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Arginine vasotocin (AVT)/mesotocin (MT) receptors in chickens: Evidence for the possible involvement of AVT-AVPR1 signaling in the regulation of oviposition and pituitary prolactin expression

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ABSTRACT

Two structurally related peptides, arginine vasotocin (AVT) and mesotocin (MT), are reported to regulate many physiological processes, such as anti-diuresis and oviposition in birds, and their actions are likely mediated by four AVT/MT receptors (AVPR1A, AVPR1B, MTR and AVPR2b), which are orthologous/paralogous to human AVPR1A, AVPR1B, OXTR and AVPR2 respectively. However, our knowledge regarding the functions of these avian AVT/MT receptors has been limited. Here, we examined the functionality and expression of these receptors in chickens and investigated the roles of AVT in the anterior pituitary. Our results showed that 1) AVPR1A, AVPR1B and AVPR2b could be preferentially activated by AVT, monitored by cell-based luciferase reporter assays and/or Western blot, indicating that they are AVT-specific receptors (AVPR1A; AVPR1B) or AVT-preferring receptor (AVPR2b) functionally coupled to intracellular calcium, MAPK/ERK and cAMP/PKA signaling pathways. In contrast, MTR could be activated by AVT and MT with similar potencies, indicating that MTR is a receptor common for both peptides; 2) Using qPCR, differential expression of the four receptors was found in chicken tissues including the oviduct and anterior pituitary. In particular, only AVPR1A is abundantly expressed in the uterus, suggesting its involvement in mediating AVT-induced oviposition. 3) In cultured chick pituitary cells, AVT could stimulate ACTH and PRL expression and secretion, an action likely mediated by AVPR1B and/or AVPR1A abundantly expressed in anterior pituitary. Collectively, our data helps to elucidate the roles of AVT/MT in birds, such as the 'oxytocic action' of AVT, which induces uterine muscle contraction during oviposition.

1. Introduction

It is well-known that two structurally-related peptide hormones named arginine vasopressin (AVP) and oxytocin (OXT) are secreted from the neurohypophysis in mammals. AVP displays pressor and anti-diuretic activities, while OXT has oxytocic and milk-ejecting activities (Acher, 1993). Similarly, AVP-like and OXT-like neurohypophysial hormones have been identified in non-mammalian vertebrates including birds, frogs and teleosts. They are named as arginine vasotocin (AVT)/vasotocin (VT) and mesotocin (MT)/isotocin (IT) (Sawyer, 1977), due to their orthology to mammalian AVP and OXT respectively. All these peptides share striking structural conservation among vertebrates, including the presence of two cysteines at position 1 and 6 for an intra-molecular disulfide bond formation (Acher, 1993). It is clear that AVP and OXT are mainly expressed in the paraventricular nuclei (PVN) and supraoptic nuclei (SON) of the hypothalamus and play a broad spectrum of actions in the central nervous system (CNS) and peripheral tissues of mammals, such as water balance, lactation, parturition, social cognition and behavior, learning and memory, sexual behavior, feeding and satiety, liver glycogenolysis, contraction of vascular smooth muscle, stress, circadian rhythm, and hormone secretion of the anterior pituitary (*e.g.* ACTH secretion) (Gillies et al., 1982; Antoni et al., 1993; Engler et al., 1999; Gimpl and Fahrenholz, 2001; Donaldson and Young, 2008; Albers, 2012; Wacker and Ludwig, 2012; Sabatier et al., 2013; Yamaguchi et al., 2013).

In mammals, the diverse actions of AVP and OXT are mediated by four membrane-spanning receptors, all of which belong to G protein-coupled receptors and share 40–45% amino acid sequence identity with each other (Gimpl and Fahrenholz, 2001). AVP has three specific receptors, namely AVPR1A, AVPR1B and AVPR2, which bind AVP with much higher affinity than OXT (Birnbaumer et al., 1992; Lolait et al., 1992; Morel et al., 1992; Sugimoto et al., 1994). Both AVPR1A and AVPR1B are reported to be functionally coupled to Gq protein and their activation can stimulate

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intracellular calcium mobilization (Morel et al., 1992; Lolait et al., 1995), whereas AVPR2 is functionally coupled to Gs protein and its activation can stimulate the intracellular cAMP/protein kinase A (PKA) signaling pathway (Birnbaumer et al., 1992). Unlike AVP, OXT possesses a single receptor named OXT receptor (OXTR) (Kimura et al., 1992), which can be specifically activated by OXT (Kimura et al., 1992). OXTR is coupled to Gq protein and its activation can increase the intracellular calcium level and stimulate MAPK/ERK signaling cascade (Gimpl and Fahrenholz, 2001). The tissue- or cell-specific expression of AVPR and OXTR are known to mediate the specific role of AVP/OXT in different target tissues. For instance, AVPR1B mainly expressed in pituitary corticotrophs mediates the effect of AVP on pituitary ACTH secretion and thus plays a crucial role in stress response (Hernando et al., 2001); OXTR is abundantly expressed in the mammary gland and uterus (Kimura et al., 1992; Kimura et al., 1996), which mediates the OXT-induced milk ejection during lactation and uterine muscle contraction during parturition.

Similar to mammalian AVP/OXT system, the AVT/MT system has been reported to play important roles in birds. Avian AVT is involved in the regulation of many physiological processes, such as anti-diuresis, oviposition, food intake, stress, reproductive behaviors, and pituitary ACTH and prolactin (PRL) secretion (Riddle, 1921; Castro et al., 1986; Proudman and Opel, 1988; Jurkevich and Grossmann, 2003; Kuenzel et al., 2013; Masunari et al., 2016; Opel and Proudman, 1988). In contrast, the roles of MT in birds remain unclear (Robinzon et al., 1988a,b). More intriguingly, there is solid evidence showing that AVT can act as both anti-diuretic and oxytocic hormone (ie a potent inducer of oviposition) (Rzasa and Ewy, 1970; Niezgoda et al., 1973; Koike et al., 1988), which is drastically different from that in mammals, in which the anti-diuretic and oxytocic actions are undertaken by AVP and OXT, respectively.

To present, four AVT/MT receptors, named AVPR1A (alternative name: VT4) (Selvam et al., 2013), AVPR1B (VT2) (Cornett et al., 2003), MTR (VT3) (Gubrij et al., 2005), and AVPR2-like receptor (VT1) (Tan et al., 2000), have been identified. Several pioneering studies have suggested their roles in mediating the actions of AVT in birds (*e.g.* chickens) such as AVT-induced ACTH secretion and oviposition (Jurkevich et al., 2005; Baeyens and Cornett, 2006; Jurkevich et al., 2008; Selvam et al., 2013). However, it should be noted that avian *AVPR2*-like gene is found to be paralogous (ie not orthologous) to human *AVPR2*, thus it is re-named as *AVPR2b* (or *AVPR2c*) in previous studies (Ocampo Daza et al., 2012; Yamaguchi et al., 2012; Lagman et al., 2013). So far, the functional similarity and difference of the four AVT/MT receptors has not been carefully evaluated in birds. This limitation undoubtedly prevents our understanding of the physiological roles of AVT and MT in birds. Therefore, using chicken as an animal model, our present study aimed to: (1) examine the functional difference of AVPR1A, AVPR1B, AVPR2b and MTR *in vitro*; (2) investigate the expression of the four AVT/MT receptors in chicken tissues, including the oviduct and anterior pituitary; (3) test whether AVT could regulate pituitary *PRL* and *POMC* (encoding ACTH) mRNA expression. Undoubtedly, results from the present study will aid to elucidate the physiological roles of AVT/MT in birds, such as the 'oxytocic action' of AVT in birds. In addition, our data will also help to reveal the conservation and change in functional roles of AVT/MT across vertebrates.

2. Materials and methods

2.1. Animal tissues

Adult chickens or 3-week-old chicks (Lohmann layer) employed in this study were purchased from local commercial companies. Sexually mature chickens were sacrificed and various tissues including oviduct (of laying hens) were collected. Anterior pituitaries were collected from 3-week-old chicks either for total RNA extraction or cell culture. All animal experiments were conducted in accordance with the Guidelines for Experimental Animals issued by the Ministry of Science and Technology of People's Republic of China. All animal experimental protocols employed in this study were approved by the Animal Ethics Committee of the College of Life Sciences, Sichuan University.

2.2. Chemicals, peptides, antibodies and primers

All chemicals were purchased from Sigma-Aldrich and restriction enzymes were obtained from TaKaRa (Takara, Dalian, China). Chicken (c-)AVT, MT, corticotrophin-releasing hormone (CRH), vasoactive intestinal peptide (VIP) were synthesized using solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). The pharmacological agents including H89, PD98059, U73122 and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Polyclonal antibodies against recombinant chicken PRL were prepared in our laboratory (Bu et al., 2016). The anti-ERK, anti-CREB or anti-β-actin antibodies were purchased from Cell Signaling Technology Inc. (CST, Beverly, MA). Rabbit anti-ACTH antibody was purchased from Abcam (Abcam, China). All primers used in this study were synthesized by Tsingke (Beijing, China) and listed in Supplementary Table 1.

2.3. Reverse transcription and quantitative real-time PCR (qPCR) assay

Total RNA was extracted from chicken tissues or cultured pituitary cells using RNAzol (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and reversely transcribed using M-MLV reverse transcriptase (TaKaRa). In brief, Oligodeoxythymide (0.5 μg) and total RNA (2 μg) 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 and 100 U Moloney murine leukemia virus (MMLV) reverse transcriptase were added into the reaction mix in a total volume of 10 μL. Reverse transcription (RT) was performed at 42 °C for 90 min. All RT negative controls were performed under the same condition, but without the addition of reverse transcriptase.

RT samples were then used to investigate the mRNA expression of genes by quantitative real-time PCR (qPCR) assay, which was conducted on the CFX96 Real-time PCR Detection System (Bio-Rad), as described in our previous study (Cai et al., 2015).

2.4. Evaluation of mRNA levels of target genes using RNA–Seq analysis

RNA–Seq data publicly available was used to evaluate the expression level of *AVPRs/MTR* in chickens (PRJNA173328) and ducks (PRJNA449259) (Yin et al., 2019). RNA-Seq data were first downloaded from the National Center for Biotechnology Information (NCBI) Website (http://www.ncbi.nlm.nih.gov/sra). Using Gallus_gallus-5.0/BGI_duck_1.0 transcriptome (Ensembl release 94) as a reference, quantification of gene expression level was performed directly using the high-speed quantification tool Salmon v0.10.2 with default parameters (Patro et al., 2017). The transcripts per million (TPM) values of the transcripts generated by Salmon were used to express the relative abundance of *AVPR1A*, *AVPR1B*, *AVPR2b* and *MTR* transcripts. Using a similar strategy, the relative expression levels of *AVT*, *MT*, *AVPR1A*, *AVPR1B*, *AVPR2b* and *MTR* were quantitated using RNA-seq data of hypothalamus or anterior pituitary from 1-week-old chicks (*unpublished data*).

2.5. Functional analysis of chicken AVPR1A, AVPR1B, AVPR2b and MTR in cultured HEK293 cells

Based on cDNA sequences of chicken (c-)*AVPR1A*, c*AVPR1B*, c*AVPR2b* and *cMTR* (NM_001110438.1, NM_001031498.1, NM_001031479.1, NM_001031569.1) published in previous studies, gene-specific primers (*see* Supplementary Table 1) and high-fidelity KOD DNA polymerase (Toyobo, Japan) were used to amplify the coding region of each receptor (Fig. S1) from adult chicken pituitaries. The amplified PCR products were cloned into the pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA) and sequenced.

According to the methods described in our previous studies (Wang et al., 2007; Wang et al., 2012; Mo et al., 2015; Mo et al., 2017), the functionality and signaling property of chicken AVPR1A, AVPR1B, AVPR2b and MTR were evaluated in HEK293 cells by three cell-based luciferase reporter (pGL3-CRE-Luciferase, pGL3-NFAT-RE-Luciferase, and pGL4-SRE-Luciferase) systems. In brief, HEK293 cells transiently expressing each receptor (AVPR1A, AVPR1B, AVPR2b and MTR) were treated with chicken AVT and MT (10^{-12} to 10^{-6} M, 6 h), and the receptor-activated cAMP/PKA, calcium mobilization and MAPK/ERK signaling pathways were then monitored by the three cell-based luciferase reporter systems.

2.6. Western blot

To test whether chicken AVPR1A, AVPR1B, AVPR2b and MTR activation can enhance ERK1/2 (44/42 kDa) and CREB (43 kDa) phosphorylation, HEK293 cells transiently expressing each receptor were treated by cAVT (100 nM) for 10 min, and the phosphorylated ERK1/2 (pERK1/2) and CREB (pCREB) levels in cell lysates were examined by Western blot, as described in our previous study (He et al., 2016; Gao et al., 2017). β-actin level was included as a loading control.

To examine whether ACTH and PRL secretion could be induced by AVT in cultured chick pituitary cells, Western blot analysis was performed in this study. The cell medium was used to detect ACTH/PRL secretion using corresponding antibodies (1:600), as described in our recent studies (Bu et al., 2016). In brief, anterior pituitaries collected from 3-week-old chicks were cut into pieces and digested by 0.25% trypsin at 37 °C for 30 min. The dispersed pituitary cells were cultured at a density of 5×10^5 cells per well in Medium 199 supplemented with 15% fetal bovine serum in a Corning CellBIND 48-well plate (Corning) at 37 °C with 5% CO_2. After 24-h culture, the culture medium was replaced with serum-free M199 medium (300 μL per well), and the cells were treated with cCRH (10 nM) or different doses of cAVT (0.1 nM − 10 nM) for 4 h. Then, the protein level of ACTH/PRL/β-actin in the culture medium/cell lysates were examined by Western blot.

2.7. Evaluation on the effect of cAVT on POMC and PRL expression in cultured chick pituitary cells

To examine whether AVT can induce *POMC* (*ACTH*) and *PRL* mRNA expression, the cultured chick pituitary cells were treated by cAVT (0.1 nM/1nM/10 nM, 24 h) in the presence or absence of drugs (U73122, 20 μM/H89, 10 μM). The total RNA extracted from cultured pituitary cells (Huang et al., 2014) was prepared for qPCR assay of *POMC* and *PRL* gene expression, according to our previously established method (Huang et al., 2014; Zhang et al., 2017).

2.8. Data analysis

Semi-quantitative analysis of band intensity from Western blot was performed using the Image J program (Image J software; National Institutes of Health), and the relative protein levels normalized by that of intracellular β-actin were then expressed as the percentage compared with respective controls (without treatment). The relative mRNA level of target gene was first calculated as the ratio to that of *β-actin/GAPDH* and then expressed as the percentage (or fold difference) compared with respective controls (without treatment) or chosen tissues. The data were analyzed by

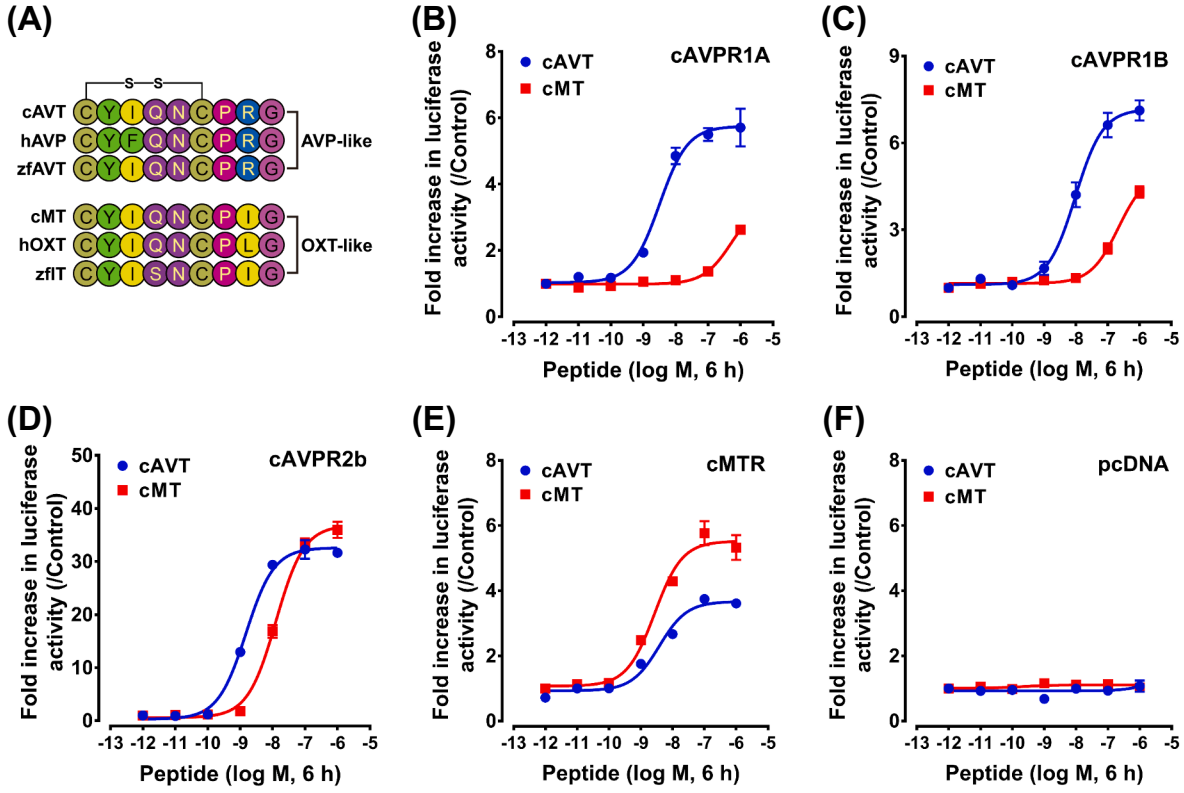

Fig. 1. (A) Amino acid alignment of chicken AVT (cAVT) and cMT with human(h-)/zebrafish(zf-) AVP/AVT and OXT/IT respectively. Two types of neurohypophysial hormones (AVP-like and OXT-like peptides) have been identified in most vertebrate groups. The cysteine residues at the position 1 and 6 form a disulphide bridge. (B–D) Effects of cAVT and cMT (10^{-12} to 10^{-6} M, 6 h) on activating chicken (c-) AVPR1A (B), AVPR1B (C), AVPR2b (D), and MTR (E) expressed in HEK293 cells, monitored by pGL3-NFAT-RE-luciferase reporter system. (F) HEK293 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 HEK293 cells at any concentration tested. Each data point represents mean ± SEM of three replicates ($N = 3$).

Table 1
EC_{50} values of cAVT and cMT in activating different signaling pathways in HEK293 cells expressing chicken (c-) AVPR1A, AVPR1B, AVPR2b and MTR.

EC_{50} values (nM)				
Peptide	cAVPR1A	cAVPR1B	cAVPR2b	cMTR
Calcium signaling pathway				
cMT	–	–	12.62	2.71
cAVT	3.17	9.61	1.5	3.81
MAPK/ERK signaling pathway				
cMT	–	–	≥100	23.35
cAVT	5.38	91.37	8.49	16.87
cAMP/PKA signaling pathway				
cMT	–	–	29.19	–
cAVT	10.36	43.15	7.64	–

Note: '–' means that the EC_{50} values could not be calculated based on the experimental data. The underlined EC_{50} values were roughly estimated based on the experimental data (shown in Figs. 2B and 3C) by GraphPad software, as the tested doses have not reached maximal response in our experiments.

the Student's *t* test (for two groups), or by one-way ANOVA followed by the Dunnett's test with the use of Graph Pad Prism 7 (Graph Pad Software). To validate our results, all experiments were repeated at least twice.

3. Results

3.1. Functional characterization of chicken AVPR1A, AVPR1B, AVPR2b and MTR

To compare the functional similarity and difference between the four chicken AVT/MT receptors, each receptor was transiently expressed in HEK293 cells and treated with synthetic chicken MT and AVT (Fig. 1A). Receptor activation was then examined by pGL3-NFAT-RE-luciferase, pGL4-SRE-luciferase, and pGL3-CRE-luciferase reporter systems established in our lab, which could monitor receptor-stimulated intracellular calcium mobilization (Wang et al., 2012), MAPK/ERK (Mo et al., 2015; Mo et al., 2017), and cAMP/PKA signaling pathways (Wang et al., 2007), respectively.

Using the pGL3-NFAT-RE-luciferase reporter system, we demonstrated that chicken AVT, not MT, could potently activate AVPR1A and AVPR1B expressed in HEK293 cells and stimulate luciferase activities dose-dependently. Since AVT is ~100-fold more potent than MT in activating the two receptors (Table 1), this indicates that both AVPR1A and AVPR1B likely function as AVT-specific receptors (Fig. 1). In contrast, AVPR2b could be preferentially activated by AVT (EC_{50}: 1.50 nM) with a potency more than 8-fold higher than MT (EC_{50}: 12.61 nM). This suggests that AVPR2b is likely an AVT-preferring receptor, and it can still cross-react with MT at a slightly higher concentration. Interestingly, chicken MTR could be potently activated by both AVT and MT (Table 1), indicating that MTR is a receptor common for both peptides (Fig. 1). Moreover, our findings suggest that activation of all four receptors could cause intracellular calcium mobilization.

Similarly, using pGL4-SRE-luciferase reporter assay, we demonstrated that both AVPR1A and AVPR1B could be potently activated by AVP and not by MT, and AVPR2b could be preferentially activated by AVT, whereas MTR could be potently activated by both peptides (Fig. 2, Table 1). These findings suggest that activation of the four AVT/MT receptors can stimulate intracellular MAPK/ERK signaling cascade. In addition, as monitored by the pGL3-CRE-luciferase reporter assay (Fig. 3, Table 1), we found that activation of AVPR1A, AVPR1B and AVPR2b can stimulate cAMP/PKA signaling pathway while MTR cannot.

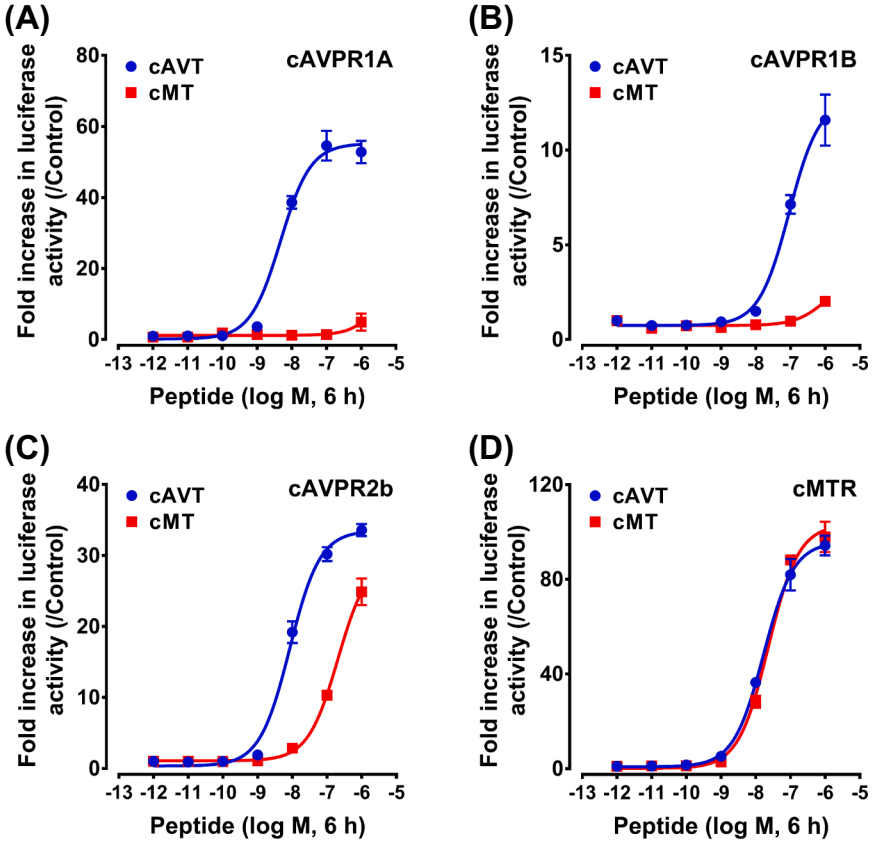

Fig. 2. (A-D) Effects of cAVT and cMT (10^{-12} to 10^{-6} M, 6 h) on activating chicken (c-) AVPR1A (A), AVPR1B (B), AVPR2b (C), and MTR (D) expressed in cultured HEK293 cells, monitored by a pGL4-SRE-luciferase reporter system. Each data point represents mean ± SEM of three replicates ($N = 3$).

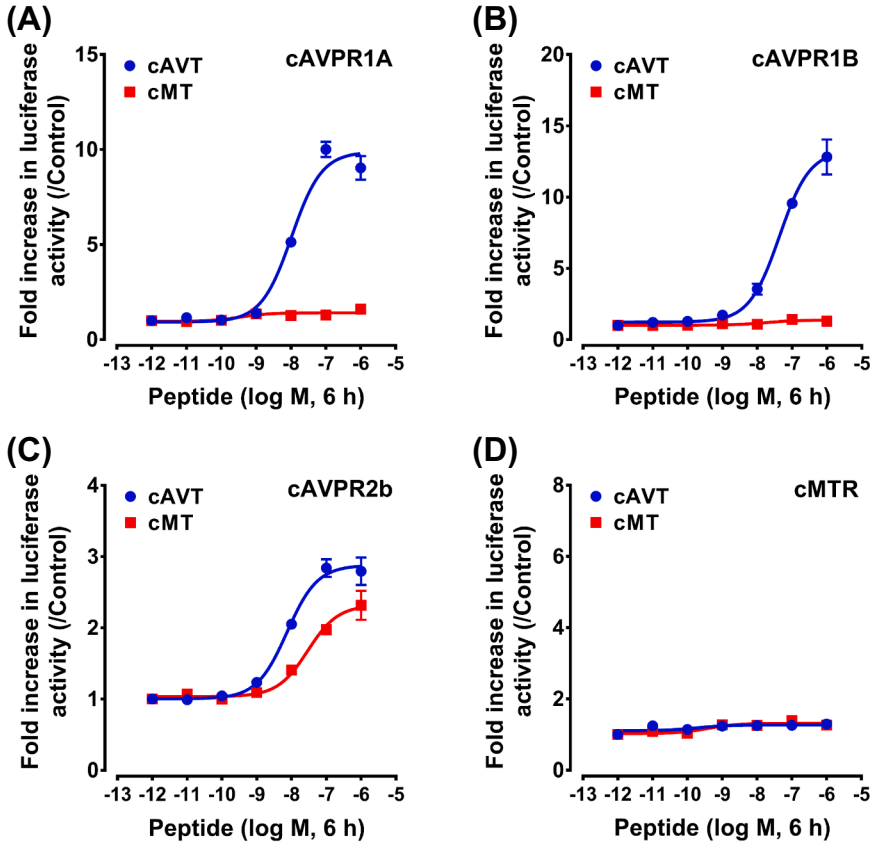

Fig. 3. (A–D) Effects of cAVT and cMT (10^{-12} to 10^{-6} M, 6 h) on activating chicken (c-) AVPR1A (A), AVPR1B (B), AVPR2b (C), and MTR (D) expressed in cultured HEK293 cells, monitored by a pGL3-CRE-luciferase reporter assay. *Note*: unlike the other 3 receptors (ie AVPR1A, AVPR1B, and AVPR2b), cMTR activation seems not capable of activating cAMP/PKA signaling pathway significantly (D). Each data point represents mean ± SEM of three replicates ($N = 3$).

To verify the functional coupling between the four receptors and intracellular calcium mobilization, MAPK/ERK, and cAMP/PKA pathways, pharmacological inhibitors of the three signaling pathways were used. As shown in Fig. 4 2-APB (an IP3 receptor antagonist, which can block IP3-induced calcium mobilization, 100 μM), U73122 [a phospholipase C (PLC) inhibitor, 20 μM], PD98059 (an inhibitor of MEK/MAPK signaling cascade, 100 μM) and H89 (a PKA inhibitor, 10 μM) could significantly inhibit AVT-induced luciferase activity of HEK293 cells expressing either receptor. These findings further confirmed the functional coupling of these receptors to multiple signaling pathways.

Using Western blot, we demonstrated that cAVT-activation of the four receptors expressed in HEK293 cells could enhance ERK1/2 (44/42 kDa) and CREB (43 kDa) phosphorylation (Fig. 5) (Note: MTR activation cannot induce CREB phosphorylation, *data not shown*). These findings further support the notion that AVPR1A, AVPR1B and AVPR2b are coupled to both MAPK/ERK and cAMP/PKA/CREB signaling pathways, and MTR to MAPK/ERK signaling cascade.

3.2. Tissue distribution of AVPR1A, AVPR1B, AVPR2b and MTR in chickens

Using quantitative real-time RT-PCR (qPCR), we examined the mRNA expression of *AVPR1A, AVPR1B, AVPR2b* and *MTR* in adult chicken tissues, including the anterior pituitary, heart, kidneys, liver, lung, breast muscle, testes, ovary, spleen, pancreas, subcutaneous fat, skin, adrenal gland, various brain regions (the telencephalon, midbrain, cerebellum, hindbrain, and hypothalamus), spinal cord, and gastrointestinal tract (including the crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum and colon).

As shown in Fig. 6, the four receptors are differentially expressed in chicken tissues. *AVPR1A* is expressed abundantly in the anterior pituitary, moderately in the midbrain, hypothalamus, kidneys, ovary, pancreas and colon, and weakly in other tissues examined. *AVPR1B* is predominantly expressed in the anterior pituitary and weakly expressed in other tissues examined. *AVPR2b* is highly expressed in the telencephalon, moderately in other brain regions, the anterior pituitary, heart, muscle, fat and skin, and weakly in other tissues examined. *MTR* is ubiquitously expressed in all tissues examined, with a relatively higher expression level noted in the anterior pituitary, midbrain, hindbrain, hypothalamus, heart, kidneys, testes, ovary, pancreas, fat, skin, cecum and colon.

3.3. Predominant expression of AVPR1A in the uterus of chickens and ducks

To determine which AVT receptor(s) is involved in mediating AVT-induced oviposition, we examined the mRNA levels of *AVPR1A*, *AVPR1B*, *AVPR2b* and *MTR* in various parts of oviducts (including the infundibulum, magnum, isthmus, uterus (shell gland), and vagina of 1-year-old laying hens by qPCR. As shown in Fig. 7A, only *AVPR1A* and *MTR* are preferentially expressed in the uterus, while *AVPR1B* and *AVPR2b* are widely distributed along the oviduct. Moreover, we found that *AVPR1A* showed the highest expression level among the four genes examined observed from the amplification plots in Fig. 7B, leading us to hypothesize that this likely represents the predominant AVT receptor in the uterus.

To elucidate this, we analyzed the transcriptomes of chicken (PRJNA173328) and duck (PRJNA449259) oviduct deposited in the Sequence ReadArchive (SRA) (Yin et al., 2019). As shown in Fig. 7C and D, only *AVPR1A* is predominantly expressed in the uterus of chickens and ducks. Moreover, the temporal expression of *AVPR1A* is shown to be in sync with oviposition in ducks, which increases gradually to a peak just before oviposition and drops afterwards.

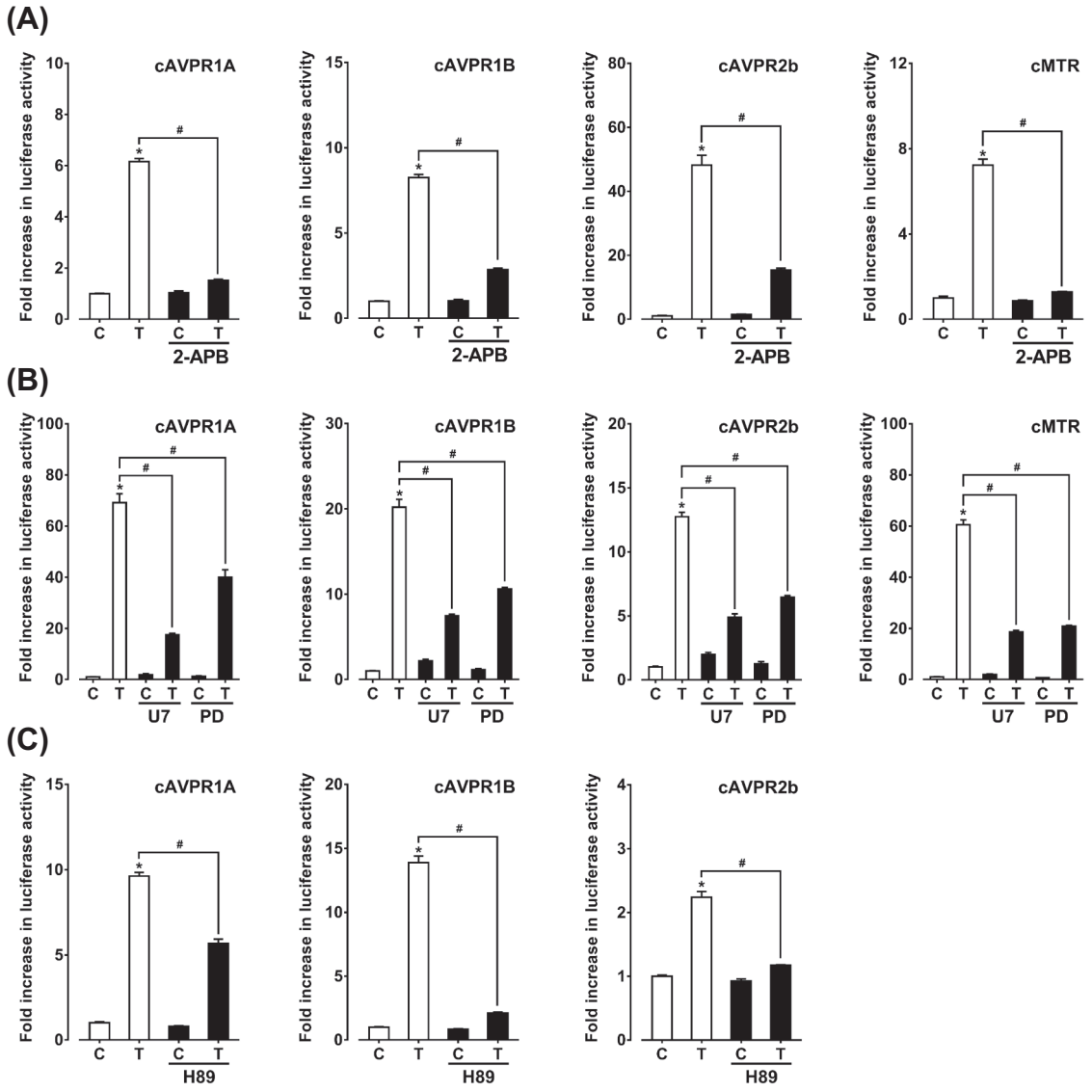

Fig. 4. (A) Effects of 2-APB (100 μM) on cAVT (100 nM, 6 h)-induced luciferase activity of HEK293 cell expressing chicken (c-) AVPR1A, AVPR1B, AVPR2b and MTR, as monitored by pGL3-NFAT-RE-luciferase reporter system. (B) Effects of U73122 (U7, 20 μM) and PD98059 (PD, 100 μM) on cAVT (100 nM, 6 h)-induced luciferase activity of HEK293 cell expressing cAVPR1A, cAVPR1B, cAVPR2b and cMTR, as monitored by pGL4-SRE-luciferase reporter system. (C) Effects of H89 (10 μM) on cAVT (100 nM, 6 h)-induced luciferase activity of HEK293 cell expressing cAVPR1A, cAVPR1B and cAVPR2b, as monitored by pGL3-CRE-luciferase reporter system. Each drug was added 0.5 h before cAVT treatment. In each graph, 'T' represents peptide treatment and 'C' represents control without peptide treatment. Each data point represents mean ± SEM of three replicates ($N = 3$). *, $P < 0.001$ *vs* control (without peptide treatment); $^{\#}$, $P < 0.001$ *vs* cAVT treatment (without drug treatment).

3.4. Spatial expression of AVPRs in chicken anterior pituitary

To evaluate the relative abundance of the four AVT/MT receptor transcripts in chicken anterior pituitaries, using RNA-seq data of chick pituitary obtained in our lab, we analyzed the transcript abundance of four genes. The results showed that *AVPR1B* has an extremely high expression level in the anterior pituitaries of chicks (Fig. 8A) and *AVPR1A* has a moderate level. In contrast, both *AVPR2b* and *MTR* have a very weak expression. As in chickens, the abundant expression of *AVPR1B* and *AVPR1A* has also been detected in adult duck pituitary by RNA-seq analysis (Fig. S2).

To further probe the spatial localization of *AVPR1A* and *AVPR1B* within the chicken anterior pituitary, we analyzed their relative mRNA levels in the cephalic (Ce) and caudal lobe (Ca) by qPCR. As shown in Fig. 8, both *AVPR1A* and *AVPR1B* are nearly exclusively expressed in the cephalic lobe of chicken anterior pituitary.

3.5. Effects of AVT on POMC and PRL mRNA expression in cultured chick pituitary cells

The abundant expression of *AVPR1A* and *AVPR1B* transcripts in the cephalic lobe where ACTH- and PRL-cells are localized, together with previous pioneering studies showing the localization of AVPR1B in ACTH- and PRL-cells and AVPR1A in ACTH-cells within the chicken anterior pituitary (Jurkevich et al., 2005, 2008; Selvam et al., 2013), led us to further examine whether AVT could regulate both the secretion and expression of ACTH (encoded by *POMC* gene) and PRL in cultured chick pituitary cells.

As shown in Fig. 9, AVT could stimulate ACTH secretion in cultured chick pituitary cells dose-dependently with minimal effect dose observed at 1 nM. Moreover, co-treatment of AVT with another corticotrophin-releasing factor, CRH, could further increase ACTH secretion, as detected by Western blot. Interestingly, AVT could also significantly

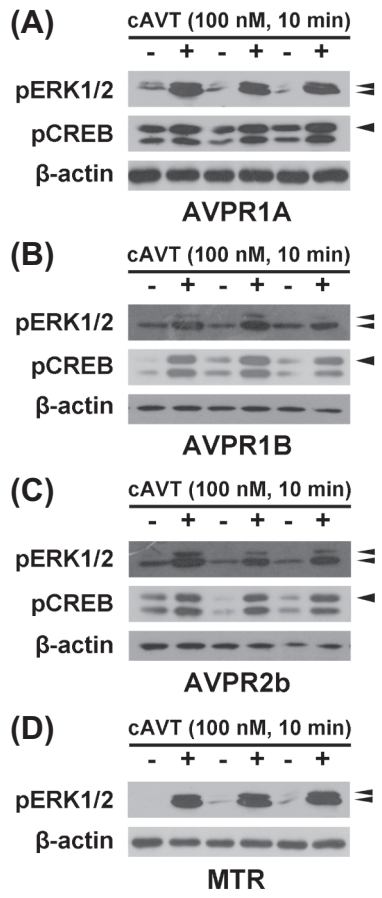

Fig. 5. Western blot showing that cAVT treatment (100 nM, 10 min) could enhance the phosphorylation levels of ERK1/2 (pERK1/2) in HEK293 cells expressing chicken AVPR1A (A), AVPR1B (B), AVPR2b (C) and MTR (D). In parallel, cAVT treatment could also enhance the phosphorylation level of CREB (marked by arrow) in HEK293 cells expressing chicken AVPR1A (A), AVPR1B (B), and AVPR2b (C). β-actin levels in the cell lysates were examined and used as loading controls. Each experiment was performed in triplicate.

induce *POMC* mRNA expression, and this stimulatory effect could be inhibited by U73122 and H89, suggesting that AVT-induced *POMC* expression is likely mediated by AVT receptor(s) coupled to both Gq-PLC and cAMP/PKA signaling pathways.

Like ACTH, AVT could stimulate PRL secretion and expression dose-dependently. Although both AVT and VIP [VIP is a potent PRL-releasing factor (PRF) in birds (Macnamee et al., 1986; Proudman and Opel, 1988; el Halawani et al., 1990)] could stimulate PRL secretion, AVT seems to be less potent than VIP (1 nM) (Fig. 10).

4. Discussion

In this study, we examined the functional similarity and difference and tissue expression of AVPR1A, AVPR1B, AVPR2b and MTR in chickens. Our study clearly indicates that AVPR1A and AVPR1B are two AVT-specific receptors, and AVPR2b is an AVT-preferring receptor, while MTR is a common receptor for both AVT and MT. qPCR assays revealed that the four AVT/MT receptors are differentially expressed in chicken tissues, such as the predominant expression of *AVPR1A* in the uterus and *AVPR1B* in the anterior pituitary. Moreover, we proved that AVT could stimulate both secretion and expression of pituitary ACTH and PRL *in vitro*. Undoubtedly, our data will help to explore the physiological role of AVT/MT in birds.

4.1. Identification of the receptor(s) for AVT (AVPR1A, AVPR1B, AVPR2b, MTR) and MT (MTR) in chickens

Although four receptors for chicken AVT/MT, named AVPR1A, AVPR1B, MTR and AVPR2b, have been cloned in previous studies (Tan et al., 2000; Cornett et al., 2003; Gubrij et al., 2005; Selvam et al., 2013), so far, the functional similarity and difference of the four AVT/MT receptors has not been sophisticatedly evaluated in birds. It is proposed that among the four chicken AVT/MT receptors identified, only *AVPR1A*, *AVPR1B*, and *MTR* are orthologous to human *AVPR1A*, *AVPR1B*, and *OXTR* respectively (Fig. 11), while avian *AVPR2b* (called *AVPR2c* in a previous study), which shares 42% a.a. identity with human *AVPR2*, is lost in humans (Fig. 11) (Ocampo Daza et al., 2012; Yamaguchi et al., 2012; Lagman et al., 2013). Due to the difference in receptor repertoire between humans and birds, the functional similarity and difference of the four avian AVT/MT receptors cannot be simply inferred from humans and warrants careful evaluation.

In this study, we proved that both AVPR1A and AVPR1B could be potently and specifically activated by AVT, indicating these receptors function as two AVT-specific receptors in chickens (Fig. 11). This is partially consistent with finding in a previous study, in which AVT can activate chicken AVPR1B (VT2) expressed in COS7 cells and increase the intracellular calcium level (Cornett et al., 2003). Similar to these findings in chickens, both AVPR1A and AVPR1B are reported to be specifically activated by AVP/vasotocin (VT) in mammals and elephant fish (Morel et al., 1992; Sugimoto et al., 1994; Yamaguchi et al., 2012). This indicates that both receptors share functional similarity and may act as AVP/AVT-specific receptors across vertebrates. It is well-documented that in mammals, both AVPR1A and AVPR1B are coupled to Gq-PLC signaling pathway and their activation triggers IP3-induced calcium mobilization (Gimpl and Fahrenholz, 2001). In this study, we demonstrated that both chicken AVPR1A and AVPR1B are likely coupled to Gq-PLC signaling pathway, since their activation can also stimulate calcium mobilization and MAPK/ERK signaling cascade, as evidenced by luciferase reporter assays and Western blot (Figs. 1–5). Moreover, we noted that both receptors are likely coupled to Gs protein and their activation may stimulate cAMP/PKA signaling pathway (Fig. 11). Consistent with our findings in chickens, Acharjee et al (2004) reported that frog and rat AVPR1A expressed in CV-1 cells is likely coupled to Gs protein (Acharjee et al., 2004), hinting the dual coupling potentials of AVPR1 to both Gq and Gs proteins exist in different classes of vertebrates.

Unlike AVPR1A and AVPR1B, chicken AVPR2b could be preferentially activated by AVT (4–8-fold more potent than MT) and its activation can stimulate calcium mobilization, MAPK/ERK and cAMP/PKA signaling pathways (Table 1). This finding indicates that chicken AVPR2b can act as an AVT-preferring receptor coupled to Gq and Gs proteins. Our finding differs slightly from a previous report, in which chicken AVPR2b (VT1) expressed in COS7 cells could bind AVT with a much higher affinity than MT (~29-fold) (Tan et al., 2000). This may be due to difference in methodology used in the two studies. However, similar to our observation in chickens, elephant fish AVPR2b expressed in CHO cells is preferentially activated by VT (~4 fold more potent than OXT) (Yamaguchi et al., 2012), leading to an elevation in intracellular calcium level. The pharmacological property of chicken AVPR2b differs significantly from that of human/rat AVPR2, which can be activated by AVP potently and specifically (~100–1000 fold more potent than OXT) and is primarily coupled to Gs-cAMP/PKA signaling pathway (Birnbaumer et al., 1992; Lolait et al., 1992), a pathway crucial for water re-absorption in the renal collecting tubules. Though chicken AVPR2b and human AVPR2 share 42% a.a. identity (Fig. S1) (Ocampo Daza et al., 2012; Yamaguchi et al., 2012), they are genes of distinct origins. Therefore, it is not surprising that chicken AVPR2b has pharmacological and signaling property different from that shown by human AVPR2 (Fig. 11).

In this study, we also proved that chicken MTR could be potently

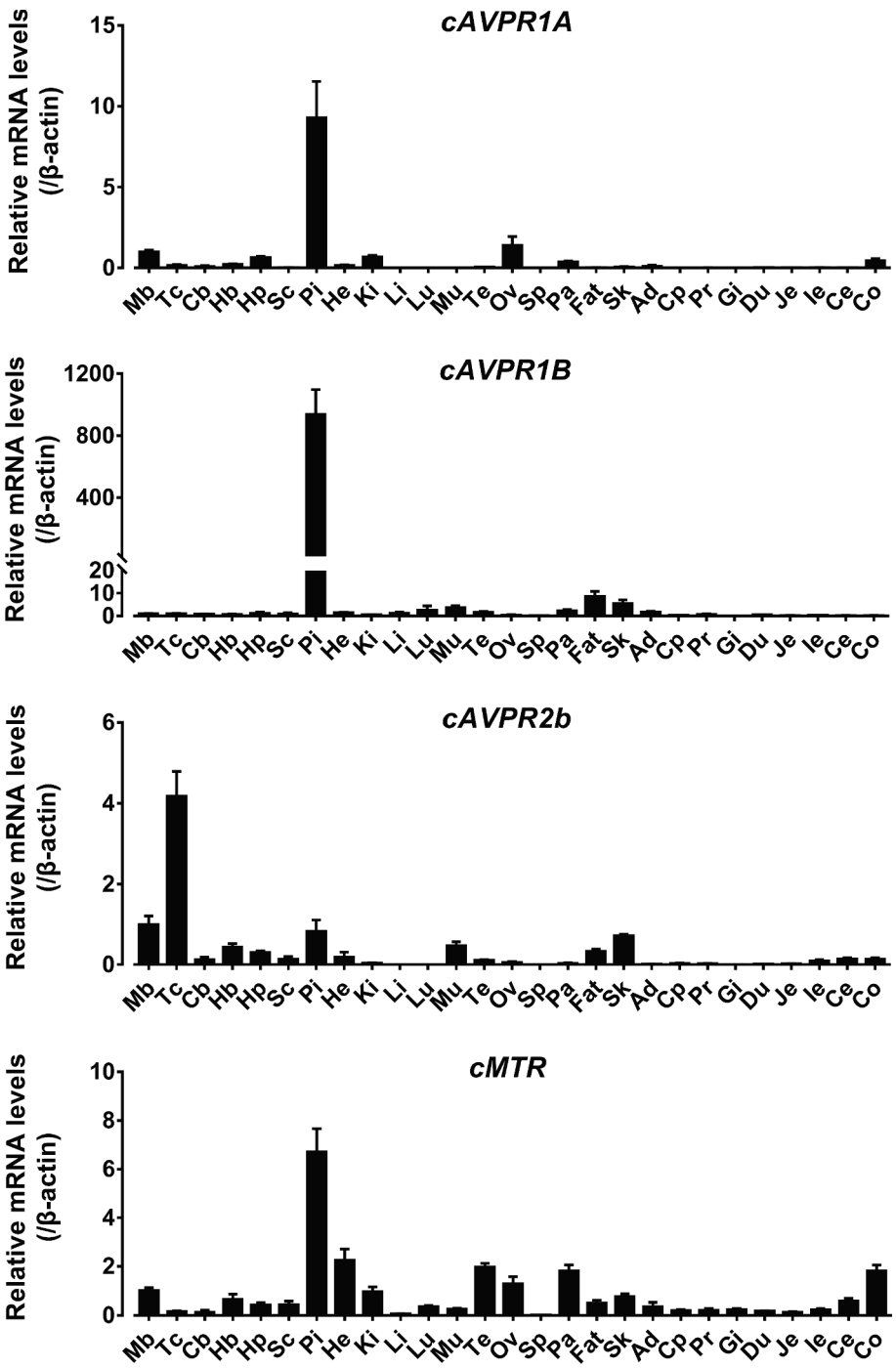

Fig. 6. Quantitative real-time RT-PCR (qPCR) assay of *AVPR1A*, *AVPR1B*, *AVPR2b* and *MTR* mRNA levels in different adult chicken tissues including the midbrain (Mb), telencephalon (Tc), cerebellum (Cb), hindbrain (Hb), hypothalamus (Hp), spinal cord (Sc), anterior pituitary (Pi), heart (He), kidneys (Ki), liver (Li), lung (Lu), muscle (Mu), testes (Te), ovary (Ov), spleen (Sp), pancreas (Pa), fat (Fat), skin (Sk), adrenal gland (Ad), crop (Cp), proventriculus (Pr), gizzard (Gi), duodenum (Du), jejunum (Je), ileum (Ie), cecum (Ce) and colon (Co). The mRNA levels of target genes were normalized by that of *β-actin* and expressed as the fold difference compared with that of the midbrain (Mb). The data point of each tissue represents the mean ± SEM of six adult chickens (3 males and 3 females) ($N = 6$), with the exception of that of testes and ovaries, which represent the mean ± SEM of three adult chickens ($N = 3$).

activated by both AVT and MT, monitored by pGL3-NFAT-RE-luciferase and pGL4-SRE-luciferase reporter assays (Figs. 1–5), indicating that chicken MTR (orthologous to human OXTR) is a common receptor for both peptides and functionally coupled to Gq-PLC signaling pathway. As in chickens, elephant fish OXTR expressed in CHO cells was demonstrated to be potently activated by VT and OXT and led to an elevation of intracellular calcium level (Yamaguchi et al., 2012). In contrast, human OXTR expressed in *Xenopus* oocytes or CV-1 cells, could be specifically activated by OXT and thus acts as an OXT-specific receptor coupled to Gq-PLC signaling pathway (Kimura et al., 1992; Acharjee et al., 2004). The ligand selectivity towards OXT of human OXTR hints that mammalian OXTR may have undergone a structural/functional change after its split from avian lineage.

In summary, in chickens, AVT possesses four receptors namely AVPR1A, AVPR1B, AVPR2b and MTR, in which AVPR1A and AVPR1B are specific to AVT only, and MT has a single receptor, MTR. The ligand-receptor interaction of avian AVT/MT system differs significantly from that of human/rat AVP/OXT system, in which AVP has three AVP-specific receptors (AVPR1A, AVPR1B, AVPR2) and OXT has an OXT-specific receptor OXTR (Fig. 11) (Gimpl and Fahrenholz, 2001). Furthermore, the identification of the four AVT receptors including MTR in birds, points to the possibility that some of the reproduction-related actions (*e.g.* oxytocic action) regulated by 'OXT-OXTR' axis in mammals may be taken over by the 'AVT-AVPR/AVT-MTR' axis in birds (Figs. 7 and 11), which was investigated in this study and discussed below.

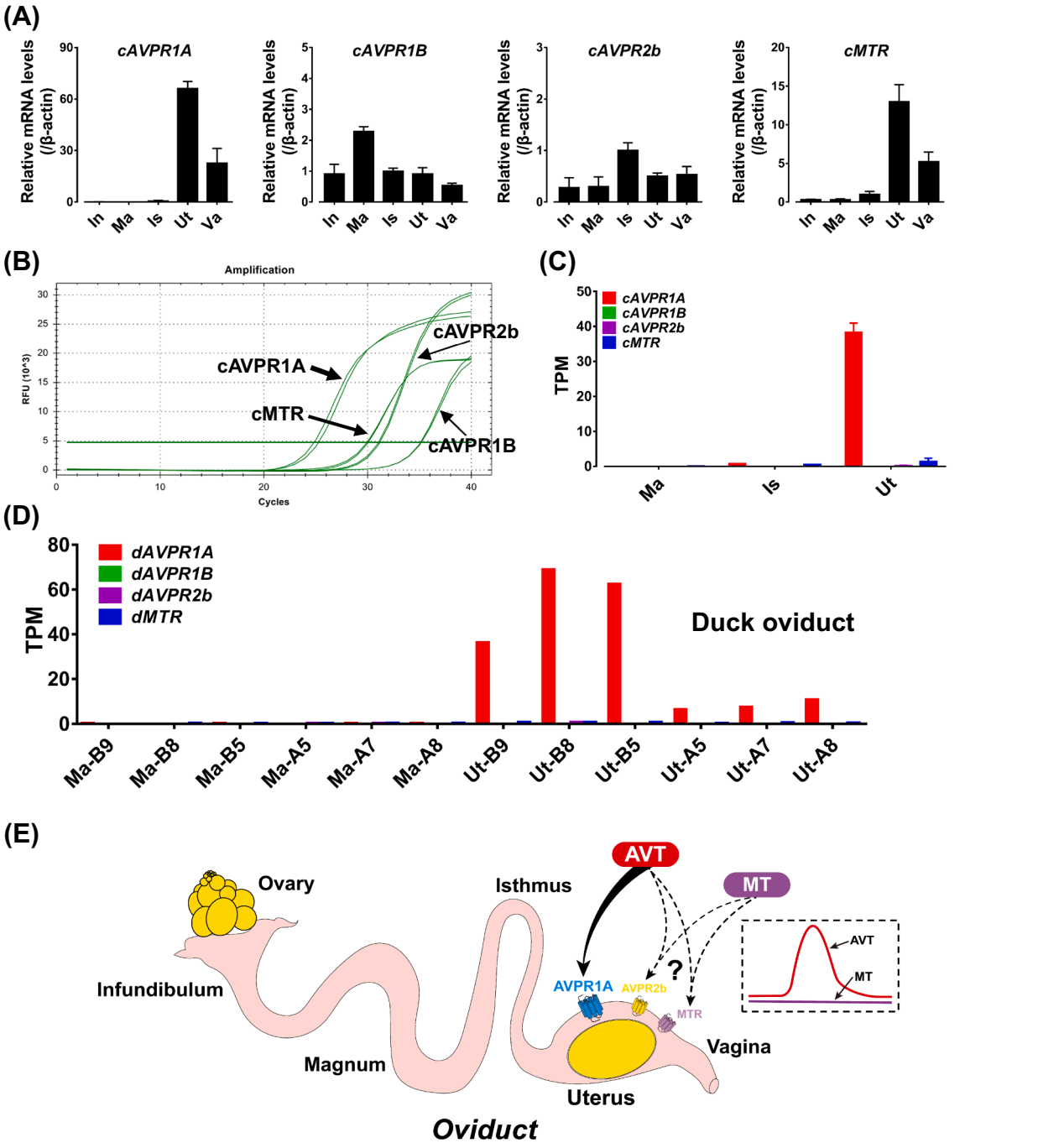

Fig. 7. (A) qPCR detection of chicken (c-) *AVPR1A*, *AVPR1B*, *AVPR2b*, and *MTR* mRNA levels in the various parts of oviduct including the infundibulum (In), magnum (Ma), isthmus (Is), uterus (Ut) and vagina (Va). Each data point represents the mean ± SEM of 3 laying hens ($N = 3$). (B) Amplification plots showing the abundant expression of *cAVPR1A*, and not the other 3 receptors (ie *cMTR*, *cAVPR2b*, and *cAVPR1B*), in the uterus of two laying hens. *Note:* the same amount of cDNA template was used for qPCR assay. (C) RNA-Seq analysis showing the abundant expression of *cAVPR1A* (and not *cAVPR1B/cAVPR2b/cMTR*) in the uterus of laying hens ($N = 2$). (D) RNA-Seq analysis showing the changes in *AVPR1A* (not *AVPR1B/AVPR2b/MTR*) mRNA level in duck (d) uterus before (9 h/8h/5h before oviposition: Ut-B9/B8/B5) and after oviposition (5 h/7h/8h after oviposition: Ut-A5/7A/A8). The expression of the four genes in the magnum (Ma) was shown in parallel. (E) Proposed model of AVT action in avian (*e.g.* chicken) uterus. The surge of plasma AVT (and not MT) (shown in the box) may induce oviposition through AVPR1A abundantly and specifically expressed in the uterus, while MT and the other three AVT receptors may play less significant roles during oviposition. However, this hypothesis needs further verification.

4.2. Differential expression of the four AVT/MT receptors in chicken tissues

In this study, we found that AVT/MT receptors are differentially expressed in adult chicken tissues (Fig. 6). *cAVPR1B* is predominantly expressed in the anterior pituitary and weakly in other tissues examined. This finding is in accordance with previous findings in chickens and mammals (Engler et al., 1999; Jurkevich et al., 2005), in which *AVPR1B* is mainly expressed in the corticotrophs and involved in mediating AVT/AVP action on ACTH secretion (Engler et al., 1999; Cornett et al., 2013).

Like *AVPR1B*, *AVPR1A* is also highly expressed in the anterior pituitary of chickens (Fig. 6). This finding, together with the localization

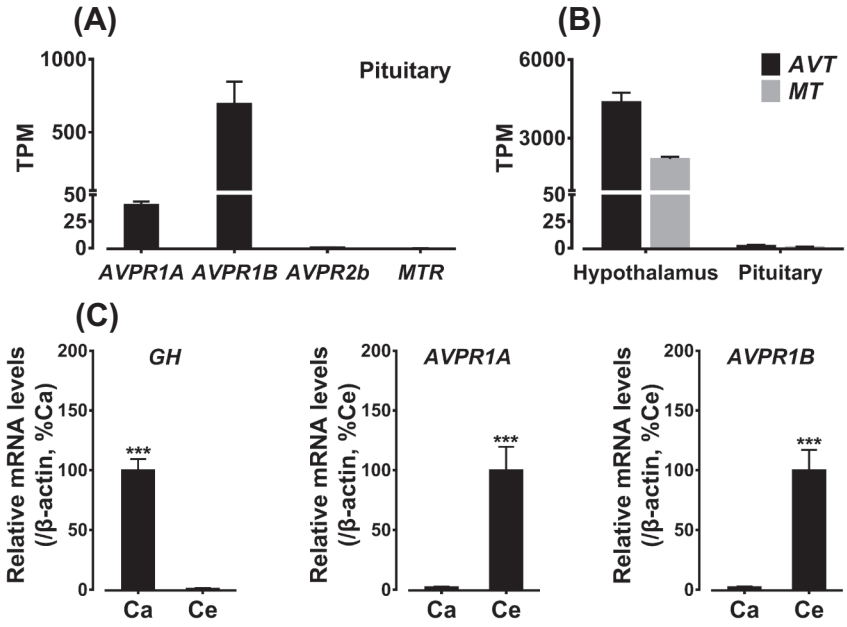

Fig. 8. (A-B) RNA-seq assays showing that (A) *AVPR1B* and *AVPR1A* (not *AVPR2b/MTR*) are abundantly expressed in chick anterior pituitaries. (B) RNA-Seq showing that *AVT* and *MT* are predominantly expressed in chicken hypothalamus (and not in the pituitary), as previously reported (Robinzon et al., 1988a,b). (C) qPCR assay showing the relative mRNA levels of growth hormone *(GH)*, *AVPR1A*, and *AVPR1B* in the caudal (Ca) and cephalic (Ce) lobes of anterior pituitaries collected from adult chickens. The mRNA levels of each gene were normalized to that of *β-actin* and expressed as the percentage to that observed in the cephalic lobe (Ce) or caudal lobe (Ca). Each data point represents the mean ± SEM of 6 individual adult male chickens ($N = 6$). ***, $P < 0.001$ vs cephalic lobe (Ce) or caudal lobe (Ca).

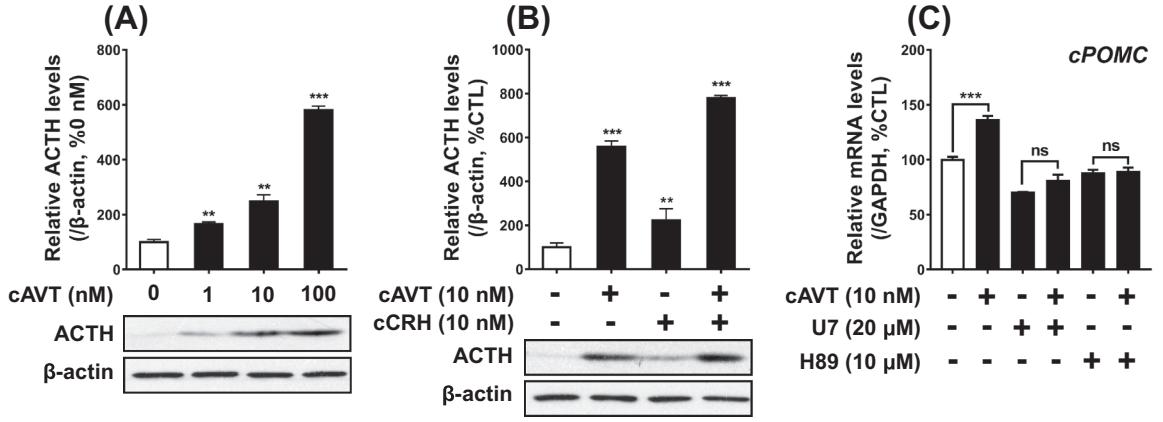

Fig. 9. (A) Western blot analysis showing that cAVT treatment (1–100 nM, 4 h) can dose-dependently increase ACTH secretion (~5kDa band) to the culture medium of the cultured chick pituitary cells. In parallel, β-actin levels in cell lysates were also examined by Western blot and used as loading controls. (B) Both cAVT (10 nM) and cCRH (10 nM) treatment for 4 h can increase ACTH secretion detected by Western blot. Co-treatment of AVT and CRH can further increase ACTH secretion, however, it remains unclear whether their actions are synergistic/additive under our experimental condition. In (A) and (B), ACTH levels in culture medium were determined using densitometric analysis and normalized by that of β-actin, and then expressed as the percentage of respective control (without peptide treatment). The representative set of Western blot is shown at the bottom of each graph. Each data point represents mean ± SEM of three replicates ($N = 3$). **, $P < 0.01$ *vs* control; ***, $P < 0.001$ *vs* control. (C) qPCR assay showing that cAVT (10 nM, 24 h)-induced *cPOMC* expression could be significantly inhibited by U73122 (U7, 20 μM) or H89 (10 μM) treatment. The expression levels of *cPOMC* were normalized by *GAPDH* and expressed as the percentage of the control (without peptide treatment). Each value represents the mean ± SEM of four replicates ($N = 4$). ***, $P < 0.001$ vs. control. 'ns' mean no statistical difference between two groups.

of *AVPR1A* (VT4) in the corticotrophs of chicken anterior pituitary (Selvam et al., 2013), indicates that both AVPR1B and AVPR1A are likely associated with neuroendocrine regulation of stress (*e.g* pituitary ACTH secretion) (Kuenzel et al., 2013). Within the CNS, *AVPR1A* is widely expressed in various brain regions including the hypothalamus and midbrain. This is consistent with a previous study, in which AVPR1A was found to be widely expressed in glia and neurons throughout the brain (including hypothalamic nuclei) (Selvam et al., 2015). This line of evidence supports the notion that AVPR1A signaling is involved in the regulation of several basic physiological and behavioral functions such as social behavior, neurogenesis and food intake, as reported in chickens and mammals (Donaldson and Young, 2008; Aoyagi et al., 2009; Masunari et al., 2016; Nagarajan et al., 2016). In the periphery, *AVPR1A* is moderately expressed in the kidneys, ovary, pancreas and colon, suggesting that AVPR1A signaling may regulate the physiological processes therein, such as AVT-induced renal anti-diuresis (Brummermann and Braun, 1995). Interestingly, *AVPR1A* mRNA is almost undetectable in chicken liver, suggesting that AVPR1A signaling is likely not important for liver glycogenolysis. This is in sharp contrast to the finding in mammals, in which AVPR1A abundantly expressed in the liver is proposed to be responsible for glycogenolysis (Morel et al., 1992).

Like *AVPR1A*, *AVPR2b* is widely distributed in the CNS, including the telencephalon and hypothalamus, suggesting that like AVPR1A, AVPR2b could mediate part of AVT/MT actions in the CNS (Selvam et al., 2015). In addition, *AVPR2b* is also moderately expressed in several peripheral tissues, including the anterior pituitary, muscle, fat and skin and weakly expressed in other tissues examined, suggesting its possible roles in these tissues. Interestingly, unlike AVPR2 abundantly expressed in mammalian kidneys (Birnbaumer et al., 1992; Lolait et al.,

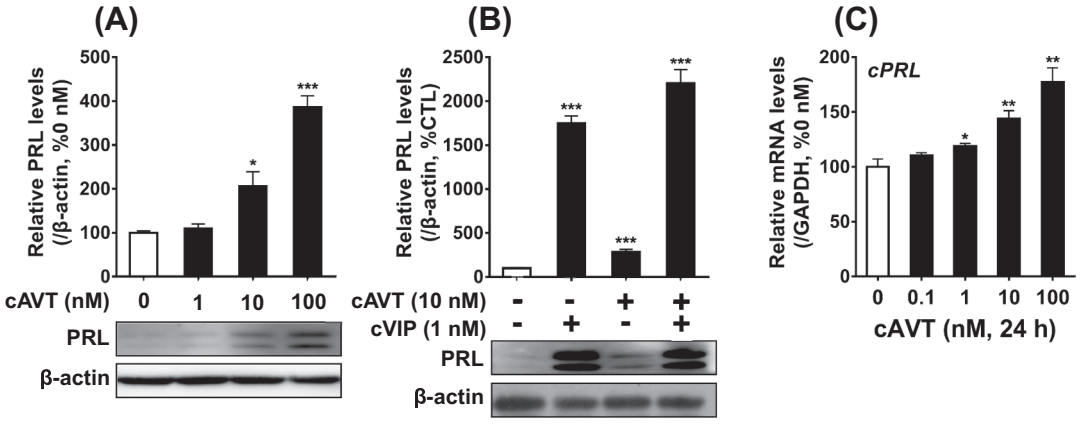

Fig. 10. (A) Western blot analysis showing that cAVT treatment (1–100 nM, 4 h) can dose-dependently increase PRL secretion to the culture medium from cultured chick pituitary cells. (B) As previously reported in turkeys (Proudman and Opel, 1988), both AVT (10 nM) and VIP (1 nM) treatment for 4 h could increase PRL secretion from cultured chick pituitary cells (detected by Western blot). Interestingly, co-treatment of both peptides could further increase PRL secretion slightly. In (A) and (B), PRL levels in culture medium were determined using densitometric analysis and normalized by that of cellular β-actin, and then expressed as the percentage of respective control (without peptide treatment). The representative set of Western blot is shown at the bottom of each graph [*Note:* two major PRL bands of ~27 kDa and ~23 kDa were detected in culture medium, as previously reported (Huang et al., 2014, Bu et al, 2016)]. Each data point represents mean ± SEM of three replicates ($N = 3$). *, $P < 0.05$ *vs* control; ***, $P < 0.001$ *vs* control; (C) qPCR assay showing that cAVT (0.1–100 nM, 24 h) could increase *cPRL* mRNA expression dose-dependently in cultured chick pituitary cells. Each value represents the mean ± SEM of four replicates ($N = 4$). *, $P < 0.05$ *vs* control; ** $P < 0.01$ *vs* control.

1992), AVPR2b is only weakly expressed in chicken kidneys. This finding, together with the significant pharmacological/signaling difference between chicken AVPR2b and mammalian AVPR2 (Fig. 11), cast doubts on whether chicken AVPR2b, like mammalian AVPR2, could mediate the anti-diuretic action of AVT on renal collecting tubules (Birnbaumer et al., 1992; Lolait et al., 1992).

Unlike the regional or tissue-specific distribution of *AVPR1A*, *AVPR1B* and *AVPR2b*, chicken *MTR* is widely distributed in all tissues examined including various brain regions, anterior pituitary, heart, kidneys, fat, pancreas, GI tract, ovary and testes. The wide expression of *MTR/OXTR* has also been found in Japanese tree frogs (Kohno et al., 2003) and elephant fish (Yamaguchi et al., 2012). Considering the fact that MTR/OXTR in chickens and elephant fish acts as a common receptor for both peptides (Fig. 11), it suggests that MTR(/OXTR) can partially mediate the diverse actions of both AVT and MT in non-mammalian vertebrates, for instance, reproductive behaviors and functions in the CNS and peripheral tissues (*e.g.* ovary, testes, and uterus) (Jurkevich and Grossmann, 2003).

4.3. *AVPR1A is likely a key receptor mediating AVT-induced oviposition in birds*

It is well-known that in mammals, OXT induces uterine contraction via OXTR during parturition, thus acting as the most potent uterotonic hormone (Gimpl and Fahrenholz, 2001). However, in birds, there are compelling evidence that AVT, and not MT, is the key regulator of avian oviposition, a process involving similar uterine contraction for egg expulsion (Munsick et al., 1960; Rzasa and Ewy, 1970; Wilson et al., 2017). In support of this notion, AVT (and not MT) is rapidly released from the posterior pituitary during oviposition, and its plasma levels transiently increase to reach a peak at oviposition and drop rapidly afterwards in chickens (Fig. 7E) (Tanaka and Nakajo, 1962; Sturkie and Lin, 1966; Niezgoda et al., 1973; Nouwen et al., 1984; Koike et al., 1988; Seth et al., 2004). Moreover, AVT, and not MT, has been shown to stimulate the contraction of uterine muscle strips *in vitro*. (Koike et al., 1988). All these findings support the hypothesis raised in the 1960s that AVT(/AVP), and not MT(/OXT), exerts 'oxytocin-like action' (oviposition) in birds (Burrows and Fraps, 1942; Munsick et al., 1960; Tanaka and Nakajo, 1962; Rzasa and Ewy, 1970). Meanwhile, this finding raises another interesting question on which AVT receptor(s) could mediate AVT-induced oviposition.

In this study, we found that only *AVPR1A* is abundantly and specifically expressed in the uterus of chicken (and ducks) (Fig. 7), whereas the other three AVT receptors (*AVPR1B*, *AVPR2b*, *MTR*) have much lower expression levels. Moreover, we noted that *AVPR1A*, and not the other three AVT receptors, shows a temporal expression pattern synchronized with oviposition in duck uterus, in which *AVPR1A* expression reaches a peak level just before oviposition and drops sharply afterwards (Fig. 7D), which may coincide with the plasma AVT trend during avian oviposition (Niezgoda et al., 1973). All these findings suggest that AVPR1A is most likely a key candidate receptor mediating AVT-induced oviposition (Fig. 7). Concurrent with our finding, a few previous studies have suggested that the binding sites specific for AVT exist on chicken uterine myometrial membrane (Koike et al., 1988) and they show a dynamic variation before and after oviposition (Takahashi et al., 1992, 1994).

In previous studies, the other two AVT receptors, ie MTR and AVPR2b (VT1), have been found to be expressed in chicken uterus and proposed to mediate AVT-induced oviposition (Tan et al., 2000; Seth et al., 2004; Gubrij et al., 2005), however, their mRNA levels are much lower than that of *AVPR1A* detected in our present study. Considering that both AVT and MT can activate MTR and AVPR2b effectively *in vitro* (Figs. 1–3), the specific action of AVT (and not MT) on uterine contraction tends to support the involvement of an AVT-specific receptor, AVPR1A, which is highly expressed in the uterus (Fig. 7). Further studies on the detailed localization of AVPR1A in the avian uterus will help to clarify whether AVPR1A alone, or in combination with AVPR2b/MTR, can mediate AVT-induced oviposition.

Interestingly, a relatively high mRNA level of *AVPR1A* has also been found in the vagina of chicken oviduct (Fig. 7). Similarly, a previous report has described the AVT-specific binding sites in the vagina of laying hens (Takahashi et al., 1998). This suggests that AVPR1A is likely a candidate receptor involved in the coordinated action of vagina during oviposition. Nevertheless, this idea needs further verification.

4.4. *AVT stimulates both the expression and secretion of ACTH and PRL in cultured pituitary cells via AVPR1(s)*

In this study, both *AVPR1B* and *AVPR1A* are found to be abundantly expressed in the cephalic lobe (Fig. 8), where ACTH-cells and PRL-cells

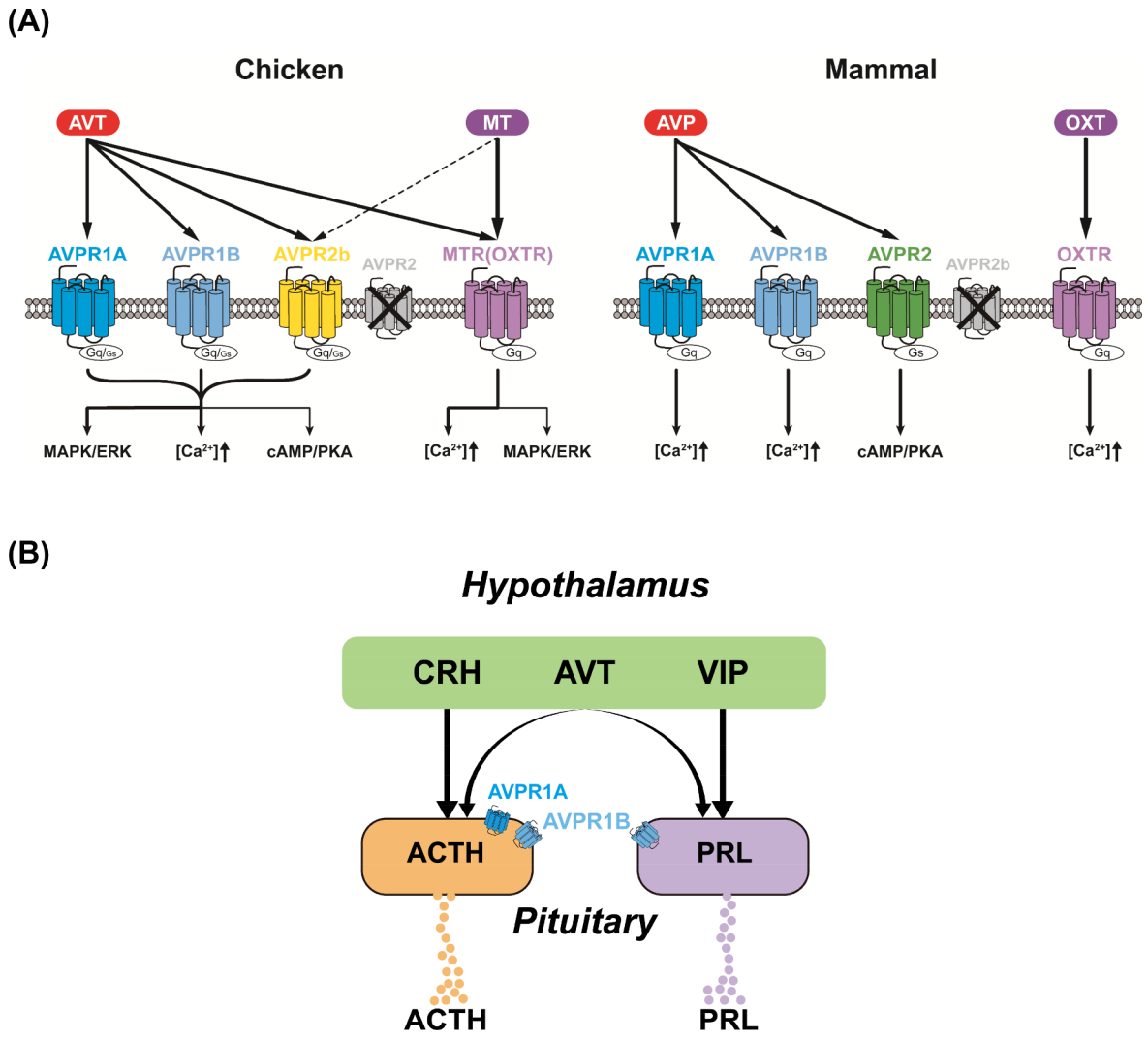

Fig. 11. (A) Schematic diagram showing the difference in the ligand-receptor interaction of AVT(AVP)/MT(OXT) systems between chickens and mammals (*e.g.* humans). In chickens, AVT has four receptors: AVPR1A, AVPR1B, AVPR2b and MTR, while MT has a single receptor MTR (OXTR), which could also be activated by AVT potently. Interestingly, AVPR2b could be preferentially activated by AVT (dotted line indicates that MT is ~4–8-fold less potent than AVT). The four AVT/MT receptors are likely coupled to Gq(/Gs) proteins and their activation can stimulate multiple signaling pathways. In contrast, in mammals (*e.g.* humans), AVP has three specific receptors, which selectively bind AVP, while OXT possesses a single OXT-specific receptor (OXTR). Among the four receptors, AVPR1A, AVPR1B, and OXTR are coupled to Gq protein and their activation increases intracellular calcium level, whereas AVPR2 is coupled to Gs protein and its activation stimulates cAMP/PKA signaling pathway. *Note: AVPR2b* and *AVPR2* are genes of distinct origins in vertebrates. *AVPR2b* exists in chickens, but is lost in humans (Yamaguchi et al., 2012; Lagman et al., 2013), while *AVPR2* exists in humans, but is lost in chickens. (B) A simplified model showing ACTH and PRL expression and secretion could be regulated by hypothalamic AVT in chickens, in addition to CRH and VIP, which function as corticotropin-releasing factor (CRF) and prolactin-releasing factor (PRF) (Macnamee et al., 1986), respectively.

reside (Scanes, 2015). Our finding is consistent with previous pioneering studies showing the localization of AVPR1B in corticotrophs and lactotrophs (Jurkevich et al., 2005, 2008) and AVPR1A in corticotrophs (Selvam et al., 2013). This suggests that AVT is likely a regulator of pituitary ACTH and PRL secretion in chickens. In agreement with this notion, we proved that AVT can stimulate ACTH secretion. It supports that besides CRH (Scanes, 2015), AVT can also act as a 'CRF' in chickens (Fig. 11B), similar to the previous study on cultured duck pituitary cells (Castro et al., 1986). As in birds, AVT(/AVP)-induced ACTH secretion has also been reported in mammals (Engler et al., 1999), frogs (Okada et al., 2016), and rainbow trout (Baker et al., 1996). All these evidence suggests that AVT-induced ACTH secretion is actively involved in stress responses, such as osmotic stress in birds and other vertebrates (Nouwen et al., 1984). In addition, we found that AVT can stimulate *POMC* mRNA expression at 24 h or 48 h (*data not shown*), which is likely mediated by AVT receptor coupled to Gq-PLC and cAMP/PKA signaling pathways (Fig. 9). The time of AVT action deviates slightly from a previous study, in which AVT can stimulate *POMC* heteronuclear RNA (hnRNA) within 6 h (Jayanthi et al., 2014). The reason for this discrepancy is unknown at present. However, our finding provides a piece of evidence that AVT can stimulate both the expression and secretion of ACTH (POMC) in chickens, distinct from the established concept in mammals, in which CRH, and not AVP, is mainly responsible for *POMC* expression (Engler et al., 1999).

In addition, we found that AVT not only induces cPRL secretion, but also stimulates c*PRL* mRNA expression *in vitro*. To our knowledge, AVT-induced *PRL* mRNA expression has not been reported in any avian species before. Our finding is partially consistent with previous reports in turkeys and mammals, in which AVT/AVP can induce PRL secretion *in vitro* (Kimura et al., 1992; Freeman et al., 2000). However, it should be noted that a relatively high concentration of AVT (≥10 nM) is required to induce PRL secretion in birds and mammals when compared to other PRF in birds [*e.g.* VIP (Macnamee et al., 1986; Proudman and Opel, 1988; el Halawani et al., 1990)] and mammals (*e.g.* thyrotropin-

releasing hormone, TRH), which functions at sub-nanomolar range (Proudman and Opel, 1988; Liu and Ben-Jonathan, 1994; Freeman et al., 2000; Bu et al., 2016). Therefore, the question whether hypothalamic AVT alone, or in combination with other factor(s), could regulate avian PRL secretion *in vivo* remains to be clarified.

Although transcripts of the four AVT receptors were detected in the anterior pituitary, only *AVPR1B* and *AVPR1A* could easily be detected by qPCR. Moreover, transcriptome analyses indicate that both *AVPR1A* and *AVPR1B* are abundantly expressed in chick (and duck) anterior pituitaries, while the other two receptors have a very low expression level. All these findings, together with the predominant expression of *AVT* in chicken hypothalamus (Fig. 8), support that AVT-induced PRL and ACTH expression and secretion is mediated by *AVPR1B* expressed in corticotrophs and lactotrophs and *AVPR1A* in corticotrophs (Jurkevich et al., 2008; Selvam et al., 2013).

In summary, we proved that AVT possesses four functional receptors (AVPR1A, AVPR1B, AVPR2b and MTR), and MT has a single receptor (MTR). The ligand-receptor interaction of AVT/MT system differs significantly from that in humans (or rats) depicted in Fig. 11. qPCR assays revealed that *AVPR1A*, *AVPR1B, AVPR2b* and *MTR* are differentially expressed in chicken tissues, suggesting the broad spectrum of AVT and MT actions in the CNS and peripheral tissues may be mediated by distinct receptors expressed in a tissue- or cell-specific manner. Within the anterior pituitary, both AVPR1A and AVPR1B are highly expressed in the cephalic lobe and likely involved in mediating AVT-induced PRL and ACTH expression (Fig. 11). Within the oviduct, *AVPR1A* is specifically and abundantly expressed in the uterus, suggesting its involvement in mediating AVT-induced oviposition. Taken together, our data will help to reveal the functional conservation and change of AVT/MT system across vertebrates, such as the 'oxytocic action' of AVT proposed in birds more than 50 years ago.

Acknowledgement

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Disclosure statement

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.05.013.

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