

The interaction of MC3R and MC4R with MRAP2, ACTH, α -MSH and AgRP in chickens

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Abstract

The interaction of melanocortin-4 (MC4R) and melanocortin-3 (MC3R) receptors with proopiomelanocortin (POMC)-derived peptides (e.g. α -MSH), agouti-related protein (AgRP) and melanocortin-2 receptor accessory protein 2 (MRAP2) is suggested to play critical roles in energy balance of vertebrates. However, evidence on their interaction in birds remains scarce. Our study aims to reveal their interaction in chickens and the results showed that (1) chicken (c-)MC3R and cMC4R expressed in Chinese hamster ovary (CHO) cells can be activated by α -MSH and ACTH_{1–39} equipotently, monitored by a pGL3-CRE-luciferase reporter system; (2) cMC3R and cMC4R, when co-expressed with cMRAP2 (or cMRAP, a cMRAP2 homolog), show increased sensitivity to ACTH treatment and thus likely act as ACTH-preferring receptors, and the interaction between cMC3R/cMC4R and cMRAP2 was demonstrated by co-immunoprecipitation assay; (3) both cMC3R and cMC4R display constitutive activity when expressed in CHO cells, as monitored by dual-luciferase reporter assay, and cMRAP2 (and cMRAP) can modulate their constitutive activity; (4) AgRP inhibits the constitutive activity of cMC3R/cMC4R, and it also antagonizes ACTH/ α -MSH action on cMC4R/cMC3R, indicating that AgRP functions as the inverse agonist and antagonist for both receptors. These findings, together with the co-expression of *cMC4R*, *cMC3R*, *cMRAP2*, *cAgRP* and *cPOMC* in chicken hypothalamus detected by quantitative real-time PCR, suggest that within the hypothalamus, α -MSH/ACTH, AgRP and MRAP2 may interact at the MC4R/MC3R interface to control energy balance. Furthermore, our data provide novel proof for the involvement of MRAP2 (and MRAP) in fine-tuning the constitutive activity and ligand sensitivity and selectivity of both MC3R and MC4R in vertebrates.

Key Words

- ▶ chicken
- ▶ MC3R and MC4R
- ▶ MRAP2
- ▶ ACTH and α -MSH
- ▶ AgRP

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Introduction

It is well documented that in mammals, proopiomelanocortin (*POMC*) gene encodes a large POMC precursor, and the tissue-specific processing of POMC precursor by two prohormone convertases (PC1/3 and PC2) gives rise to several structurally related melanocortin

peptides, including adrenocorticotropin (ACTH), α -melanocyte-stimulating hormone (α -MSH), β -MSH and γ -MSH (Cone 2006). In the anterior pituitary, POMC can generate ACTH of 39 amino acids (ACTH_{1–39}) via PC1/3 processing, while in the hypothalamus and intermediate

lobe of the pituitary, POMC mainly yields α -MSH via PC1/3 and PC2 processing (Cawley *et al.* 2016). These bioactive peptides exert diverse actions, such as regulation of skin pigmentation, adrenal gland function and energy balance, through interaction with 5 melanocortin receptors (MCR), namely MC1R, MC2R (also called ACTHR), MC3R, MC4R and MC5R respectively (Chhajlani & Wikberg 1992, Mountjoy *et al.* 1992, Yaswen *et al.* 1999, Cone 2006). All these MCRs belong to G protein-coupled receptor (GPCR) and their activation can trigger multiple signaling pathways, including cAMP signaling pathway (Cone 2006, Ghamari-Langroudi *et al.* 2015).

Among the 5 MCRs studied in mammals, MC4R and MC3R and their ligand (i.e. α -MSH) have attracted much attention due to their pivotal roles in the regulation of food intake, energy expenditure, body weight, metabolism and obesity (Krude *et al.* 1998, Cone 2006). It is clear that within the hypothalamus, α -MSH derived from POMC neurons in the arcuate nucleus can bind to and activate MC3R and MC4R expressed in different hypothalamic nuclei and thus regulate energy balance (Roselli-Rehfuß *et al.* 1993, Mountjoy *et al.* 1994). Targeted disruption of MC4R causes hyperphagia, obesity, hyperinsulinemia and longitudinal growth in mice (Huszar *et al.* 1997). Similarly, inactivation of mouse MC3R causes an increase in fat mass and obesity (Butler *et al.* 2000, Chen *et al.* 2000). These findings emphasize the importance of MC4R/MC3R signaling in energy homeostasis. Apart from α -MSH, agouti-related protein (AgRP), an endogenous antagonist of MC4R/MC3R, can also bind to MC4R/MC3R and control energy balance mainly via antagonizing α -MSH actions (Fong *et al.* 1997, Ollmann *et al.* 1997, Rossi *et al.* 1998). Like α -MSH, AgRP is highly expressed in the arcuate nucleus (Ollmann *et al.* 1997, Shutter *et al.* 1997), where it is mainly co-localized to neuropeptide Y (NPY) neurons (Hahn *et al.* 1998, Chen *et al.* 1999). Transgenic mice over-expressing AgRP recapitulate many features of MC4R-null mice, including obesity, increased body length and hyperinsulinemia (Graham *et al.* 1997, Ollmann *et al.* 1997). These findings also highlight the importance of the competitive interaction between α -MSH and AgRP at the MC4R/(MC3R) interface to control energy balance. Recently, two melanocortin-2 receptor accessory proteins, named MRAP (also called MRAP1) and MRAP2, respectively, have been shown to modulate MCR functions (Metherell *et al.* 2005, Chan *et al.* 2009, Sebag & Hinkle 2009a,b). For instance, MRAP2 can interact with MC4R and modulate MC4R-mediated cAMP signaling (Chan *et al.* 2009, Asai *et al.* 2013), while MRAP is crucial

for MC2R trafficking, ACTH binding and signaling (Sebag & Hinkle 2007, 2009b, Dores *et al.* 2016a). Mice with whole-body and brain-specific deletion of MRAP2 develop early-onset severe obesity, due to the impaired MC4R signaling (Asai *et al.* 2013). Taken together, all these findings outline a more complex interaction of MC4R (or MC3R) with α -MSH, AgRP and MRAP2 in the regulation of energy homeostasis.

As in mammals, the interaction of MC4R with α -MSH, AgRP and MRAP2 has also been suggested to play a critical role in energy balance in teleosts (Forlano & Cone 2007, Cerda-Reverter *et al.* 2011, Sebag *et al.* 2013). In goldfish and rainbow trouts, i.c.v. injection of MC4R/(MC3R) agonist and antagonist can inhibit and stimulate food intake, respectively (Cerda-Reverter *et al.* 2003, Schjolden *et al.* 2009). Transgenic zebrafish over-expressing AgRP exhibit obesity and increased linear growth (Song & Cone 2007, Zhang *et al.* 2012). Moreover, two MRAP2s, named MRAP2a and MRAP2b, have been identified in zebrafish (Agulleiro *et al.* 2010). MRAP2a is expressed at larval stage and stimulates growth of zebrafish by blocking the action of MC4R, while MRAP2b expressed at a later developmental stage enhances the responsiveness of MC4R to α -MSH, thus increasing its capacity for regulating food intake and growth (Sebag *et al.* 2013). In addition, an interesting study has shown that in the presence of MRAP2a, MC4R becomes an ACTH receptor, which may mediate ACTH-induced anorexic action (Josep Agulleiro *et al.* 2013). All these findings also depict an interaction network of MC4R with α -MSH/ACTH, AgRP and MRAP2 involved in the control of energy balance and growth of teleosts.

As in mammals and teleosts, POMC, AgRP, MC4R, MC3R and MRAP2 also exist in birds (Takeuchi & Takahashi 1998, 1999, Takeuchi *et al.* 1999, 2000, Dores & Lecaude 2005, Boswell & Dunn 2015, Dores 2016, Ren *et al.* 2017). Central administration of α -MSH or ACTH can inhibit food intake in chicks and ring doves (Kawakami *et al.* 2000, Strader *et al.* 2003, Cline *et al.* 2008, Shipp *et al.* 2015), while central injection of AgRP stimulates food intake or attenuates the anorexic action of α -MSH (Tachibana *et al.* 2001, Strader *et al.* 2003). It is reported that both POMC and AgRP are expressed in avian hypothalamic infundibular nucleus, a structure equivalent to mammalian arcuate nucleus, and food deprivation/restriction can induce hypothalamic AgRP expression in chickens or quails (Boswell *et al.* 2002, Phillips-Singh *et al.* 2003, Dunn *et al.* 2013, 2015). These findings suggest that POMC-derived peptides (α -MSH/ACTH) and AgRP are likely involved in the control of avian

energy balance, presumably through interaction with MC4R/MC3R expressed in the hypothalamus (Ling *et al.* 2004, Boswell & Dunn 2015, Wang *et al.* 2016). However, there has been no direct evidence showing the complex interaction of MC4R/(MC3R) with α -MSH/ACTH, AgRP and MRAP2 in birds prior to current report. Chicken is an animal model widely used for agricultural and biomedical research (Hen *et al.* 2006, Xu *et al.* 2016). Broiler chickens display rapid body weight gain, hyperphagia, ovarian dysfunction and other metabolic disorders, whereas layer chickens are lean with efficient egg production (Hen *et al.* 2006, Resnyk *et al.* 2013). These unique features render chickens an excellent model to decipher the conserved roles of MC4R/(MC3R) signaling in energy balance, metabolism, growth and reproduction across vertebrates. Therefore, using chickens as the model, our present study aimed to investigate: (1) whether α -MSH/ACTH and AgRP have opposite actions on MC4R/MC3R signaling; (2) whether MC4R/MC3R functions can be modulated by MRAP2 (or MRAP). As a result, we demonstrated that as in mammals/teleosts, α -MSH/ACTH can activate MC4R and MC3R, while AgRP can antagonize α -MSH/ACTH action on MC4R/MC3R and inhibit the constitutive activity of MC4R/MC3R. Strikingly, we also observed that MRAP2 can enhance the sensitivity of MC4R and MC3R for ACTH and block the constitutive activity of both receptors. These findings, together with the co-expression of *MC4R*, *MC3R*, *AgRP*, *POMC* and *MRAP2* detected in chicken hypothalamus, suggest that within the hypothalamus, α -MSH/ACTH, AgRP and MRAP2 may interact with MC4R/(MC3R) to control energy balance in birds, as demonstrated in mammals/teleosts (Asai *et al.* 2013, Josep Agulleiro *et al.* 2013, Sebag *et al.* 2013). Meanwhile, the increased sensitivity of MC4R/MC3R for ACTH in the presence of MRAP2 also points out the possibility that in addition to being a key player in the pituitary–adrenal axis, ACTH may play a yet-appreciated role in energy balance via interaction with MC4R/MC3R in birds, and possibly in other vertebrates as well.

Materials and methods

Chemicals, primers, peptides and antibodies

All chemicals were purchased from Sigma-Aldrich and restriction enzymes were obtained from TaKaRa. Chicken (c-)ACTH_{1–39}, α -MSH (acetyl- α -MSH), β -MSH and γ -MSH were synthesized by GL Biochem Ltd (Shanghai, China). The purity of synthesized peptides is more than 95%

(analyzed by HPLC) and their structures were verified by mass spectrometry. Recombinant human AgRP protein (Cat no. 704-AG) was purchased from R&D Systems. Anti-Flag Affinity Gel beads were purchased from BioTool company (BioTool, Shanghai, China). Rabbit anti-Myc polyclonal antibody was purchased from Abclonal Technology (Abclonal, Wuhan, China). Rabbit anti-Flag monoclonal antibody and mouse anti-Myc-monoclonal antibody were from Cell Signaling Technology. All primers used in this study were synthesized by Beijing Genome Institute (BGI, China) and listed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article).

Animals and tissues

Adult chickens or chicks (Lohmann layer) of both sexes used in this study were purchased from a local commercial company. Chickens were killed and various tissues were collected, frozen in liquid nitrogen and stored at -80°C until use. All animal experimental protocols used in this study were approved by the Animal Ethics Committee of College of Life Sciences, Sichuan University.

Total RNA extraction and quantitative real-time PCR assays

Total RNA was extracted from chicken tissues using RNAzol (Molecular Research Center, Cincinnati, OH, USA) and dissolved in DEPC-treated H_2O . These RNA samples were reversely transcribed by Moloney murine leukemia virus (MMLV) reverse transcriptase (Takara) and were either used for PCR amplification of target genes, or for quantitative real-time PCR assay of gene expression, as described in our previous study (Cai *et al.* 2015).

Cloning of chicken *MRAP2* cDNA

Using human *MRAP2* cDNA as a reference, we performed a search in the chicken genome database (http://www.ensembl.org/gallus_gallus) and identified a DNA fragment of *MRAP2*. According to this sequence information, gene-specific primers were designed to amplify the 5'- and 3'-cDNA ends of *MRAP2* from chicken brain using SMART-RACE cDNA amplification kit (Clontech). The PCR product was cloned into pTA2 vector and sequenced by BGI. Finally, the full-length cDNA of *cMRAP2* was determined based on the sequences of 5'- and 3'-cDNA ends with an overlapping region.

Functional analysis of chicken MC3R, MC4R, MC2R, MRAP and MRAP2

Based on the cloned cDNA sequences of chicken *MRAP2* and *MRAP*, or the cDNA sequences of chicken melanocortin-2 receptor (cMC2R, NM_001031515), melanocortin-3 receptor (cMC3R, BAA32555.1) and melanocortin-4 receptor (cMC4R, BAA25252.1) published in previous studies (Takeuchi & Takahashi 1998, 1999, Takeuchi *et al.* 1998), the coding regions of these genes were amplified by PCR with high-fidelity Taq DNA polymerase and cloned into pcDNA3.1(+) vector.

According to our established method (Wang *et al.* 2007, 2012), each receptor (cMC4R/cMC3R) transiently expressed in Chinese hamster ovary (CHO) cells was treated by chicken ACTH₁₋₃₉/α-MSH/β-MSH/γ-MSH (10⁻¹²–10⁻⁶ M, 6 h), and the receptor-activated cAMP signaling pathway was then monitored by a pGL3-CRE-Luciferase reporter system, which is capable of monitoring receptor-mediated cAMP production (Wang *et al.* 2007, 2012).

To test whether cMRAP2 and its homolog, cMRAP, could alter the pharmacological property of cMC3R/cMC4R (cMC2R used as a control), CHO cells co-transfected with cMRAP2 (or cMRAP) and receptor expression plasmids (at a ratio of 5:1, w/w) were treated by chicken ACTH₁₋₃₉ or α-MSH, the relative potencies of the two peptides in activating these receptors were evaluated by the same reporter system.

Co-immunoprecipitation (Co-IP) assay

To investigate the interaction of chicken MC3R/MC4R with MRAP2, we first prepared the pcDNA3.1(+) expression plasmids encoding an N-terminally Myc-tagged MRAP2 (Myc-MRAP2) and N/C-terminally Flag-tagged receptors by PCR. These MRAP2 and receptor expression plasmids were used to co-transfect CHO cells cultured in a 60-mm dish (Nunc, Rochester, NY, USA) at 37°C by jetPRIME transfection reagent according to the manufacturer's instruction (Polyplus transfection, France). After 24 h of transfection, CHO cells were lysed for 30 min on ice in a lysis buffer (containing 0.1% n-dodecyl-β-maltoside and protease inhibitors). The cell lysates were centrifuged at 9600 *g* for 10 min at 4°C and supernatants were used either for Western blot, or Co-IP assay. For Co-IP, the cell lysates were added to anti-Flag Affinity Gel beads and incubated overnight at 4°C. After incubation, the agarose beads were washed with lysis buffer, the supernatant was removed and 2× Laemmli sample buffer was added. After boiling for 5 min, samples were run in SDS-polyacrylamide

gel and rabbit anti-Myc antibodies (1:2000) were used for Western blot.

Immunofluorescence microscopy

CHO cells grown in a 24-well plate were co-transfected with Flag-cMC3R (or Flag-cMC4R) and Myc-cMRAP2 expression plasmids. After 24 h of transfection, cells were fixed by 4% paraformaldehyde, washed with PBS and blocked by 3% BSA and 10% donkey serum in PBS for 45 min. After blocking, cells were incubated with rabbit anti-Flag (1:200) and mouse anti-c-Myc antibodies (1:200) overnight at 4°C. Primary antibodies were detected by goat anti-rabbit secondary antibodies coupled to Alexa-Fluor 488 or goat anti-mouse secondary antibodies (1:500) coupled to Alexa-Fluor 594 (Invitrogen). Cells were washed with PBS and stained by DAPI for 1 min. The fluorescence was then examined by a Laser-scanning confocal microscope (LeicaTCS SP5 II).

Detection of the basal constitutive activity of cMC3R and cMC4R

The basal constitutive activity of cMC3R and cMC4R expressed in CHO cells was detected by dual-luciferase reporter assay, established in our previous studies (He *et al.* 2016, Gao *et al.* 2017). In brief, CHO cells were cultured in a 48-well plate at a density of 1×10⁵ cells per well before transfection. After 24 h of incubation, a mixture containing 480 ng of pGL3-CRE-luciferase reporter construct (which is capable of monitoring receptor-stimulated cAMP production), 5 ng of pRL-TK construct (used to normalize transfection efficiency), 120 ng of cMC3R/cMC4R expression plasmid, various amounts (0–600 ng) of cMRAP2 (or cMRAP) expression plasmid and 0.5 μL of JetPRIME reagent, was prepared in transfection buffer and used to transfect CHO cells, following the manufacturer's instructions. 24 h later, culture medium was removed and 150 μL 1× passive lysis buffer (Promega) was added to each well. Luciferase activities of 15 μL cellular lysates were measured by using Dual-Luciferase Reporter Assay Kit (Promega). The cells transfected with empty pcDNA3.1(+) vector or the expression plasmid encoding chicken neuropeptide S receptor (NPSR, a Gs protein-coupled receptor characterized in our laboratory: Fang & Wang, unpublished observations) were used as controls. Luciferase activity of CHO cells expressing cMC4R/cMC3R was normalized to *Renilla* luciferase activity derived from the pRL-TK vector and then expressed as relative fold increase as compared

with the control (CHO cells transfected with the empty pcDNA3.1(+) vector).

Detection of the inverse agonistic and antagonistic actions of AgRP

To evaluate whether AgRP could act as an inverse agonist to inhibit the basal constitutive activity of cMC3R/cMC4R, or act as an antagonist to block α -MSH/ACTH action on cMC3R/cMC4R activation, CHO cells transfected with cMC3R/cMC4R expression plasmid, or co-transfected with cMC3R/cMC4R and cMRAP2/cMRAP expression plasmid (at a ratio of 1:5), were treated by recombinant human AgRP (10^{-12} – 10^{-7} M, 6 h) alone, or co-treated by both AgRP (10^{-10} – 10^{-7} M) and chicken α -MSH/ACTH (10 nM). Then, the receptor-mediated cAMP production was monitored by pGL3-CRE-luciferase reporter system.

Detection of cell surface expression of cMC2R, cMC3R and cMC4R by ELISA

To investigate whether cMRAP2 (or cMRAP) can alter the cell surface expression of cMC3R/cMC4R (cMC2R used as a control), the expression of Flag-cMC3R, Flag-cMC4R and Flag-cMC2R on cell membrane were measured by cell surface enzyme-linked immunosorbent assay (ELISA). In brief, CHO cells cultured in a 24-well plate were transfected with receptor expression plasmid or co-transfected with receptor and cMRAP2/cMRAP expression plasmids (at a ratio of 1:5). After 24 h of transfection, cells were fixed by 4% paraformaldehyde, washed with PBS and incubated with 5% milk in PBS for 45 min to block non-specific binding. These cells were incubated with rabbit anti-Flag antibody (diluted 1:5000) in 5% milk in PBS for 1 h, washed with PBS and incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5000) for 1 h. Finally, CHO cells were washed with PBS and incubated with the substrate 3,5,3',5'-tetramethylbenzidine for 15 min, and the reaction was stopped by adding an equal volume of 10% sulphuric acid. The absorbance was measured at 450 nm (OD_{450}) on a Tri-Star LB 941 Multimode Reader (Berthold, Germany). Total expression of Flag-tagged receptor was measured by the same protocol, except the cells were permeabilized by 1% Triton X-100 in the blocking buffer. Absorbance of CHO cells transfected with empty pcDNA3.1 (+) vector was used to subtract the background. Finally, the surface expression was first calculated as the ratio to that of total expression of each receptor, and then expressed as the percentage of the

control group (CHO cells expressing cMC3R/cMC4R/cMC2R only).

Investigate the effect of fasting on AgRP, POMC, MRAP2 and NPY mRNA expression

In this experiment, chicks (Lohmann layer, 4 week old) were maintained at 22°C on a 14-h light, 10-h dark (14L:10D) photoperiod and fed a commercial mixed diet with free access to water. To determine whether fasting can regulate mRNA expression of AgRP (NM_001031457), POMC (NM_001031098), MRAP2 and NPY (NM_205473) in the hypothalamus, 4-week-old male chicks of similar body weight were randomly divided into three groups: two experimental groups (8 individuals in each group) and one control group (8 individuals). In the experimental groups, chicks had free access to water but were deprived of food for 24 h or 36 h. In the control group, all chicks had free access to food and water for 36 h. At the end of the experiments, the hypothalamus from all chicks was collected to examine the mRNA levels of target genes by quantitative real-time PCR assay.

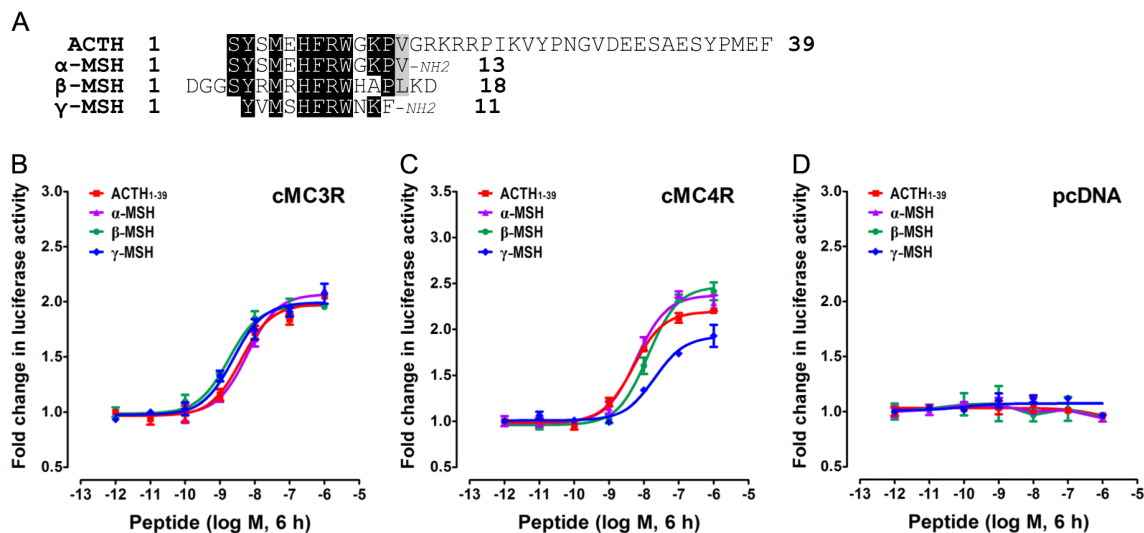
Data analysis

The mRNA level of each gene was first normalized by that of β -actin and then expressed as fold difference compared to control group or chosen tissues. The gene expression data or the reporter assay data were analyzed by one-way ANOVA followed by Dunnett's test. The dose–response curves were constructed using nonlinear regression models, and the corresponding half-maximal effective concentration (EC_{50}) values were evaluated with GraphPad Prism 5 (GraphPad Software). All experiments were repeated at least twice to validate our results.

Results

Chicken MC3R and MC4R can be activated by α -MSH and ACTH equipotently

To examine the relative potencies of chicken α -MSH, β -MSH, γ -MSH and ACTH_{1–39} in activating chicken MC3R and MC4R, each receptor was transiently expressed in CHO cells and treated by synthetic chicken peptides (Fig. 1). Receptor activation was then monitored by a pGL3-CRE-luciferase reporter system established in our laboratory (Wang *et al.* 2007, 2012), which is capable of monitoring receptor-induced cAMP production.

**Figure 1**

Functional analysis of chicken MC3R and MC4R. (A) Amino acid sequences of chicken ACTH₁₋₃₉, α-MSH (acetyl-α-MSH with an amidated C-terminus), β-MSH and γ-MSH (with amidated C-terminus) used in this study. (B, C) Effects of chicken ACTH₁₋₃₉, α-MSH, β-MSH and γ-MSH (10⁻¹²–10⁻⁶ M) in activating cMC3R (B) and cMC4R (C) expressed in CHO cells, as monitored by a pGL3-CRE-luciferase reporter system. (D) As a negative control, CHO cells transfected with the empty pcDNA3.1 (+) vector showed no response to peptide treatment. Each data point represents the mean ± s.e.m. of 3 replicates (*N* = 3). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0131>.

As shown in Fig. 1, all peptides could stimulate luciferase activities of CHO cells expressing cMC3R dose dependently. Since the EC₅₀ values of all peptides tested (α-MSH: 3.59 ± 1.01 nM; ACTH: 4.29 ± 1.22 nM; β-MSH: 1.78 ± 0.43 nM; γ-MSH: 2.43 ± 0.78 nM) are within the nanomolar range, it suggests that they may act as the endogenous ligands for cMC3R.

Like cMC3R, cMC4R could also be activated by α-MSH (EC₅₀: 4.01 ± 0.75 nM) and ACTH₁₋₃₉ (5.34 ± 1.21 nM) with similarly high potencies (Fig. 1). Although β-MSH and γ-MSH could also activate cMC4R, their potencies are 3- to 5-fold lower (β-MSH: 13.34 ± 2.33 nM; γ-MSH: 20.31 ± 4.73 nM) than those of α-MSH/ACTH (Supplementary Table 2).

Cloning of chicken MRAP2

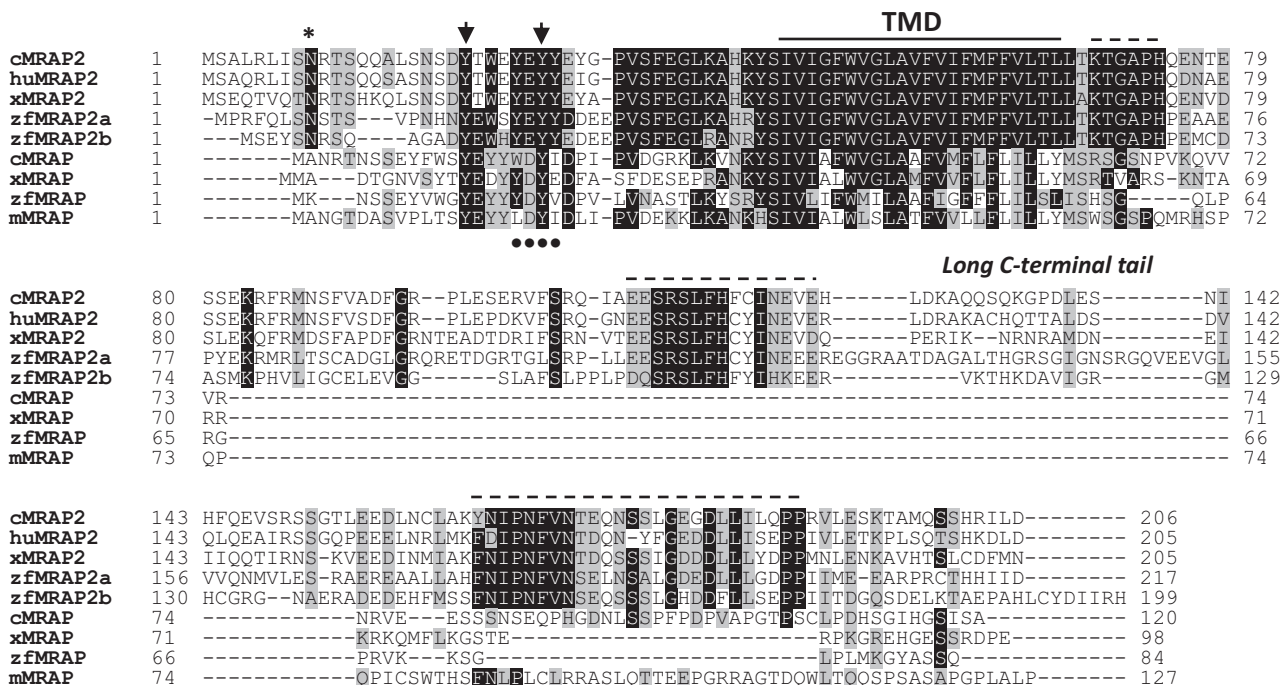
To examine whether chicken MRAP2 could interact with cMC4R/cMC3R *in vitro*, we first cloned the full-length cDNA of MRAP2 from chicken brain using RACE PCR. The cloned cMRAP2 cDNA consists of 7 exons (accession no.: KX447647) and is predicted to encode a protein of 206 amino acids (KT183012) (Supplementary Fig. 1). Sequence alignment revealed that chicken MRAP2 shares high amino acid sequence identity with that of humans (74%), *Xenopus tropicalis* (63%) and zebrafish (MRAP2a, 48%; MRAP2b, 45%), and a comparatively low identity (24–30%) with MRAP of chickens, *Xenopus tropicalis*,

zebrafish, and mice (Fig. 2). Like human MRAP2, chicken MRAP2 contains a transmembrane domain, a putative glycosylation site (Asn⁹) at the N-terminus, a putative domain (LKAHKYS) crucial for the formation of antiparallel homodimers (Sebag & Hinkle 2009b), and a long C-terminal tail with many conserved residues (or regions) observed among vertebrates (Fig. 2). However, chicken MRAP2 lacks the typical 'LDYI' motif present in mouse MRAP, which is required for ACTH binding (Sebag & Hinkle 2009b).

cMC4R and cMC3R can be preferentially activated by ACTH in the presence of cMRAP2

To test whether chicken MRAP2 (or MRAP) could alter the responsiveness of cMC3R and cMC4R to ACTH and α-MSH, CHO cells co-expressing receptor and cMRAP2 (or cMRAP) were treated by ACTH₁₋₃₉ and α-MSH, and the receptor activation was monitored by a pGL3-CRE-luciferase reporter system.

As shown in Fig. 3, in the presence of cMRAP2, both cMC3R and cMC4R could be preferentially activated by chicken ACTH₁₋₃₉, and the potencies of ACTH₁₋₃₉ in activating cMC3R and cMC4R increase ~9-fold and ~21-fold, respectively, as indicated by the significant changes in EC₅₀ values of ACTH₁₋₃₉ from nanomolar to subnanomolar ranges (Fig. 3 and Table 1). Likewise, in the presence of cMRAP, both cMC3R and cMC4R could also

**Figure 2**

Amino acid alignment of chicken MRAP2 (cMRAP2, KT183012) with that of humans (hMRAP2, NP_612418), *Xenopus tropicalis* (xMRAP2, XP_002933963), zebrafish (zfMRAP2a, F8W4H9.1; zfMRAP2b, P0DM64), or with MRAP of chickens (cMRAP), *Xenopus tropicalis* (xMRAP, XP_002938489), zebrafish (zfMRAP, BC122349) and mice (mMRAP, NP_084120). The horizontal line marks the transmembrane domain (TMD). The asterisk indicates a potential *N*-glycosylation site; Dots indicate the 'LDYI' motif presented in mouse MRAP, which is crucial for ACTH activation of MC2R (Sebag & Hinkle 2009b). Triangles denote the domain (LKAHKYS) required for the formation of antiparallel MRAP2 homodimer (Sebag & Hinkle 2009b). The conserved regions and amino acid residues noted in the long C-termini of vertebrate MRAP2 are indicated by horizontal dashed lines and black boxes respectively. Arrows indicate the two tyrosine (Y) residues fully conserved in MRAPs across vertebrates. Dashes denote gaps in the alignment.

be preferentially activated by ACTH₁₋₃₉, and the potencies of ACTH₁₋₃₉ in activating both receptors increase ~7-fold. In sharp contrast, cMRAP2 (or cMRAP) causes a slight increase (<2.3-fold) or no increase in the potencies of α -MSH in activating cMC3R/cMC4R (Table 1). These findings indicate that in the presence of MRAP2 (or MRAP), both cMC3R and cMC4R show increased sensitivity and selectivity towards ACTH, and thus likely act as two ACTH-preferring receptors.

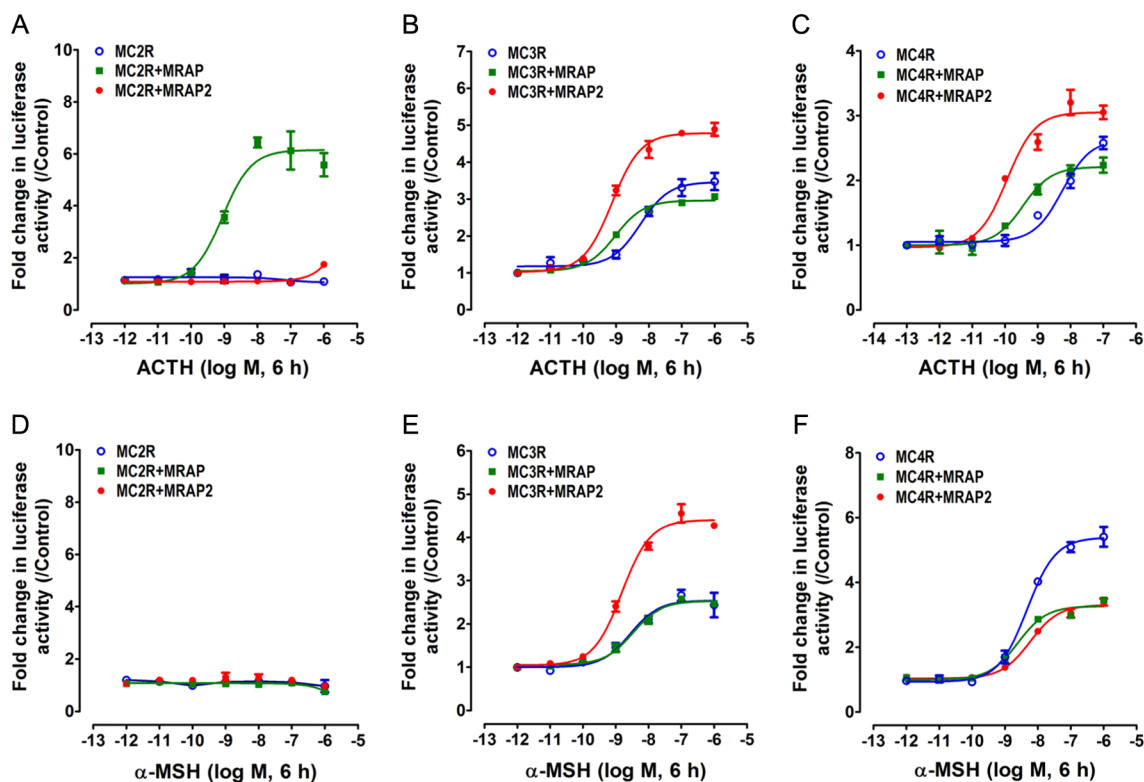
In contrast to cMC4R/cMC3R, cMC2R could only be activated by ACTH₁₋₃₉ (EC₅₀: 0.89 ± 0.05 nM) potently in the presence of cMRAP, but not MRAP2 (Fig. 3 and Table 1), indicating that cMC2R acts as an ACTH-specific receptor, as previously reported (Barlock *et al.* 2014).

Since cMRAP2 is more effective than cMRAP in enhancing the sensitivity of cMC4R and cMC3R for ACTH (Fig. 3 and Table 1), we further examined whether cMRAP2 could interact with cMC4R/cMC3R *in vitro*. Using double-fluorescence staining, we observed that Myc-cMRAP2 and Flag-cMC4R (or Flag-cMC3R) expressed in CHO cells were co-localized on the cell

surface (Fig. 4). Co-immunoprecipitation assay further elucidates a direct interaction of Myc-cMRAP2 with cMC3R-Flag/cMC4R-Flag (Fig. 4). Although cMRAP is less effective than cMRAP2 in enhancing sensitivity of cMC4R/cMC3R for ACTH, Co-IP assay still showed that cMRAP could interact with cMC3R/cMC4R *in vitro* (data not shown).

Chicken MRAP2 (and MRAP) can modulate the constitutive activity of cMC4R/cMC3R

To test whether cMC4R/cMC3R displays constitutive activity similar to mammalian MC4R (Ollmann *et al.* 1997, Nijenhuis *et al.* 2001), we examined the basal luciferase activity of CHO cells expressing cMC4R or cMC3R using dual-luciferase reporter assay. As shown in Fig. 5, the basal luciferase activity of CHO cells expressing cMC3R is ~3-fold higher compared to that of cells transfected with the empty pcDNA3.1(+) vector. In contrast, CHO cells expressing chicken NPSR, a Gs protein-coupled receptor, display no basal activity under the same condition.

**Figure 3**

(A, B and C) Effects of ACTH₁₋₃₉ in activating chicken MC2R (A), MC3R (B) and MC4R (C) expressed in CHO cells co-transfected with (or without) MRAP2/MRAP expression plasmid, monitored by a pGL3-CRE-luciferase reporter system. (D, E and F) Effects of α-MSH in activating chicken MC2R (D), MC3R (E) and MC4R (F) expressed in CHO cells co-transfected with (or without) MRAP2/MRAP, monitored by a pGL3-CRE-luciferase reporter system. Each data point represents the mean ± S.E.M. of 3 replicates (N=3). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0131>.

Likewise, the basal luciferase activity of CHO cells expressing cMC4R is slightly, but significantly, higher (~1.4 fold) than that of control. These findings indicate

Table 1 EC₅₀ values of chicken α-MSH/ACTH in activating cMC3R/cMC4R/cMC2R expressed in CHO cells, in the absence or presence of cMRAP2/cMRAP.

	EC ₅₀ (nM)		
	cMC3R	cMC3R + cMRAP	cMC3R + cMRAP2
α-MSH	3.59 ± 1.01	3.26 ± 0.65	1.55 ± 0.24
ACTH ₁₋₃₉	4.29 ± 1.22	0.55 ± 0.39*	0.47 ± 0.28*
AgRP	3.62 ± 1.04	9.61 ± 5.12	—
	cMC4R + cMRAP		
	cMC4R	cMC4R + cMRAP	cMC4R + cMRAP2
α-MSH	4.01 ± 0.75	2.42 ± 0.06*	3.96 ± 1.79
ACTH ₁₋₃₉	5.34 ± 1.21	0.74 ± 0.24*	0.25 ± 0.21*
AgRP	—	—	—
	cMC2R + cMRAP		
	cMC2R	cMC2R + cMRAP	cMC2R + cMRAP2
α-MSH	n	n	n
ACTH ₁₋₃₉	n	0.89 ± 0.05	—

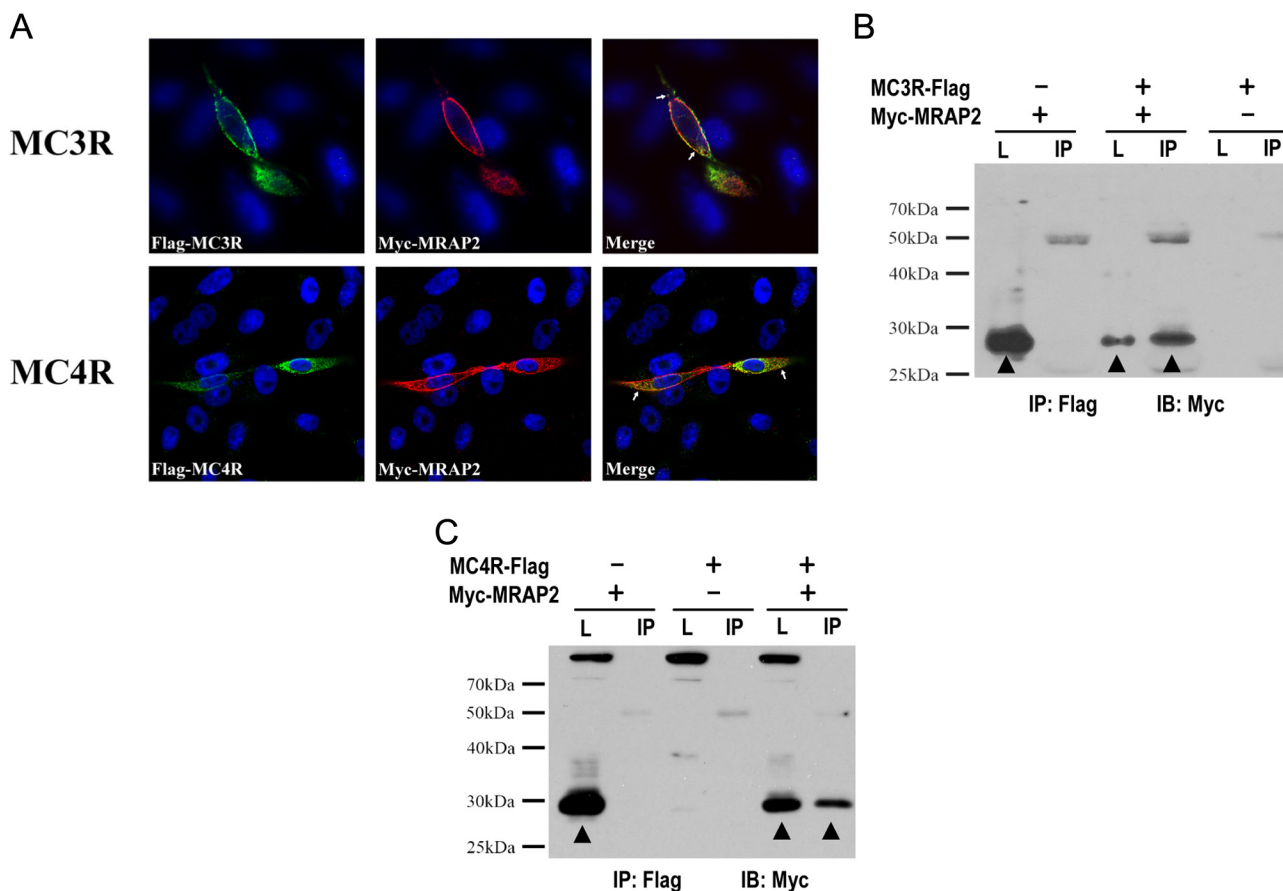
Data shown are the mean ± S.E.M. of two to five independent experiments performed in triplicate. EC₅₀ values of human AgRP in inhibiting the constitutive activity of cMC3R/cMC4R are also shown above.

*P < 0.05 vs. cMC4R/cMC3R (analyzed by the Student t-test using GraphPad Prism 5 software). '—' indicates that EC₅₀ values could not be estimated based on the experimental data; 'n' indicates no response.

that both cMC3R and cMC4R are constitutively active receptors (Fig. 5).

Using dual-luciferase reporter assay, we further examined whether cMRAP2 (or cMRAP) could modulate the basal constitutive activity of cMC4R/cMC3R. As shown in Fig. 5, cMRAP2 could dose dependently inhibit the basal constitutive activity of cMC3R, when co-transfected, and the maximal inhibition was observed at the ratio of 1:5 (cMC3R:cMRAP2) plasmids used. In contrast, cMRAP has no significant effect on the basal activity of cMC3R. Although the constitutive activity of cMC4R is relatively weak, cMRAP2 could still reduce its basal constitutive activity significantly. Interestingly, unlike cMRAP2, cMRAP could enhance the basal constitutive activity of cMC4R in a dose-dependent manner.

To determine whether the suppressed (or enhanced) basal constitutive activity of cMC3R or cMC4R is due to alterations in their cell surface expression, surface expression of Flag-cMC3R/Flag-cMC4R in CHO cells was examined by whole-cell ELISA assay. As shown in Fig. 6, co-expression of cMRAP2 (or cMRAP) with cMC3R did not alter surface expression of cMC3R significantly,

**Figure 4**

(A) Confocal microscopy showing the co-localization of Flag-cMC3R/Flag-cMC4R and Myc-cMRAP2 on the cell surface. Flag-cMC3R/Flag-cMC4R and Myc-cMRAP2 were transiently expressed in CHO cells and their expression was detected by Alexa-Fluor 488-conjugated anti-Flag antibody (green fluorescence) and Alexa-Fluor 594-conjugated anti-Myc antibody (red fluorescence) respectively. White arrows indicate the co-localization (yellow color) of MC3R/MC4R with MRAP2 on the cell surface. (B, C) Co-IP assay showing the interaction of Myc-cMRAP2 with cMC3R-Flag (B), or with cMC4R-Flag (C), in CHO cells co-transfected with cMRAP2 and cMC3R (or cMC4R) expression plasmids. Arrow heads in (B, C) indicate the positive MRAP2 band (~27 kDa) in immuno-precipitated samples (IP) and cell lysates (L) detected by anti-Myc antibody. L, cell lysates; IP, immuno-precipitated samples (Anti-Flag antibody used); IB, immuno-blotting (anti-Myc antibody used).

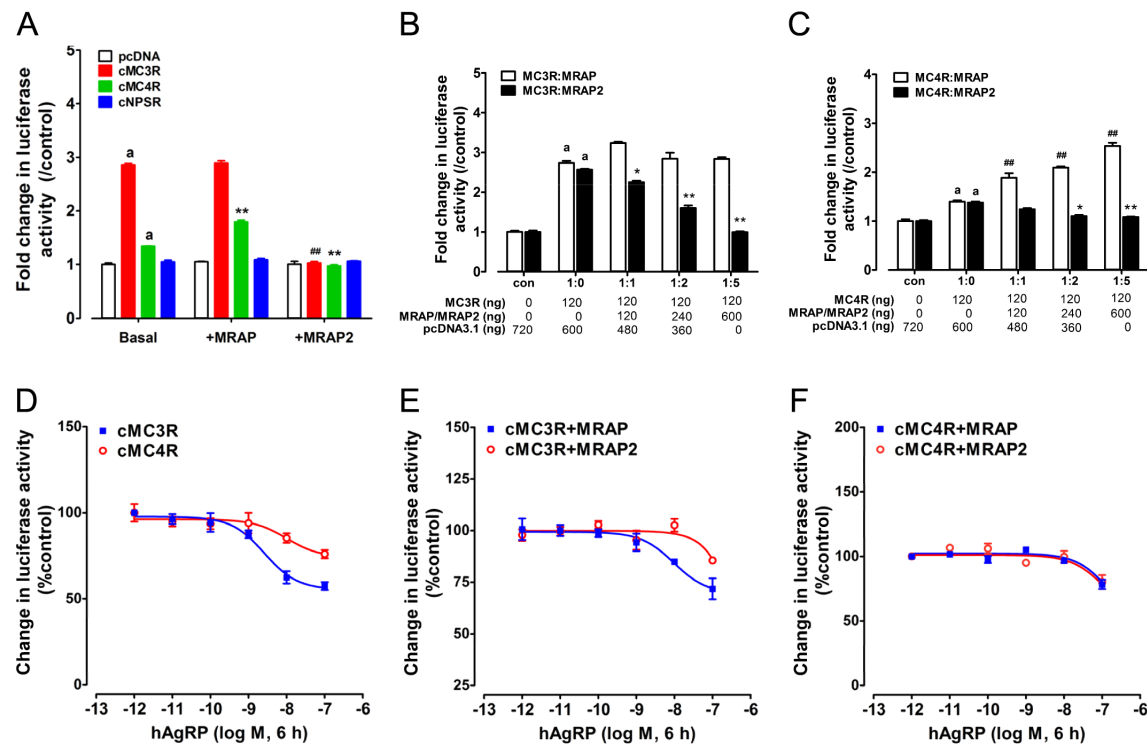
when compared with CHO cells expressing cMC3R alone. Similarly, co-expression of cMRAP2 (or cMRAP) with cMC4R did not alter surface expression of cMC4R significantly, although a slight decrease in cMC4R surface expression was noted in the presence of cMRAP2. In sharp contrast, both cMRAP and cMRAP2 could enhance the surface expression of cMC2R significantly (Fig. 6), as previously reported in mammals (Chan *et al.* 2009, Sebag & Hinkle 2009b).

AgRP can act as an inverse agonist of cMC3R and cMC4R

The strong constitutive activity of cMC3R led us to examine whether AgRP can act as an inverse agonist to inhibit the basal constitutive activity of cMC3R *in vitro* using pGL3-CRE-luciferase reporter system. As shown in

Fig. 5, human AgRP could effectively inhibit the basal luciferase activity of CHO cells expressing cMC3R with an EC_{50} value at 3.62 ± 1.04 nM, indicating that AgRP can act as a potent inverse agonist of cMC3R. However, we noted that in the presence of cMRAP2, the inhibitory action of AgRP diminished considerably (EC_{50} , >100 nM). In contrast, in the presence of cMRAP, AgRP could still inhibit the basal activity of cMC3R effectively (EC_{50} : $\sim 9.16 \pm 5.12$ nM) (Table 1).

Unlike cMC3R, cMC4R basal activity could only be inhibited significantly by AgRP at a high concentration (100 nM) (Fig. 5), suggesting that AgRP may be a weak inverse agonist for cMC4R. Since the basal constitutive activity of cMC4R is relatively weak, it is rather difficult to judge whether cMRAP2 (or cMRAP) can modulate the inverse agonistic action of AgRP on cMC4R (Fig. 5).

**Figure 5**

(A) The constitutive activity of cMC3R and cMC4R detected by dual-luciferase reporter assay. Both cMC3R and cMC4R demonstrate constitutive activity, and cMRAP2/cMRAP can modify (inhibit/enhance) their activity. In contrast, under the same conditions, chicken NPSR (cNPSR, a Gs protein-coupled receptor) shows no basal activity. CHO cells transfected with empty pcDNA3.1(+) vector was used as a control. ^a $P < 0.001$ vs pcDNA3.1(+) control. ^{##} $P < 0.001$ vs cMC3R-expressing CHO cells; ^{**} $P < 0.001$ vs cMC4R-expressing CHO cells. The ratio of receptor to MRAP/MRAP2 plasmid used in these experiments is 1:5 (w/w). (B) The basal constitutive activity of cMC3R could be dose dependently inhibited by cMRAP2 (not cMRAP) plasmid, as indicated. (C) The basal constitutive activity of cMC4R could be dose dependently enhanced by cMRAP2 plasmid, as indicated, while its basal activity was enhanced by cMRAP dose dependently. In graphs (B) and (C), CHO cells transfected with empty pcDNA3.1(+) vector were used as a control (con). ^a $P < 0.001$ vs control (con). ^{*} $P < 0.05$, ^{**} $P < 0.001$ vs cMC3R/cMC4R-expressing CHO cells; ^{##} $P < 0.001$ vs cMC4R-expressing CHO cells. Each data point in (A, B and C) represents the mean $\pm$ S.E.M. of 4 replicates ($N=4$). (D, E and F) The effect of human AgRP (10^{-12} – 10^{-7} M, 6 h) on the basal luciferase activities of CHO cells expressing cMC3R/cMC4R (D), or CHO cells co-transfected with cMC3R and cMRAP2/cMRAP plasmids (E), or CHO cells co-transfected with cMC4R and cMRAP2/cMRAP plasmids (F), as monitored by pGL3-CRE-luciferase reporter assay. Each data point in (D, E and F) represents the mean $\pm$ S.E.M. of 3 replicates ($N=3$). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0131>.

AgRP antagonizes ACTH/ α -MSH actions on cMC3R and cMC4R

To determine whether AgRP could antagonize α -MSH/ACTH action on cMC4R/cMC3R activation, CHO cells expressing cMC4R (or cMC3R) were co-treated by ACTH (or α -MSH) (10 nM) and human AgRP (0.1–100 nM). As shown in Fig. 7, AgRP could block ACTH-induced luciferase activities of CHO cells expressing cMC3R or cMC4R dose dependently, as monitored by pGL3-CRE-luciferase reporter system. Similarly, AgRP could also effectively suppress ACTH-induced luciferase activities of CHO cells co-expressing cMC3R and cMRAP2 (or cMRAP) or CHO cells co-expressing cMC4R and cMRAP2 (or cMRAP).

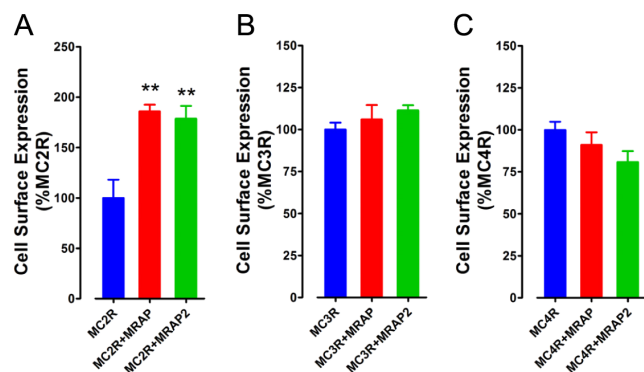
In addition, AgRP could also suppress α -MSH-induced luciferase activity of CHO cells expressing cMC4R/cMC3R,

or CHO cells co-expressing cMC4R (or cMC3R) and cMRAP2 (or cMRAP) in a dose-dependent manner (Fig. 8).

Tissue expression of MC3R, MC4R, AgRP, POMC, MRAP and MRAP2 in chickens

Using quantitative real-time PCR, we further examined the mRNA expression of cMC4R, cMC3R, cAgRP, cPOMC, cMRAP and cMRAP2 in adult chicken tissues, including various brain regions.

As shown in Fig. 9, cMC4R is expressed predominantly in the hypothalamus and weakly in other tissues, including the midbrain, hindbrain, adrenal gland, anterior pituitary, fat, heart and small intestine. Like cMC4R, cMC3R is expressed predominantly in the hypothalamus, and weakly in remaining tissues,

**Figure 6**

Surface expression of Flag-cMC2R (A), Flag-cMC3R (B) and Flag-cMC4R (C) in the absence or presence of cMRAP/cMRAP2. Surface expression levels of each receptor were detected by whole-cell ELISA assay and expressed as the percentage of CHO cells expressing Flag-cMC2R (or Flag-cMC3R/Flag-cMC4R) alone. Each data point represents mean $\pm$ S.E.M. of four replicates ($N=4$). ** $P<0.001$ vs CHO cells expressing cMC2R only. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0131>.

including anterior pituitary, ovary, muscle, adrenal gland and other brain regions.

Unlike the predominant expression of *cMC3R* and *cMC4R* in chicken hypothalamus, a differential expression of *cMRAP2* and *cMRAP* was observed in chicken tissues. *cMRAP2* mRNA is highly expressed in various brain regions (including the hypothalamus) and adrenal gland, while only a moderate or weak expression of *cMRAP2* was detected in other tissues, including the testes, pancreas and anterior pituitary. In contrast, *cMRAP* is expressed predominantly in the adrenal gland and weakly in other tissues examined.

cAgRP was detected to be expressed predominantly in the hypothalamus, moderately in the anterior pituitary, and weakly in the midbrain, cerebellum, hindbrain and spleen, while it was almost undetectable in other tissues examined. Unlike *cAgRP*, *cPOMC* is highly expressed in the anterior pituitary, moderately in the hypothalamus, and weakly in other brain regions, lung, skin, fat and spleen. *cPOMC* mRNA was barely detectable in other tissues examined.

Regulation of hypothalamic *cMRAP2* and *cPOMC* expression by fasting

The abundant expression of *cMRAP2* and *cPOMC* in chicken hypothalamus led us to further examine whether fasting could alter their expression in chick hypothalamus by quantitative real-time PCR. Since fasting is reported to upregulate the mRNA expression of *NPY* and *AgRP*, both

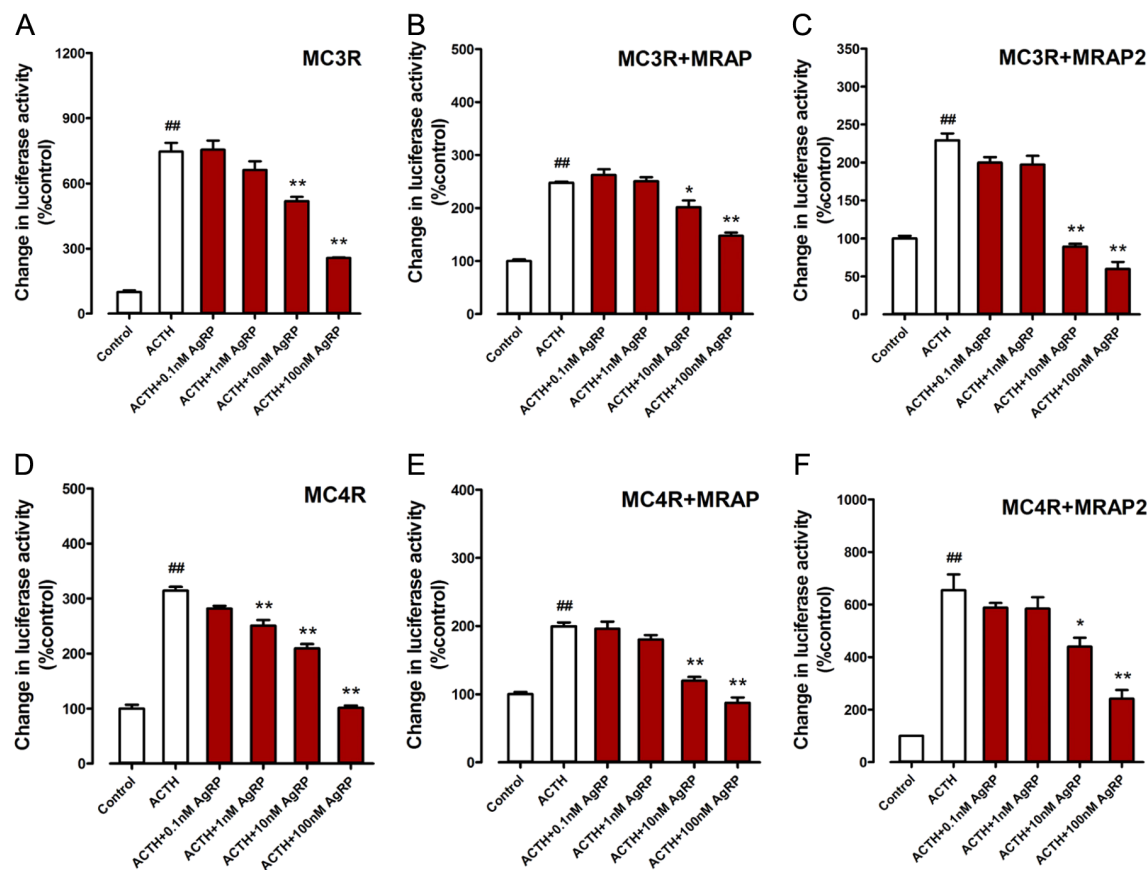
of which are mainly co-expressed in the same neurons of avian hypothalamic infundibular nucleus (Boswell *et al.* 2002, Phillips-Singh *et al.* 2003), therefore, their expression was also examined in parallel and used as positive controls. As shown in Supplementary Fig. 2, 24-h and 36-h fasting caused a 6- to 8-fold increase in *cAgRP* expression and a 2- to 3-fold increase in *cNPY* expression. Interestingly, *cMRAP2* mRNA levels also showed a slight, but significant, increase after 24-h or 36-h fasting. In contrast, *POMC* expression showed no significant variation after 24-h fasting, but 36-h fasting caused a slight, but significant downregulation on its expression.

Discussion

MC4R/(MC3R) signaling has long been implicated to control energy balance in birds (Boswell & Dunn 2015); however, the complex interaction of avian MC4R/MC3R with MRAP2, AgRP and POMC-derived peptides remains unclear. Here, we elucidated that chicken MC4R and MC3R, when co-expressed with MRAP2 (or MRAP), show increased sensitivity and selectivity toward ACTH, and MRAP2 (and MRAP) can modulate their constitutive activity. Moreover, we demonstrated that AgRP can act as an inverse agonist for MC3R and MC4R, and it also antagonizes α -MSH/ACTH action on MC4R/MC3R. These findings, together with the co-expression of MRAP2, POMC, AgRP and MC4R/MC3R in chicken hypothalamus, strongly suggest that these molecules may interact at the hypothalamic level to control avian energy balance. To our knowledge, this study represents the first to reveal an intricate interaction of MC3R/MC4R with MRAP2, α -MSH/ACTH and AgRP in an avian species and provide novel proof that MRAP2 (and MRAP) can modulate the constitutive activity and ligand sensitivity and selectivity of both MC3R and MC4R in vertebrates.

Differential tissue expression of MRAP2 and MRAP in chickens

In this study, MRAP2 cDNA was cloned from chicken brain. It encodes a single-pass membrane protein, which shows a remarkable conservation (45–74% identity) with human MRAP2 or zebrafish MRAP2a/MRAP2b, and a relatively low sequence identity (24–30%) with chicken, *Xenopus* and human MRAP (Fig. 2). qPCR revealed that *cMRAP2* is highly expressed in the brain and adrenal gland. In contrast, *cMRAP* is predominantly expressed in the adrenal gland

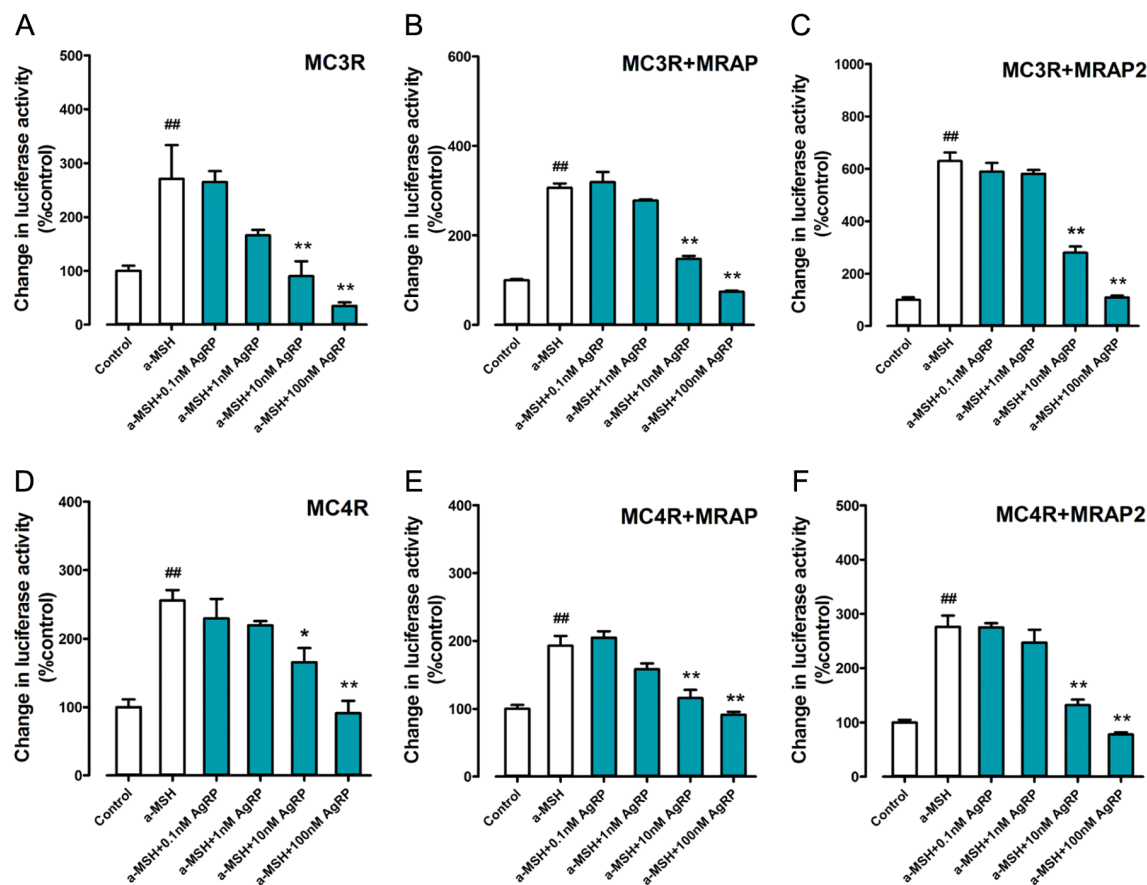
**Figure 7**

(A, B and C) Human AgRP (0.1–100 nM, 6 h) could dose dependently block ACTH_{1–39} (10 nM)-stimulated luciferase activity of CHO cells expressing cMC3R (A), or CHO cells co-expressing cMC3R and cMRAP (B), or CHO cells co-expressing cMC3R and cMRAP2 (C), as monitored by a pGL3-CRE-luciferase reporter system. (D and E) Human AgRP (0.1–100 nM, 6 h) could dose dependently inhibit ACTH_{1–39} (10 nM)-stimulated luciferase activity of CHO cells expressing cMC4R (D), or CHO cells co-expressing cMC4R and cMRAP (E), or CHO cells co-expressing cMC4R and cMRAP2 (F), as monitored by a pGL3-CRE-luciferase reporter system. Each data point represents the mean $\pm$ S.E.M. of 3 replicates ($N=3$). * $P<0.05$; ** $P<0.001$ vs ACTH_{1–39} treatment; ## $P<0.001$ vs control (without peptide treatment). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0131>.

(Fig. 9). Our finding coincides with the observations in mammals, in which MRAP is highly expressed in the adrenal cortex, and MRAP2 is mainly expressed in the brain and adrenal gland (Metherell *et al.* 2005, Sebag & Hinkle 2007, Chan *et al.* 2009, Asai *et al.* 2013). The conserved expression patterns of MRAP and MRAP2 between birds and mammals suggest that as in mammals (Metherell *et al.* 2005, Sebag & Hinkle 2007, Chan *et al.* 2009, Asai *et al.* 2013), MRAP is crucial for MC2R trafficking, ACTH binding (Fig. 3) and normal functions of the adrenal gland in chickens (Barlock *et al.* 2014), whereas MRAP2 may be crucial for MC4R/MC3R (and other non-melanocortin GPCR) functions in the brain (Chaly *et al.* 2016) and other non-brain tissues, such as the adrenal gland.

MC4R and MC3R may act as potential ACTH-prefering receptors in the presence of MRAP2

In this study, we found that chicken MC3R expressed in CHO cells can be potently activated by chicken α -MSH, β -MSH, γ -MSH and ACTH_{1–39} with similarly high potencies. Our finding differs from a previous report, in which chicken MC3R expressed in HEK293 cells can bind to human γ -MSH ($K_i=3.4$ nM) with a much higher affinity than to ACTH_{1–39} ($K_i=15.4$ nM), α -MSH ($K_i=24$ nM) and β -MSH ($K_i=151$ nM) (Ling *et al.* 2004). This discrepancy may be due to the sequence difference between chicken and human melanocortins used in the two studies. Similar to our finding in chickens, MC3R can be activated by α -MSH, β -MSH, γ -MSH and ACTH with similarly high

**Figure 8**

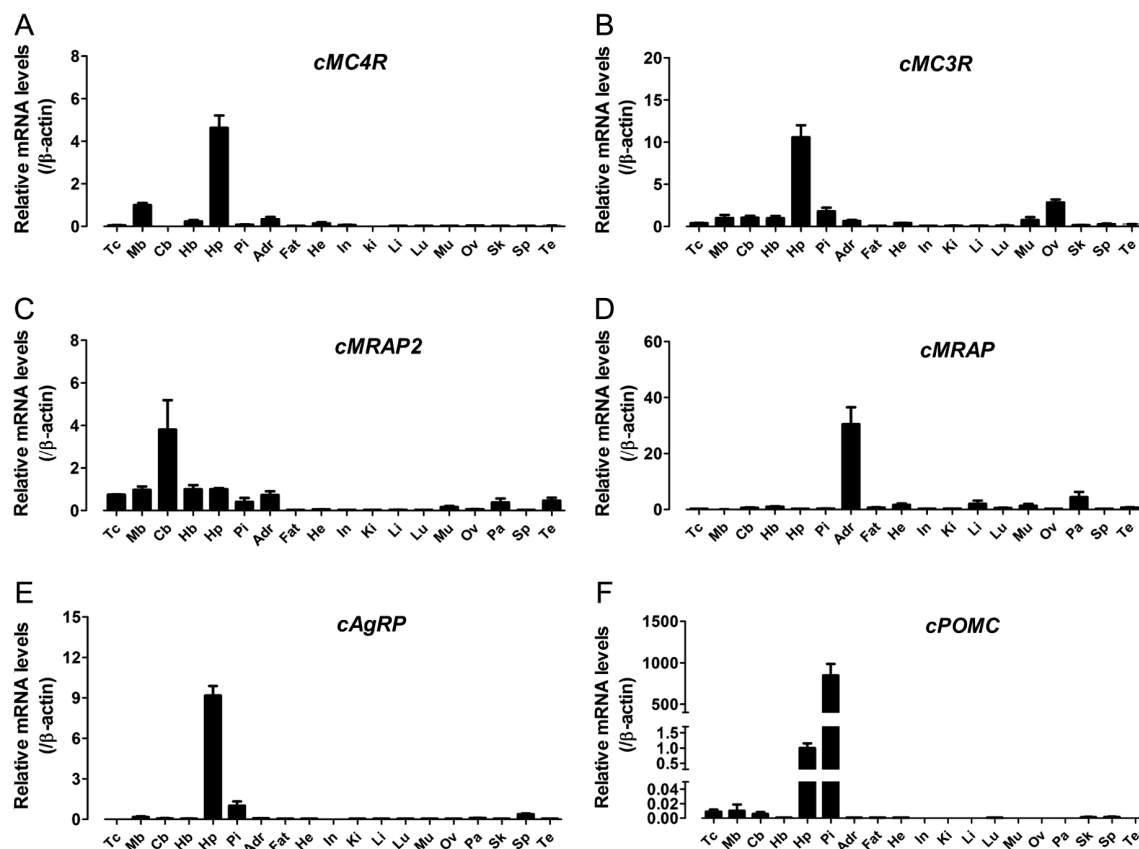
(A, B and C) Human AgRP (0.1–100 nM, 6 h) could dose dependently inhibit α -MSH (10 nM)-stimulated luciferase activity of CHO cells expressing cMC3R alone (A), or CHO cells co-expressing cMC3R and cMRAP (B), or CHO cells co-expressing cMC3R and cMRAP2 (C), as monitored by a pGL3-CRE-luciferase reporter system. (D, E and F) Human AgRP (0.1–100 nM, 6 h) could dose dependently inhibit α -MSH (10 nM)-stimulated luciferase activity of CHO cells expressing cMC4R alone (D), or CHO cells co-expressing cMC4R and cMRAP (E), or CHO cells co-expressing cMC4R and cMRAP2 (F), as monitored by a pGL3-CRE-luciferase reporter system. Each data point represents the mean $\pm$ S.E.M. of 3 replicates ($N=3$). * $P<0.05$; ** $P<0.001$ vs α -MSH treatment; *** $P<0.001$ vs control (without peptide treatment). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0131>.

potencies in mammals, indicating the conservation of pharmacological property between avian and mammalian MC3R (Gantz *et al.* 1993a, Roselli-Reh fuss *et al.* 1993, Cone 2006).

Unlike cMC3R, cMC4R displays a weak selectivity towards α -MSH and ACTH_{1–39}. Both α -MSH and ACTH_{1–39} can activate cMC4R equipotently, with EC₅₀ values in the nanomolar range, whereas β -MSH and γ -MSH are less potent (EC₅₀ >10 nM). Our data partly agree with the finding in humans, in which human MC4R expressed in COS-1 or HEK293 cells can be activated by α -MSH, ACTH_{1–39} and β -MSH with similarly high potencies (Gantz *et al.* 1993b, Mountjoy *et al.* 1994).

There is growing evidence supporting that MRAP2 (and MRAP) can interact with all 5 MCRs, and by which they can modify MCR trafficking, ligand binding or signaling (Sebag & Hinkle 2007, Chan *et al.* 2009, Asai *et al.* 2013,

Josep Agulleiro *et al.* 2013, Sebag *et al.* 2013, Dores 2016). In this study, we demonstrated that cMC3R and cMC4R, when co-expressed with cMRAP2 (or cMRAP), can be preferentially activated by ACTH, and the potencies of ACTH increase 7- to 21-fold, as revealed by the significant shifts of EC₅₀ values from nanomolar to sub-nanomolar ranges. By contrast, the potencies of α -MSH in activating cMC3R/cMC4R only show a slight variation (Table 1). Moreover, we also found that cMRAP2 is more effective than cMRAP in enhancing the sensitivity of both receptors for ACTH in all experiments performed. These findings indicate that in the presence of MRAPs, particularly MRAP2, cMC3R and cMC4R can act as two potential ACTH-preferring receptors. The interaction between cMC3R/cMC4R and cMRAP2 *in vitro* was further confirmed by co-immunoprecipitation assay. Although the action of MRAP(s) in enhancing the sensitivity of MC3R/MC4R

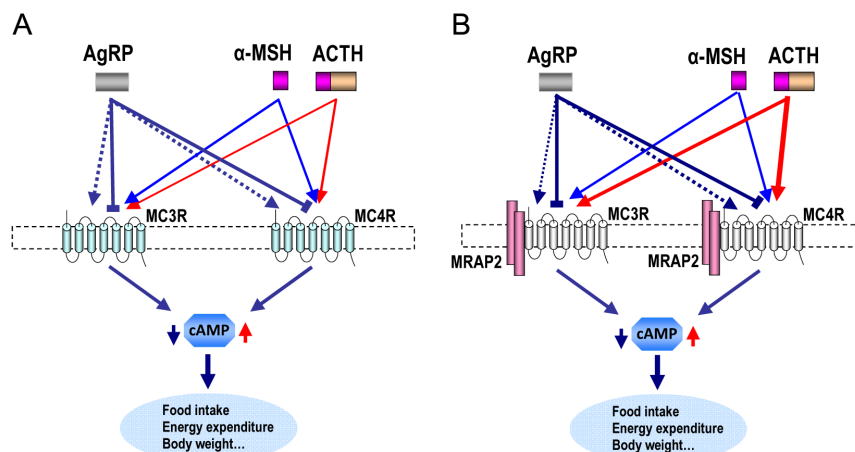
**Figure 9**

Quantitative real-time RT-PCR assay of *cMC4R* (A), *cMC3R* (B), *cMRAP2* (C), *cMRAP* (D), *cAgRP* (E) and *cPOMC* (F) mRNA expression in various chicken tissues, including the telencephalon (Tc), midbrain (Mb), cerebellum (Cb), hindbrain (Hb), hypothalamus (Hp), anterior pituitary (Pi), adrenal gland (Adr), subcutaneous fat (fat), heart (He), duodenum (in), kidneys (Ki), liver (Li), lung (Lu), muscle (Mu), ovary (Ov), pancreas (Pa), skin (Sk), spleen (Sp) and testes (Te). The mRNA levels of target genes were normalized to that of β -actin and expressed as the fold difference compared with that of the midbrain (A, B), or hindbrain (C, D), or pituitary (E), or hypothalamus (F). Each data point represents the mean $\pm$ s.e.m. of 6 adult chickens (3 males and 3 females) ($N=6$).

for ACTH has not been reported in any mammalian species, there is solid evidence showing the interaction of MRAP2/MRAP with MC4R/MC3R (Chan *et al.* 2009). For instance, MRAP2 can interact with MC4R and enhance its cAMP signaling in mice, and MRAP2 deletion or mutation causes severe obesity, possibly due to impaired MC4R signaling in mice and humans (Asai *et al.* 2013). In zebrafish, both MRAP2a and MRAP2b have been shown to interact with MC4R (Sebag *et al.* 2013). MRAP2a can reduce the binding of MC4R to α -MSH, possibly by decreasing its binding sites, while MRAP2b can regulate MC4R activity by increasing α -MSH sensitivity and suppressing receptor constitutive activity (Sebag *et al.* 2013). Interestingly, in partial accordance with our findings in chickens, Josep Agulleiro and coworkers reported that co-expression of zebrafish MC4R with MRAP2a, and not MRAP2b (or MRAP), can increase the sensitivity of MC4R for ACTH₁₋₂₄, thus becoming an ACTH receptor

(Josep Agulleiro *et al.* 2013). Our findings, together with the previous report in zebrafish, suggest that in addition to modifying the signaling of MC4R and MC3R, MRAP(s), particularly MRAP2, can also increase the sensitivity of MC4R and MC3R towards ACTH in birds, and maybe in some other vertebrates as well (Josep Agulleiro *et al.* 2013).

The cMRAP2-associated enhancement on potencies of ACTH in cMC4R and cMC3R activation (Table 1), together with the co-expression of cMRAP2, cMC4R and cMC3R in chicken hypothalamus, led us to hypothesize that like α -MSH, ACTH may play active roles via interaction with cMC4R/cMC3R–cMRAP2 complex in the hypothalamus, such as controlling food intake and body weight in chickens (Fig. 10). Consistent with this speculation, central injection of ACTH can inhibit food intake in chicks (Shipp *et al.* 2015), as reported in rats and zebrafish (Poggioli *et al.* 1986, Vergoni *et al.* 1986, Al-Barazani *et al.* 2001, Josep Agulleiro *et al.* 2013).

**Figure 10**

Proposed interaction of MC3R/MC4R with MRAP2, POMC-derived peptides (α -MSH/ACTH), and AgRP in chicken hypothalamus. (A) In the absence of MRAP2, both MC3R and MC4R are constitutively active receptors, and they can be further activated by α -MSH and ACTH equipotently (arrows). AgRP can function either as an inverse agonist to inhibit the basal constitutive activity of MC3R/MC4R (dotted arrows), or as an antagonist to block α -MSH/ACTH action on MC4R/MC3R (bold bars). (B) In the presence of MRAP2 (homodimer), MRAP2 can not only block the basal constitutive activity of MC3R/MC4R, possibly by altering their conformation into an inactivated state (Sebag *et al.* 2013), but also enhance the sensitivity of both receptors for ACTH (bold arrows) (Table 1). Although the inverse agonistic action of AgRP on MC3R is likely attenuated by MRAP2, AgRP can still antagonize α -MSH/ACTH action on MC4R/MC3R effectively. In brief summary, MRAP2 can interact with MC4R (and MC3R), and thus, fine-tune their constitutive activity, ligand sensitivity and selectivity, and downstream signaling intensity (e.g. cAMP and other signaling pathways). This interaction may work in chicken hypothalamus to regulate food intake, energy expenditure, metabolism and body weight gain (Qiu *et al.* 2006, Boswell & Dunn 2015). Moreover, our data imply that like α -MSH, ACTH may play active roles in other physiological processes, such as energy balance, metabolism and body weight gain via interaction with MC4R/MC3R (A) or MC4R/MC3R-MRAP2 complex (B), in addition to being a key player in the pituitary–adrenal axis in chickens. Interestingly, MRAP can also enhance the sensitivity of MC4R/MC3R for ACTH; however, it seems less effective than MRAP2. Moreover, unlike MRAP2, MRAP cannot inhibit basal constitutive activity of MC4R/MC3R, it enhances the basal activity of MC4R instead. Therefore, the question whether the interaction of MC4R/MC3R with MRAP is physiologically relevant remains open to discussion. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-17-0131>.

Moreover, there are substantial evidence showing that repeated peripheral injections of ACTH into immature chickens usually decrease their growth rate and body weight gain, accompanied by unaltered (or increased) plasma growth hormone levels, adrenal hypertrophy and reduction in size of their lymphoid organs (Freeman & Manning 1975, Davison *et al.* 1979). Since POMC is abundantly expressed in both hypothalamus and anterior pituitary (Fig. 9), it is tempting to speculate that ACTH derived from avian hypothalamus or pituitary may be capable of interacting with cMC4R/(cMC3R)–cMRAP2 complex expressed in the hypothalamus to control energy balance (Fig. 10). Moreover, it should be noted that unlike other vertebrates, birds lack an intermediate lobe of pituitary (Scanes 2015), where α -MSH is mainly produced in most vertebrates. This unique anatomical feature of the avian pituitary also highlights the importance of pituitary ACTH actions on avian tissues, including its actions on the CNS (e.g. hypothalamus) and peripheral tissues. Presumably, chicken pituitary ACTH may be transported to the brain, as demonstrated in mammals (Bergland & Page 1979, Bergland *et al.* 1980), and thus, regulate certain CNS-associated

physiological processes, such as food intake in chickens (Shipp *et al.* 2015). Future studies on these aspects may help to reveal the novel facets of ACTH, derived both centrally (e.g. hypothalamus) and peripherally (e.g. anterior pituitary), in the control of energy balance, metabolism and body weight, in addition to its classic action on the pituitary–adrenal axis (Davison *et al.* 1979, Scanes 2015). Meanwhile, our findings also highlight the need to rethink the role of ACTH in energy balance of mammals and teleosts, as the existence and/or anorexic action of ACTH in the hypothalamus have been reported in both groups of vertebrates (Krieger *et al.* 1977, O'Donohue & Dorsa 1982, Vergoni *et al.* 1986, Josep Agulleiro *et al.* 2013).

cMRAP2/(cMRAP) can modulate the constitutive activity of cMC3R and cMC4R

In this study, we revealed that both cMC3R and cMC4R display constitutive activity, as their mammalian orthologs (Ollmann *et al.* 1997, Nijenhuis *et al.* 2001, Wang *et al.* 2016). This finding suggests that cMC4R or cMC3R alone may regulate the physiological processes in the CNS and

peripheral tissues, such as the tonic inhibition of feeding behavior in the hypothalamus, without the involvement of their endogenous agonists, or antagonist.

Interestingly, we noted that cMRAP2, but not cMRAP, can completely inhibit the strong constitutive activity of cMC3R. To our knowledge, this finding has not been reported in any vertebrate species before. However, analogous to our finding in chickens, the constitutive activity of MC4R can be inhibited by MRAP2 (MRAP2b) in mice and zebrafish (Asai *et al.* 2013, Josep Agulleiro *et al.* 2013, Sebag *et al.* 2013). Since cMC3R could still be activated by ACTH preferentially and potently in the presence of cMRAP2 (Fig. 3), we speculated that cMRAP2 may play an active role in stabilizing or locking cMC3R in an inactive conformation and conferring the sensitivity of cMC3R to ACTH (Fig. 10), rather than reducing its cell surface expression (Fig. 6).

Although cMC4R has a weak constitutive activity, cMRAP2 still shows considerable inhibition on its activity. Our finding is in accordance with the findings in mice and zebrafish, in which MRAP2 (or MRAP2b) can block the constitutive activity of MC4R (Asai *et al.* 2013, Sebag *et al.* 2013). Interestingly, we also observed that cMRAP can enhance the basal luciferase activity of CHO cells expressing cMC4R. Similar to our finding, Kay and coworkers also reported that MRAP can increase the constitutive activity of human MC4R transiently expressed in HEK293 cells (Kay *et al.* 2013). These findings indicate the distinct roles of MRAP and MRAP2 in modulating the constitutive activity of MC4R in birds and mammals (Fig. 5).

AgRP functions as inverse agonist and antagonist of cMC3R and cMC4R

In this study, we found that AgRP can potently inhibit the constitutive activity of cMC3R, indicating that AgRP can act as a potent inverse agonist for MC3R. Interestingly, we also noted that in the presence of cMRAP2, AgRP is much less potent in its inhibition on MC3R basal activity. This may be due to the masking of the inhibitory effect of AgRP by cMRAP2, rather than the reduction in the affinity of cMC3R for AgRP, since AgRP could still antagonize α -MSH/ACTH action effectively on CHO cells co-expressing cMC3R and cMRAP2 (Figs 7 and 8).

Unlike that of cMC3R, the weak basal activity of cMC4R could only be inhibited by AgRP at a high concentration (100 nM). Our finding differs slightly from that in mammals, in which AgRP can inhibit the strong

constitutive activity of MC4R effectively (Ollmann *et al.* 1997, Haskell-Luevano & Monck 2001, Nijenhuis *et al.* 2001, Ghamari-Langroudi *et al.* 2015). The weak basal activity of cMC4R also prevented us from drawing a conclusion on whether cMRAP2/cMRAP could modulate the inverse agonistic action of AgRP on cMC4R. In spite of this uncertainty, our data suggest that AgRP alone can inhibit the constitutive activity of MC4R/MC3R and thus regulate some physiological processes, such as induction of food intake in birds and mammals (Rossi *et al.* 1998, Tachibana *et al.* 2001, Strader *et al.* 2003), in an α -MSH/ACTH-independent manner.

It was hypothesized that AgRP can block the anorexic action of α -MSH, possibly through interaction with MC4R/(MC3R) in avian hypothalamus (Tachibana *et al.* 2001, Strader *et al.* 2003, Boswell & Dunn 2015, Dunn *et al.* 2015); however, this hypothesis has been without experimental validation until now. Here, we demonstrated that AgRP can effectively antagonize α -MSH/ACTH actions on cMC4R/(cMC3R) *in vitro* (Figs 7 and 8), indicating that as in mammals and teleosts (Ollmann *et al.* 1997, Song & Cone 2007), AgRP can function as a potent antagonist for MC4R and MC3R in birds as well. Furthermore, we proved that in the presence of cMRAP2 (or cMRAP), AgRP can still antagonize α -MSH/ACTH actions on cMC4R/cMC3R effectively. Similar to our findings in chickens, AgRP can antagonize ACTH₁₋₂₄ action on MC4R in the presence of MRAP2a in zebrafish (Song & Cone 2007). These findings suggest that *in vivo*, AgRP can act as an antagonist to block α -MSH/ACTH actions on MC4R/MC3R effectively, regardless of the presence or absence of MRAP2 (or MRAP).

Co-expression of MRAP2, AgRP, POMC, MC3R and MC4R in chicken hypothalamus

The intricate interaction of MRAP2, MRAP, ACTH/ α -MSH and AgRP with MC4R/MC3R *in vitro* led us to further examine their spatial expression in chickens. We found that both MC3R and MC4R are predominantly expressed in chicken hypothalamus. The predominant expression of cMC4R/cMC3R, together with the abundant expression of cMRAP2 in chicken hypothalamus (Fig. 9), strongly suggests that as in mammals and zebrafish (Asai *et al.* 2013, Josep Agulleiro *et al.* 2013, Sebag *et al.* 2013), cMRAP2 may be co-expressed with cMC4R/(cMC3R) in some neurons, and thus, can fine-tune the constitutive activity, ligand sensitivity and selectivity, and signaling of cMC4R/(cMC3R) and orchestrate the actions of α -MSH/ACTH and AgRP on energy balance (Fig. 10). As cMRAP

expression is extremely low in chicken hypothalamus, it is unlikely that it is involved in this action therein.

Apart from *cMC3R*, *cMC4R* and *cMRAP2*, *cAgRP* is also predominantly expressed in chicken hypothalamus (Fig. 9), where abundant expression of *cPOMC* was found therein. Our findings further support the notion that AgRP(NPY) neurons and POMC neurons residing in the infundibular nucleus (Boswell *et al.* 2002, Phillips-Singh *et al.* 2003) may act as two key signaling centers to control energy balance through interaction with MC4R/(MC3R) (Boswell & Dunn 2015), or MC4R/(MC3R)–MRAP2 complex, in chicken hypothalamus (Fig. 10).

Regulation of MRAP2, POMC, AgRP and NPY expression by fasting: implications for their involvement in energy balance

Since *POMC* and *MRAP2* are abundantly expressed in chicken hypothalamus (Fig. 9), thus, we further examined whether *MRAP2* and *POMC* expression in chicken hypothalamus, like *AgRP* and *NPY*, could also be regulated by fasting (Boswell *et al.* 2002). We found that both 24-h and 36-h fasting can induce *cAgRP* and *cNPY* mRNA expression (Supplementary Fig. 2), as previously reported (Boswell *et al.* 2002, Phillips-Singh *et al.* 2003, Dunn *et al.* 2013, 2015). In contrast, 24-h fasting cannot alter *cPOMC* mRNA level, while 36-h fasting downregulates *cPOMC* expression (Supplementary Fig. 2). These findings, together with evidence showing the expression of the functional receptors for NPY (Yi *et al.* 2015, He *et al.* 2016, Gao *et al.* 2017) and α -MSH/ACTH (*cMC4R/cMC3R*) in chicken hypothalamus, supports the notion that the elevated *cAgRP* (and *cNPY*) production may stimulate food intake by activating neuronal feeding pathways via NPY receptor(s) and blocking *cMC4R/(cMC3R)*-regulated anorexic pathways under negative energy status (Kuenzel *et al.* 1987, Tachibana *et al.* 2001, Boswell *et al.* 2002, Phillips-Singh *et al.* 2003, Boswell & Dunn 2015). Meanwhile, the downregulated *cPOMC* expression may further reduce the anorexic action of α -MSH/ACTH simultaneously. Interestingly, we also noted that fasting can increase *cMRAP2* expression, as reported in zebrafish (*MRAP2b*) (Josep Agulleiro *et al.* 2013), implying that the increased *cMRAP2* expression may inhibit the basal constitutive activity of *cMC4R/cMC3R*, thus also driving food intake under negative energy status.

It is well documented that in mammals, hypothalamic *AgRP* and *POMC* expression is regulated by peripheral

metabolic signals, such as leptin (*LEP*) derived from adipose tissue (Morton *et al.* 2006). However, recently, we and others both revealed that little or no *LEP* expression could be detected in adipose tissue of zebra finches, chickens and ducks (Huang *et al.* 2014, Seroussi *et al.* 2016). These findings hint that birds harbor a regulatory network of energy balance different from the model established in mammals (Huang *et al.* 2014, Boswell & Dunn 2015, Seroussi *et al.* 2016), in which avian leptin may not act as an adipocyte-derived signal to control energy balance (Huang *et al.* 2014, Seroussi *et al.* 2016). However, the co-expression of *MC4R/(MC3R)*, *AgRP*, *POMC* and *MRAP2* in the hypothalamus, together with the interaction of MC4R/MC3R with *AgRP*, α -MSH/ACTH and *MRAP2*, noted in zebrafish, chickens (Figs 9 and 10) and mammals (Asai *et al.* 2013, Josep Agulleiro *et al.* 2013, Sebag *et al.* 2013), still highlight the conserved roles of this interaction network (Fig. 10) in energy balance, metabolism and growth across vertebrates (Cone 2006, Cerda-Reverter *et al.* 2011, Boswell & Dunn 2015). Undoubtedly, our study sets a cornerstone towards uncovering the conserved roles of MC4R/MC3R signaling in energy homeostasis of birds, a group of endothermic vertebrates adopting diverse and fascinating energy balance strategies (Huang *et al.* 2014).

In summary, we revealed an intricate interaction network of MC4R/MC3R with α -MSH/ACTH, *AgRP* and *MRAP2* (/MRAP) in chickens. As in mammals/teleosts, α -MSH/ACTH can activate MC4R and MC3R in chickens, while *AgRP* can antagonize α -MSH/ACTH actions on MC4R/MC3R and lower the constitutive activity of both receptors. Interestingly, we also found that *MRAP2* can enhance the sensitivity of MC4R (and MC3R) for ACTH and block the constitutive activity of both receptors. These findings, together with evidence showing the co-expression of *POMC*, *AgRP*, *MC4R*, *MC3R* and *MRAP2* in chicken hypothalamus and the opposite central actions of *AgRP* and α -MSH/(ACTH) on food intake, suggest that within the hypothalamus, *AgRP* and α -MSH/(ACTH) may act in a coordinated manner on the MC4R/(MC3R)–MRAP2 complex, or MC4R/MC3R, to control energy balance in birds (Fig. 10), in a way similar to that in mammals/teleosts. Meanwhile, the increased sensitivity of *cMC4R/(cMC3R)* for ACTH in the presence of *MRAP2/(MRAP)* also implies that like α -MSH, ACTH, via interaction with MC4R/MC3R, may be involved in the regulation of energy balance and other physiological processes, representing a potential new facet of ACTH actions, which may have long been ‘masked’

by the actions of α -MSH and warrant our attention in future studies.

Notes

During the course of our manuscript preparation, Does and coworkers also described that chicken MRAP and MRAP2 can enhance the sensitivity of chicken MC3R and MC4R expressed in CHO cells for human ACTH₁₋₂₄ in an abstract (Does *et al.* 2016b), which partially coincides with some findings presented in our study. Chicken MRAP2 cDNA sequence was submitted to GenBank in 2015 under an accession no.: KT183012.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-17-0131>.

Declaration of interest

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

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