3b* and *EDN4*) have been identified (Braasch and Schartl, 2014). The variation in copy number and structure of EDNs and EDNRs during evolution hints that we cannot simply transfer our knowledge on mammalian EDN-EDNR signaling to the avian or fish model, thus, it is essential to investigate the ligand-receptor interaction in other vertebrate groups including birds and teleosts.

Since the information regarding the functionality of avian EDN-EDNR is incomplete (Kempf et al., 1998; Lecoin et al., 1998), we first cloned the three EDNRs (EDNRA, EDNRB and EDNRB2) from chickens and systematically tested whether they could be activated by chicken EDNs *in vitro*. We found that cEDNRA expressed in HEK293 cells could be potently activated by cEDN1 and cEDN2, and not by cEDN3. This finding partially agrees with a previous study, in which cEDNRA expressed in COS-7 cells could preferentially bind EDN1 and EDN2, but not EDN3 (Kempf et al., 1998). Consistent with the finding in chickens, EDNRA could also be potently activated by EDN1 and EDN2 and not by EDN3 in mammals (Arai et al., 1990), and *Xenopus laevis* (also named ETAx) (Kumar et al., 1994), suggesting that as in other vertebrates, EDNRA is a functional receptor common for both EDN1 and EDN2 in chickens (Fig. 8). Unlike cEDNRA, cEDNRB could be potently activated by cEDN1, cEDN2 and cEDN3 with similarly high potencies. To present, there has been no study so far regarding the functionality of EDNRB in non-mammalian vertebrates. Our study proved that cEDNRB can function as a common receptor for the three EDNs with resemblance to mammalian EDNRB (Sakurai et al., 1990), which could also be potently activated by all three cEDNs (Fig. 8). Like cEDNRB, cEDNRB2 could be

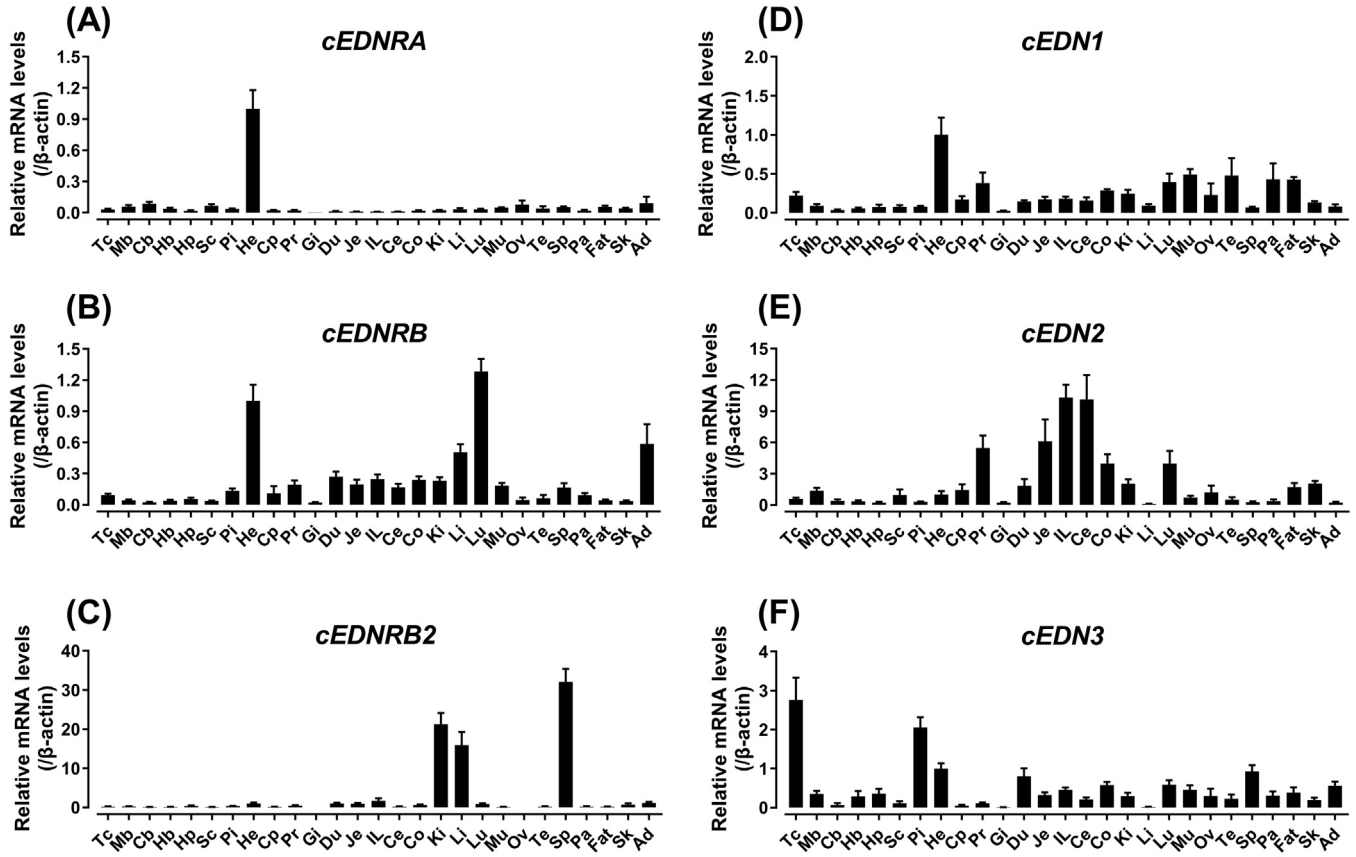


**Fig. 7.** Quantitative real-time RT-PCR assays of *cEDNRA* (A), *cEDNRB* (B), *cEDNRB2* (C), *cEDN1* (D), *cEDN2* (E), and *cEDN3* (F) mRNA expression in adult chicken tissues, including the telencephalon (Tc), midbrain (Mb), cerebellum (Cb), hindbrain (Hb), hypothalamus (Hp), spinal cord (Sc), anterior 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), skin (Sk) and adrenal gland (Ad). The mRNA levels of each gene were normalized to that of *β-actin* and expressed as the fold difference compared with that of heart (He). Each data point represents the mean ± SEM of 6 adult chickens ($N = 6$) (3 males and 3 females).

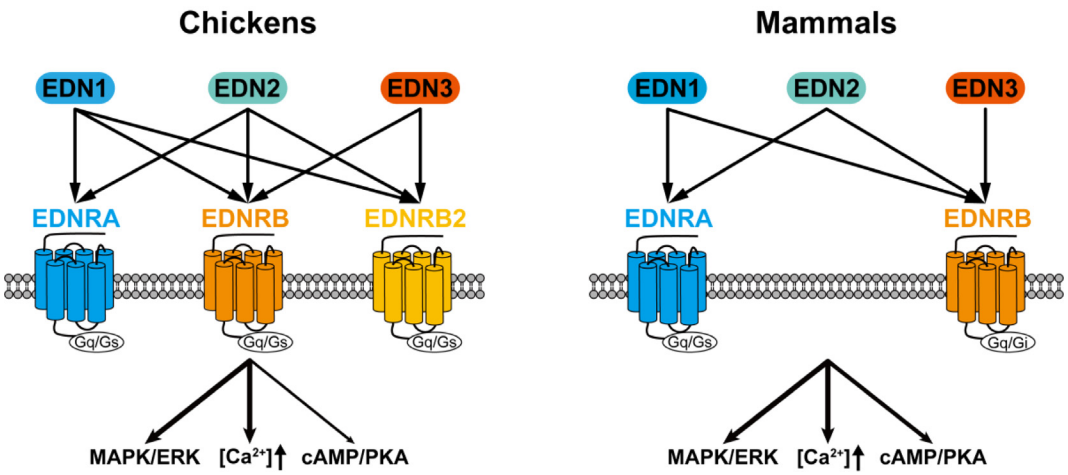


**Fig. 8.** Comparison of EDN-EDNR system between chickens and mammals. In chickens, three EDNs (cEDN1, cEDN2 and cEDN3) and three EDNRs (cEDNRA, cEDNRB and cEDNRB2) have been identified including the novel EDNRB2, which is absent in humans and mice. cEDNRA could be potently activated by cEDN1 and cEDN2, but not by cEDN3, indicating that as in mammals, EDNRA can function as a common receptor for EDN1 and EDN2. Both cEDNRB and cEDNRB2 could be potently activated by all three cEDNs, indicating that both receptors can function as receptors common for the three EDNs and resemble mammalian EDNRB. Chicken EDNRA, EDNRB and EDNRB2 are likely coupled to Gq and Gs protein, and their activation stimulates multiple downstream pathways including calcium mobilization, MAPK/ERK, and cAMP/PKA signaling pathways. In mammals, only two EDN receptors (EDNRA and EDNRB) and three EDNs have been identified. Both EDNRA and EDNRB are likely coupled to Gq and Gs/Gi protein and their activation triggers multiple signaling pathways similar but not identical to those in chickens.

potently activated by cEDN1, cEDN2 and cEDN3, indicating that cEDNRB2 can also function as a common receptor for all three cEDNs. Our finding is partially consistent with reports in quails, in which EDNRB2 expressed in Ltk$^-$-1 cells could be activated by EDN1 and EDN3 with high potencies (Lecoin et al., 1998). However, this contrasts the previous report in *Xenopus laevis*, in which EDNRB2 (also named ETC) has been proposed as an EDN3-specific receptor based on the higher efficiency of EDN3 in pigment dispersion of melanophores than that of EDN1 (Karne et al., 1993). The potent activation of both cEDNRB and cEDNRB2 by the three cEDNs, for the first time, proves the functional similarity shared between EDNRB2 and EDNRB in birds (Fig. 8). Our finding also supports the idea that EDNRB2 and EDNRB were likely generated by the second round of genome duplication event during vertebrate evolution. The presence of two EDNRBs, together with their differential expression pattern shown in Fig. 7, strongly suggests that EDN actions are partitioned by the two EDNRBs in birds. For instance, mammalian EDN3 can activate a single EDNRB to control the migration of trunk NCCs and their differentiation into the enteric nervous system and melanocytes (Baynash et al., 1994; Hosoda et al., 1994), whereas in birds, EDN3 actions on trunk NCCs are likely mediated by the two EDNRBs (EDNRB and EDNRB2), i.e. EDN3-EDNRB signaling may regulate avian NCC migration (entering ventral pathway) and formation of the enteric nervous system (Nagy and Goldstein, 2006), while EDN3-EDNRB2 signaling may be crucial for the proliferation, migration (entering dorso-lateral pathway) and differentiation of melanoblasts (Lahav et al., 1996; Pla et al., 2005) and important for pigmentation and plumage in birds (Dorshorst et al., 2011; Han et al., 2014; Kinoshita et al., 2014; Lecoin et al., 1998; Li et al., 2015; Miwa et al., 2007; Miwa et al., 2006; Wu et al., 2017).

It is reported that in mammals, both EDNRA and EDNRB are coupled to Gq protein, and their activation stimulates phospholipase C (Gq-PLC), which subsequently triggers multiple signaling pathways such as calcium mobilization and MEK/ERK signaling cascade (Davenport et al., 2016). In this study, we proved that activation of cEDNRA, cEDNRB and cEDNRB2 could strongly stimulate calcium mobilization and MAPK/ERK signaling pathway, as revealed by cell-based luciferase reporter assays and Western blot, similar to mammalian EDNRs (Fig. 8) (Aquilla et al., 1996; Aramori and Nakanishi, 1992; Davenport et al., 2016). In addition, we found that the three cEDNRs are likely also coupled to Gs protein, since their activation could stimulate AC/cAMP/PKA/CREB signaling pathway. Our finding partially agrees with mammalian studies, in which EDNRA activation by EDNs can stimulate Gs-cAMP/PKA signaling pathway, while EDNRB activation can inhibit cAMP pathway (Aquilla et al., 1996; Aramori and Nakanishi, 1992; Davenport et al., 2016; Eguchi et al., 1993). The discrepancy in G protein-coupling noted between human EDNRB and chicken EDNRB and B2 are perhaps due to the structural change of EDNRB(s) during evolution (Fig. 8).

### 4.3. Tissue expression pattern of EDN-EDNRs in chickens

In this study, the mRNAs of *EDN-EDNRs* were detected to be widely distributed in adult chicken tissues (Fig. 7). The wide tissue distribution of *EDN-EDNRs* also suggests that EDN-EDNRs have a broad spectrum of actions in adult stages, in addition to their reported actions during embryogenesis. The wide expression of EDN-EDNR system, *eg* EDN1-EDNRA/EDNRB, is not surprising, considering the wide distribution of blood vessels in various organs, where EDN1-EDNRA/EDNRB signaling has been proven to be crucial for vasoconstriction and blood perfusion in mammals (Davenport et al., 2016).

Interestingly, we found that either the *cEDNRs* or the *cEDNs* are differentially expressed in adult chicken tissues. This is consistent with the finding in mammals, in which EDNRA and EDNRB (or EDNs) are differentially expressed (Davenport et al., 2016; Firth and Ratcliffe, 1992). In chicken heart, *cEDNRA*, *cEDNRB* and *cEDN1* were detected to be abundantly expressed, where *cEDNRB2* was barely detectable. This is similar to mammalian findings that *EDNRA*, *EDNRB* and *EDN1* are highly or moderately expressed in the heart (Arai et al., 1990; Sakurai et al., 1990). These findings suggest that as in mammals, EDN1-EDNRA/EDNRB signaling is not only critical for embryonic heart development, but may also be associated with heart function (*e.g.* cardiac myocyte contractility) and disease (Bezie et al., 1996; Hassanpour et al., 2010; Khimji and Rockey, 2010). In chicken lungs, *cEDNRB* is highly expressed, where the three c*EDNs* are moderately expressed. This concurs with mammalian finding that EDNRB is abundantly expressed in the lung endothelium. The abundant expression of cEDNRB in chicken lungs not only suggests that EDNRB may function as a clearing receptor to remove circulating EDN1, but may also be associated with the physiological or etiologic processes in the lung such as pulmonary hypertension observed in broiler chickens (Teng et al., 2016), similar to its mammalian counterparts (Dupuis et al., 1996; Fukuroda et al., 1994). In chicken kidneys, both EDNRB2 and EDNRB were detected to

be highly or moderately expressed. Similarly, EDNRB and not EDNRA is abundantly expressed in mammalian kidneys (Ahn et al., 2004; Fukuroda et al., 1994; Ge et al., 2006). This hints that EDNRB/B2 signaling may play conserved roles in renal sodium and water excretion and clearance of circulating EDN in birds, thus being critical for the regulation of blood pressure. In chicken liver, both *cEDNRB* and *cEDNRB2* are highly expressed, where EDNRB/B2 signaling may be an important regulator of sinusoid vascular resistance, as reported in mice (Ling et al., 2012). Since the three *EDN*s are weakly expressed in the liver, therefore, it is tempting to speculate that as in rats, liver EDNRBs may also function as clearing receptor(s) to remove circulating EDN in chickens (Fukuroda et al., 1994).

Of particular interest to note is that *EDN2* has the highest expression level in many parts of the GI tract, including the proventriculus, jejunum, ileum, cecum and colon (Fig. 7E). Our finding is consistent with previous reports in mammals, in which *EDN2* is highly expressed in the intestinal epithelial cells (Chang et al., 2013; Firth and Ratcliffe, 1992). The abundant expression of *EDN2* in chicken GI tract suggests that cEDN2 may play important roles in controlling GI tract function via EDNR, such as GI tract motility, nutrient absorption, and immunity. As in mammals, *EDN*s and *EDNR*s are found to be expressed in chicken ovary (Fig. 7). This finding suggests the involvement of EDN-EDNR system in controlling ovarian functions such as steroidogenesis and ovulation, as demonstrated in mammals (Meidan and Levy, 2007). Besides the aforementioned tissues, *EDN-EDNR*s are also expressed in other tissues examined including spleen, various brain regions (including the hypothalamus), fat, pancreas, adrenal gland, pituitary, spinal cord, muscle and skin. This suggests that further extensive studies are required to reveal the autocrine/paracrine actions of avian EDN-EDNR system in these tissues.

In summary, we cloned the cDNA of *EDN1*, *EDN2* and *EDN3* genes in chickens. Functional studies proved that cEDNRA is a receptor common for both cEDN1 and cEDN2, and cEDNRB and cEDNRB2 are the two receptors common for the three EDNs and share functional similarity. Moreover, activation of the three cEDNRs can trigger multiple downstream signaling pathways (Fig. 8). qPCR assays revealed that *EDN*s and *EDNR*s are widely, but differentially, expressed in adult chicken tissues. Collectively, our findings will facilitate our better understanding of the physiological and pathological roles of EDN-EDNR system across vertebrates, such as their roles in NCC migration and differentiation and cardiovascular development, function and diseases.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113231.

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