General and Comparative Endocrinology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

Analyzing the effects of co-expression of chick (*Gallus gallus*) melanocortin receptors with either chick MRAP1 or MRAP2 in CHO cells on sensitivity to ACTH(1–24) or ACTH(1–13)NH_2: Implications for the avian HPA axis and avian melanocortin circuits in the hypothalamus

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ARTICLE INFO

Article history:
Received 10 April 2017
Revised 29 August 2017
Accepted 3 September 2017
Available online xxxx

Keywords:
Gallus gallus
ACTH
α-MSH
Melanocortin receptors
MRAP1
MRAP2

ABSTRACT

In order to better understand the roles that melanocortin receptors (cMCRs) and melanocortin-2 receptor accessory proteins (cMRAP1 and cMRAP2) play in the HPA axis and hypothalamus, adrenal gland and hypothalamus mRNA from 1 day-old white leghorn chicks (*Gallus gallus*), were analyzed by real-time PCR. mRNA was also made for kidney, ovary, and liver. *Mrap1* mRNA could be detected in adrenal tissue, but not in any of the other tissues, and *mrap2* mRNA was also detected in the adrenal gland. Finally, all five melanocortin receptors mRNAs could be detected in the adrenal gland; *mc2r* and *mc5r* mRNAs were the most abundant. To evaluate any potential interactions between MRAP1 and the MCRs that may occur in adrenal cells, individual chick *mcr* cDNA constructs were transiently expressed in CHO cells either in the presence or absence of a chick *mrap1* cDNA, and the transfected cells were stimulated with hACTH(1–24) at concentrations ranging from 10^{-13} M to 10^{-6} M. As expected, MC2R required co-expression with MRAP1 for functional expression; whereas, co-expression of cMC3R with cMRAP1 had no statistically significant effect on sensitivity to hACTH(1–24). However, co-expression of MC4R and MC5R with MRAP1, increased sensitivity for ACTH(1–24) by approximately 35 fold and 365 fold, respectively. However, co-expressing of cMRAP2 with these melanocortin receptors had no effect on sensitivity to hACTH(1–24). Since the real-time PCR analysis detected *mrap2* mRNA and *mc4r* mRNA in the hypothalamus, the interaction between cMC4R and cMRAP2 with respect to sensitivity to ACTH(1–13)NH_2 stimulation was also evaluated. However, no effect, either positive or negative, was observed. Finally, the highest levels of *mc5r* mRNA were detected in liver cells. This observation raises the possibility that in one-day old chicks, activation of the HPA axis may also involve a physiological response from liver cells.

1. Introduction

Studies on mammalian melanocortin receptors have revealed a number of interesting features of this G Protein-Coupled Receptor gene family. For example, while all of the mammalian melanocortin receptors are activated by melanocortin peptides that have the HFRW motif, these receptors segregate into two groups based on ligand selectivity. MC2R can only be activated by ACTH, but not by any of the MSH-sized ligands (i.e., α-MSH, β-MSH, γ-MSH); whereas as MC1R, MC3R, MC4R, or MC5R can be activated by either ACTH or MSH-sized ligands to varying degrees (Cone, 2006). The melanocortin peptides are all derived from the precursor protein, POMC (proopiomelanocortin; Nakanishi et al., 1979). As a result of tissue specific deferential posttranslational processing, ACTH is the major melanocortin end-product in corticotropic cells of the anterior pituitary of mammals; whereas, the MSH-sized ligands are the major end-products in melanotropic cells of the intermediate pituitary, and subsets of nuclei in the central nervous system of mammals (Eipper and Mains, 1980; D'Agostino and Diane, 2010).

Another intriguing feature of some of the mammalian melanocortin receptors is the interaction with the accessory proteins, MRAP1 and MRAP2 (melanocortin-2 receptor accessory protein 1

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http://dx.doi.org/10.1016/j.ygcen.2017.09.002
0016-6480/

& 2; for review see: Ramachandrappa et al., 2013). MRAP1 and MRAP2 are single chain polypeptides, with one transmembrane domain, that form a homodimer with reverse topology (Hinkle and Sebag, 2009; Webb and Clark, 2010). As seen for the human MC2R ortholog, interaction with MRAP1 is an obligatory requirement that not only facilitates the trafficking of the receptor from the endoplasmic reticulum to the plasma membrane, but also is required for activation of the receptor following an ACTH binding event (Metherell et al., 2005). While co-expression of MRAP1 with either human MC1R or human MC3R in CHO cells had no negative effect on the trafficking or activation of these receptors, co-expression of MRAP1 with either human MC4R or human MC5R decreased trafficking of these receptors to the plasma membrane (Ramachandrappa et al., 2013).

MRAP2 is the paralog of MRAP1 (Chan et al., 2009), and although MRAP2 is present in mammalian adrenal cortex cells (Vinson, 2016), this accessory protein can only mediate the trafficking of MC2R to the plasma membrane, but cannot facilitate the activation of the receptor following stimulation with ACTH (Ramachandrappa et al., 2013). The inability to facilitate activation of MC2R is due to the absence of an activation motif in the N-terminal of MRAP2 that is present in the N-terminal of MRAP1 (Hinkle and Sebag, 2009; Webb and Clark, 2010). Chan et al. (2009) observed that co-expression of either human MC1R or human MC3R with human MRAP2 in CHO cells had no effect on the trafficking of these receptors to the plasma membrane. However, human MRAP2 had a negative effect on the activation of human MC3R following stimulation with NDP-MSH. In addition, co-expression of either human MC4R or human MC5R with human MRAP2 in CHO cells had negative effects on both trafficking of these receptors to the plasma membrane and activation by NDP-MSH (Chan et al., 2009). In this regard, it is interesting that *mrap2* mRNA levels are highest in the hypothalamus (Chan et al., 2009). Studies on the mouse MC4R circuit in the hypothalamus indicate that knocking out the MC4R gene results in obesity. In addition, co-expression of mouse MC4R with mouse MRAP2 in CHO cells increases the sensitivity of this receptor for stimulation by α-MSH (Asai et al., 2013). Hence, the interaction between MRAP2 and at least MC4R appears to be species specific.

When the preceding observations are taken collectivity, it is clear that MRAP1 plays a critical role in adrenal cortex cells through a compulsory interaction with MC2R to facilitate binding of ACTH to the receptor to initiate the production of glucocorticoids (Metherell et al., 2005). In the human adrenal cortex MC2R is the "ACTH" receptor (Noveselosa et al., 2013). However, in the rat adrenal cortex while MC2R is clearly the "ACTH" receptor, MC5R is also expressed in these cells, but to a lesser extent than MC2R (Vinson, 2016). In addition, the high expression levels of MRAP2 in the hypothalamus (Chan et al., 2009), point to a role for a MC4R/MRAP2 interaction in the regulation of feeding behavior at least in some mammals (Asai et al., 2013). To investigate whether these generalizations apply to other tetrapod melanocortin networks, the following study was done on the melanocortin receptors of the chick, *Gallus gallus*.

The chick melanocortin system seems ideally suited for this type of study. All five melanocortin receptor genes are present in the chick genome as well as the sequences of the *mrap1* and *mrap2* genes (http://uswest.ensembl.org/Gallus_gallus/Info/Index). In an earlier study, prior to the discovery of the MRAPs, Ling et al. (2004) observed that chick (c) cMCIR, cMC3R, cMC4R, and cMC5R had higher affinity for ACTH than for α-MSH. This outcome may reflect co-evolution between avian melanocortin receptors and their primary melanocortin ligand, ACTH. The avian pituitary lacks an intermediate lobe. Hence, the only cells that express the *pomc* gene are the corticotropic cells of the anterior pituitary, and the major melanocortin end-product is ACTH (Takahashi and Mizusawa, 2013). Barlock et al. (2014) subsequently showed that functional expression of cMC2R in CHO cells required co-expression with cMRAP1. However, the potential interactions between the other chick melanocortin receptors and either cMRAP1 or cMRAP2 have not been analyzed. As a result, this study was undertaken to: a) use real-time PCR to determine the tissue distribution of *cmrap1*, *cmrap2*, *cmc1r*, *cmc2r*, *cmc3r*, *cmc4r*, and *cmc5r* mRNA levels in the chick adrenal gland, hypothalamus, kidney, ovary, and liver; b) use co-expression of cMC2R, cMC3R, cMC4R, or cMC5R with either cMRAP1 or cMRAP2 in CHO cells to determine whether interactions with these accessory proteins have any effect on sensitivity to stimulation with ACTH; and finally, c) to use co-expression of cMC4R with cMRAP2 in CHO cells to determine whether this accessory protein had any effect on sensitivity to stimulation with ACTH(1–13)NH_2 the expected melanocortin ligand in the avian hypothalamus. The operating assumptions at the start of this study were that the chick melanocortin receptors would respond in essentially the same manner to the MRAP accessory proteins as seen in the mammalian studies (Chan et al., 2009). However, for some of the melanocortin receptors the outcomes were unexpected, and raise interesting phylogenetic questions about the evolution of the interactions between the melanocortin receptors and the MRAPs in tetrapods.

2. Methods and materials

2.1. Melanocortin receptors and synthetic peptides

The nucleotide sequences of *G. gallus* melanocortin receptors (MCRs) and MRAP sequences were obtained from GenBank, and the accession numbers for the chick melanocortin receptors and MRAPs are: MC1R (KF379749.1), MC2R (XM_015282470.1), MC3R (XM_004947236.2), MC4R (NM_001031514.1), MC5R (XM_015282466.1), MRAP1 (XR_001470382.1), and MRAP2 (XM_015282466.1). Each cDNA sequence was individually inserted into a pcDNA3+ expression vector (GenScript; Piscataway, NJ). Synthetic human ACTH(1–24) and synthetic mammalian ACTH(1–13)NH_2 were purchased from New England Peptide, (Garner, MA).

2.2. Real-time PCR

For real-time PCR, 1 day-old SPF White Leghorn chicken chicks (Nisseiken, Co, Japan) were sacrificed by decapitation. The experimental protocols followed the guideline of the National Institutes of Health, and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and were approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by National Institute for Environmental Studies (Tsukuba, Japan). All efforts were made to minimize animal suffering and reduce the number of animals used in this study.

Total RNA taken from adrenal glands, kidneys, ovaries, hypothalami, and livers were extracted by RNeasy Mini Kit (QIAGEN KK) with DNase treatment following the manufacturer's instruction. cDNA was synthesized by reverse transcriptase XL (Takara Bio Inc.) using 100 ng total RNA as template and oligo (dT) as a primer. Expression of target genes and β-actin was quantified by real-time PCR using LightCycler 480 SYBR Green I Master on a LightCycler Instrument (Roche Diagnostics). PCR settings were as follows: 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s. Amplification in experimental samples during the log linear phase was compared with the standard curve from the dilution series of a control cDNA using LightCycler quantification software (Version 3.5). The primer sequences used for real-time PCR are shown in Supplemental Table 1. The DNA length of end product

Please cite this article in press as: Thomas, A.L., et al. Analyzing the effects of co-expression of chick (*Gallus gallus*) melanocortin receptors with either chick MRAP1 or MRAP2 in CHO cells on sensitivity to ACTH(1–24) or ACTH(1–13)NH_2: Implications for the avian HPA axis and avian melanocortin circuits in the hypothalamus. Gen. Comp. Endocrinol. (2017), http://dx.doi.org/10.1016/j.ygcen.2017.09.002