General and Comparative Endocrinology 272 (2019) 42–51

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Elephant shark melanocortin receptors: Novel interactions with MRAP1 and implication for the HPI axis

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ARTICLE INFO

Keywords:
Melanocortin receptors
MRAP1
MRAP2
Elephant shark
HPI

ABSTRACT

The presence of Mrap1 and Mrap2 orthologs in the genome of the elephant shark (es), a cartilaginous fish, presented an opportunity to evaluate the potential interactions between these accessory proteins and melanocortin receptors of a cartilaginous fish. RT-PCR analysis indicated that Mrap1 mRNA was present in interrenal, brain, and pituitary tissue with mRNA for Mc2R, Mc3R, Mc4R, and Mc5r. Co-expression of esMrap1 cDNA with esMc2r cDNA or esMc5r cDNA in CHO cells increased sensitivity to stimulation with ACTH(1–24) 10 fold and 100 fold, respectfully, but had no effect on sensitivity to stimulation with DesAc-αMSH [i.e., ACTH(1–13)NH_2] for either receptor, and had no effect on the ligand sensitivity of either esMc3r or esMc4r. Fluorescence image analysis indicated co-localization of esMrap1/esMc2r, and esMrap1/esMc5r on the plasma membrane; however, cell surface ELISA analysis indicated that co-expression with esMrap1 had no effect, positive or negative, on the trafficking of either esMc2r or esMc5r to the plasma membrane. RT-PCR analysis also indicated that Mrap2 mRNA, as well as, mRNAs for Mc2r, Mc3r, Mc4r, and Mc5r could be detected in brain tissue, however no Mrap2 mRNA was detected in interrenal tissue. Co-expression of esMrap2 in CHO cells with, respectively, esMc2r, esMc4r, or esMc5r had no effect on ligand sensitivity. However, co-expression of esMrap2 with esMc3r did lower sensitivity to stimulation by DesAc-αMSH 10 fold. These observations are discussed in the context of the parallel evolution of melanocortin receptors and their accessory proteins, and the hypothalamus/pituitary/adrenal axis and the hypothalamus/pituitary/interrenal axis in bony vertebrates and cartilaginous fishes.

1. Introduction

A defining feature of teleost and tetrapod melanocortin-2 receptor (Mc2r) orthologs is the formation of a heterodimer with Mrap1 (melanocortin receptor-2 accessory protein 1) (Metherell et al., 2005; Hinkle and Sebag, 2009; Webb and Clark, 2010). This obligatory interaction facilitates the trafficking of the Mc2r ortholog from the endoplasmic reticulum to the plasma membrane. This interaction also places the Mc2r ortholog in the proper conformation for activation by ACTH, but not by any of the MSH-sized peptides (i.e., αMSH, βMSH, or γMSH) (Dores and Garcia, 2015).

The Mc2r ortholog of the cartilaginous fish, *Callorhynchus milii* (subclass Holocephali; elephant shark; es) is an exception to the preceding generalization. When esMc2r was expressed in Chinese Hamster Ovary (CHO) cells in the absence of an exogenous Mrap1 ortholog, the receptor trafficked to the plasma membrane. In addition, esMc2r could be activated by ACTH(1–24) as well as αMSH, βMSH, γMSH or δMSH (Renick et al., 2012). These observations suggested that the functional activation of esMc2r was Mrap1 independent (Dores and Garcia, 2015). However, a recent update of the elephant shark genome project revealed an Mrap1-like ortholog (Dores, 2016) in addition to an Mrap2-like ortholog (Vastermark and Schiöth, 2011). The presence of both Mrap1 and Mrap2 genes in the elephant shark genome would suggest that these genes were present in the ancestral gnathostomes prior to the divergence of the ancestral cartilaginous fish and the ancestral bony fish lineages (Dores, 2016). Hence, during the early evolution of the ancestral cartilaginous fish lineage, interactions between melanocortin receptors (Mcrs) and the Mraps may have developed independent of the interactions that emerged between Mcrs and Mraps in the common ancestors to the modern bony fishes and tetrapods.

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https://doi.org/10.1016/j.ygcen.2018.11.009
Received 10 September 2018; Received in revised form 15 November 2018; Accepted 16 November 2018
Available online 20 November 2018

To address this issue, various elephant shark tissues were analyzed by RT-PCR to determine whether esMrap1, esMrap2, and any of the other esMcrs mRNAs are found in the same tissues. Next pharmacological studies were done to determine whether co-expression of esMcrs with either esMrap1 or esMrap2 had any effect on sensitivity for activation by either a cartilaginous fish ACTH(1–24) or Des-Acetyl-αMSH. Experiments were also done to determine whether an esMc2r/esMrap1 heterodimer or an esMc5r/esMrap1 heterodimer could be visualized immunocytochemically, and to determine whether co-expression of esMc2r or esMc5r with esMrap1 has any effect on the trafficking of these receptors to the plasma membrane.

2. Materials and methods

2.1. Elephant shark cDNA sequences

The nucleotide sequences for elephant shark melanocortin receptors were obtained from the elephant shark genome database (http://esharkgenome.imcb.a-star.edu.sg): esMC1R (AAVX0145647.1), esMC2R (AAVX01069419.1), esMC3R (AAVX01131453.1), esMC4R (XM_007895520), and esMC5R (KI 635891.1). The nucleotide sequences for esMc2r, esMc3r, esMc4r, and esMc5r were individually synthesized with an N-terminal V5 epitope tag, and the cDNA constructs were individually inserted into a pcDNA3.1 + expression vector by GenScript (Piscataway, NJ). The nucleotide sequences for esMrap1 (XM_007903550.1) and esMrap2 (XP_007906624.1) were synthesized with an N-terminal FLAG epitope tag, and individually inserted into a pcDNA3.1 + expression vector by GenScript.

2.2. RT-PCR analysis

Sampling of the elephant shark tissues was described previously (Hasegawa et al., 2016). Elephant sharks of both sexes were collected in Western Port Bay, Victoria, Australia, using recreational fishing equipment, and were transported to the Primary Industries Research Queenscliff station (Queenscliff, Victoria, Australia). Fish were kept in a 10,000 L round tank with running seawater under a natural photoperiod. For sampling, fish were anesthetized in 0.1% 3-amino benzoic acid ethyl ester (Sigma-Aldrich, St. Louis, MO). After decapitation of the fish, tissues were dissected out and quickly frozen in liquid nitrogen and stored at −80 °C. All animal experiments were conducted according to the "Guidelines for the Care and Use of Animals" approval for this project was obtained from the Animal Care and Use Committee of the University of Tokyo.

Total RNA was extracted from the obtained tissues with Isogen (Nippon Gene, Toyama, Japan). Two micrograms of total RNA was treated using a TURBO DNA-free kit (Life Technologies, Carlsbad, CA) and reverse-transcribed to first-strand cDNA using a high-capacity cDNA reverse transcription kit (Life Technologies), following the manufacturer's instructions. To examine tissue distribution of mRNAs, RT-PCR was carried out with Kapa Taq Extra DNA polymerase (Kapa Biosystems, Boston, MA). Primer sets were designed using Primer Express software, and their sequences are shown in Supplemental Table 1. As internal controls, elephant shark β-actin (esACTB) mRNA was used. PCR products were separated on 1% agarose gel, and stained with ethidium bromide.

2.3. Transient expression of esMcrs in CHO cells & CRE-Luciferase reporter assay

The elephant shark cDNAs were transiently transfected into Chinese Hamster Ovary (CHO) cells as described in Liang et al. (2011) and maintained at 37 °C in a CO_2 incubator. To analyze the potential effect of esMrap1 and esMrap2 on the ligand selectivity of esMc2r, esMc3r, esMc4r, and esMc5r, each receptor was individually expressed in CHO cells, with the cAMP reporter construct, CRE-Luciferase (Chepurny and Holz, 2007), either in the presence or absence of an esmrap1 cDNA or an esmrap2 cDNA. The transient transfections were done using a Solution T kit (Lonza, Portsmouth, NH) and the Amaxa Cell Line Nucleofector II system (Lonza, Portsmouth, NH). The transfected CHO cells were grown in a white 96-well plate (Corning Life Sciences, Manassas, VA) at a final density of 1×10^5 cells/well. After a 48-hour incubation at 37 °C, the cells were stimulated with either synthetic stingray (subclass Elasmobrachii; *Dasyatis akajei*; sr) srACTH(1–24) or srDes-Acetyl-αMSH [i.e., srACTH(1–13)NH_2] (New England Peptide, Gardiner, MA) in serum-free CHO media at concentrations ranging from 10^{-13} M to 10^{-6} M. The rationale for using stingray derived melanocortin peptides was that these sequences are nearly identical in elasmobranchs and holocephalans. The amino acid sequence of stingray ACTH(1–24) is SYSMEHFRWGKP**K**GRKRRPIKV**Y**P (Amemiya et al., 2000) and the sequence of elephant shark ACTH(1–24) is SYSMEHFRWGKP**V**GRKRRPIKV**S**P (accession number: XP_0070905). In addition, the non-acetylated form of αMSH [i.e., Des-Acetyl-αMSH = ACTH(1–13)NH_2] was used due to the fact that the intermediate pituitary cells of cartilaginous fishes apparently do not N-acetylate this peptide (Takahashi et al., 2004).

Following a 4-hour incubation at 37 °C, luciferase substrate reagent (Bright GLO; Promega, WI) was added to each well as described in Liang et al. (2011). A Bio-Tek Synergy HTX plate reader (Winooski, VT) measured the luminescence generated after a five-minute incubation period at room temperature. Transfected CHO cells incubated with serum-free media, but no ACTH(1–24) or Des-Acetyl-αMSH, were analyzed along with each experimental group to determine basal cAMP levels.

Luminescence readings were corrected by subtracting the average basal cAMP readings (serum-free media/no ligand) for each transfection dose response curve. The data for each dose response curve were fitted to the Michaelis-Menton equation to obtain EC_{50} values using Kaleidograph software (www.synergy.com). Data points are expressed as the mean ±SEM (n = 3). To analyze the treatment effects of co-expression with esMRAP1 and esMRAP2 on ligand sensitivity, the corrected data sets were analyzed using a one-way ANOVA followed by Tukey's multi-comparison test using GraphPad Prism 2 software (GraphPad Inc, La Jolla, CA, USA). Significance was set at $p \leq 0.05$.

2.4. Cell surface ELISA protocol

COS-7 cells were plated at 0.75×10^5 cells/well in a 24-well culture dish, and grown overnight. Cells were transfected with cDNAs encoding esMc2r or esMc5r in combination with pcDNA3.1 vector, esMrap1 or esMrap2 using jetPRIME transfection reagents (Polyplus transfection, Illkirch, France). After 48 h, cells were fixed in 4% Paraformaldehyde, washed and then incubated with polyclonal V5-epitope antibody (Genetex, Irvine, CA, USA) or HA-epitope antibody (Rockland Immunochemicals, Limerick, PA, USA) followed by secondary HRP-conjugated goat anti-rabbit antibody. Cells were washed and treated with one-step 2,2′azinobis-3ethylbenzthiazoline-6-sulfonic acid (one-step ABTS) (Thermo Fisher Scientific, Waltham, MA, USA). An aliquot of supernatant was removed and absorbance at 405 nm was determined using a Spectramax i3 plate reader (Molecular Devices, San Jose, CA, USA). Data was analyzed using a one-way ANOVA with Tukey's multi-comparison post-test using GraphPad Prism software (GraphPad Inc, La Jolla, CA, USA) and the threshold for significance was set at $p < 0.05$.

2.5. Imaging of esMC2R and esMC5R with esMRAP1 and esMRAP2

COS-7 cells were seeded on fibronectin-coated glass coverslips in a 12-well culture dish at a density of 1.5×10^5 cells/well. Cells were transfected with cDNAs encoding esMc2r or esMc5r in combination with pcDNA3.3 + vector, esMrap1 or esMrap2 using jetPRIME transfection reagents (Polyplus transfection). After 48 hr, cells were fixed in 4% Paraformaldehyde and permeabilized using 100% methanol. Cells

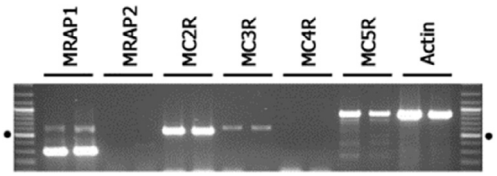

Fig. 1. Expression of esMrap and esMcr mRNAs in interrenal tissue. Interrenal tissues from two individuals were subjected to RT-PCR analysis. Expected sizes of amplicons are 294 bp (Mrap1), 345 bp (Mrap2), 552 bp (Mc2r), 596 bp (Mc3r), 755 bp (Mc4r), 872 (Mc5r), and 882 bp (Actin). GeneRuler Mix (ThermoFisher) was used as a molecular marker, and a filled circle represents 500 bp.

were then incubated with monoclonal FLAG-epitope antibody (Sigma-Aldrich, St. Louis, MO, USA) and either polyclonal V5-epitope antibody (Genetex) or HA-epitope antibody (Rockland Immunochemicals). Cells were then incubated with DyLight 488 and DyLight 594 secondary antibodies (Rockland Immunochemicals). Stained coverslips were preserved on glass slides using FluorSave Reagent (Millipore Sigma, St. Louis, MO, USA) and images were collected using a Fluoview FV10i confocal microscope (Olympus, Center Valley, PA, USA).

3. Results

3.1. RT-PCR analysis of elephant shark tissues

Earlier studies have provided evidence for a hypothalamus/pituitary/interrenal axis (HPI) in cartilaginous fishes (for review see Geisleicher, 2004), and more recently Mc2r and Mc5r orthologs were detected in the interrenal cells of the red stingray, *Dasyatis akajei* (Takahashi et al., 2016). To gain insights into the melanocortin components associated with elephant shark interrenal cells, an RT-PCR analysis was done. As shown in Fig. 1, intense signals for esmc2r and esmc5r mRNAs, and to a lesser extent esmc3r mRNA were detected in the elephant shark interrenal tissue. In addition, esmrap1 mRNA was also detected in this tissue, however esmrap2 mRNA was not detected (Fig. 1).

RT-PCR analysis was also done on other elephant shark tissues, and the results are presented in Fig. 2. In these analyses, repeated attempts to detect an esmc1r cDNA using different primer combinations were unsuccessful. In the elephant shark genome data base, the cDNA sequence for an esmc1r paralog is incomplete (http://esharkgenome.imcb.a-star.edu.sg). Hence, it would appear that Mc1r may be a pseudogene in this species.

Since studies on a teleost and a mammal have shown interactions between Mrap2 and Mc4r in the hypothalamus (Sebag et al., 2017; Agulleiro et al., 2013; Asai et al, 2013), the intact brain of the elephant shark was separated into hypothalamus and brain minus hypothalamus. In the hypothalamus (Fig. 2A and B), a strong signal was detected for mRNAs corresponding to esmrap1, esmrap2, esmc3r, esmc4r and esmc5r, respectively. A much weaker signal was detected for the mRNA corresponding to esmc2r. In the brain minus hypothalamus (Fig. 2A and B), mRNAs corresponding to esmrap1, esmrap2, and esmc4r were clearly detected.

The pituitary was another tissue in which multiple mRNAs were intensely detected including esmrap1, esmrap2, esmc2r, esmc4r and esmc5r (Fig. 2A and B), and to a lesser intensity, esmc3r (Fig. 2A). These observations are in contrast to the gill, spleen, kidney, and muscle (male only) in which only the esmrap1 mRNA was intensely detected. Finally, no mRNAs were apparent in the liver, intestine, muscle (female), and rectal gland.

In the remaining tissues, an esmc2r was the only melanocortin receptor mRNA detected in the atrium (female only) and the ventricle (both sexes) (Fig. 2A and B). In the ovary and uterus, the only melanocortin receptor mRNA detected was esmc3r and esmrap1mRNA.

3.2. Co-Expression of elephant shark Mcrs with elephant shark Mrap1

Since esmc2r, esmc5r, esmc3r, esmc4r, as well as esmrap1 cDNAs were detected in interrenal tissue or hypothalamus by RT-PCR analysis (Figs. 1 and 2Aand B), the objective of this phase of the study was to determine whether co-expression with esMrap1 had any effect on the ligand selectivity of the esMcrs. To this end, individual esMcrs were transiently transfected into CHO cells and stimulated with either srACTH(1–24) or srDes-Acetyl-αMSH.

As shown in Fig. 3A, esMc2r expressed alone could be stimulated with either srACTH(1–24) (EC_{50} value = 6.2×10^{-10} M ± 1.0×10^{-10}) or srDes-Acetyl-αMSH (EC_{50} = 8.7×10^{-11} M ± 1.9×10^{-11}); an outcome in agreement with our previous study on esMc2r (Reinick et al., 2012). Note that when expressed alone, esMc2r has higher sensitivity for Des-Acetyl-αMSH than for srACTH (1–24) ($p > 0.0002$).

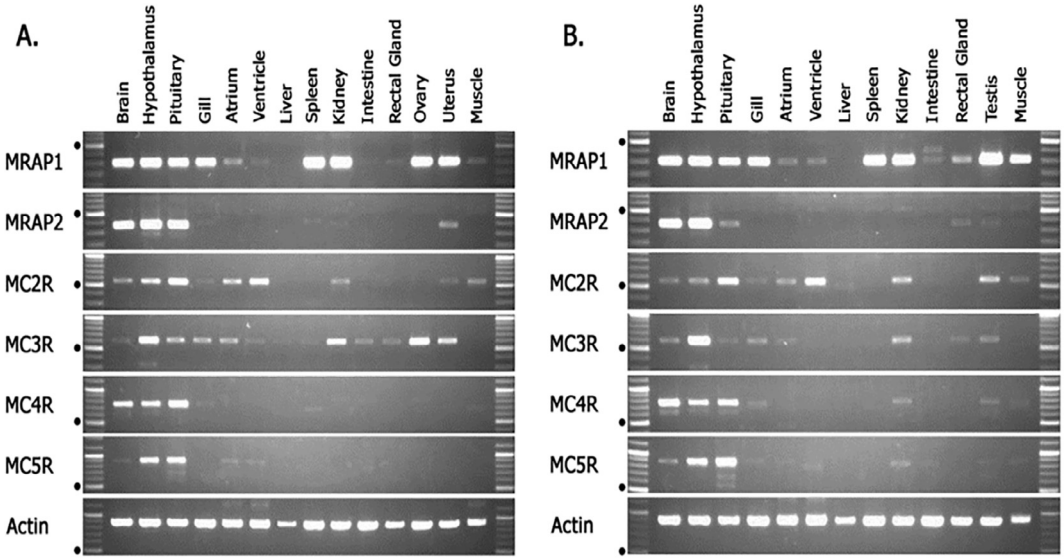

Fig. 2. RTPCR other tissues. Various tissues from two individuals (A, female; B, male) were subjected to RT-PCR analysis. Expected sizes of amplicons are 294 bp (Mrap1), 345 bp (Mrap2), 552 bp (Mc2r), 596 bp (Mc3r), 755 bp (Mc4r), 872 (Mc5r), and 882 bp (Actin). GeneRuler Mix (ThermoFisher) was used as a molecular marker, and a filled circle represents 500 bp.

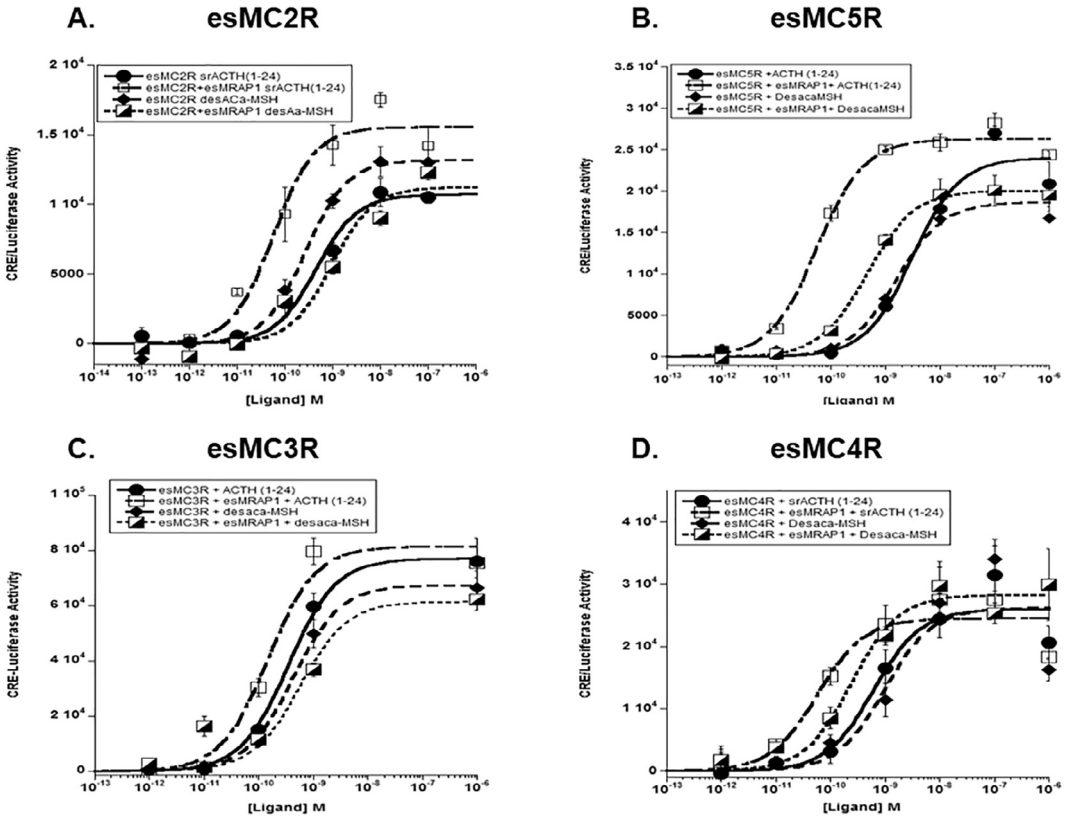

Fig. 3. Co-expression of esMc2r, esMc3r, esMc4r, and esMc5r with esMrap1. CHO cells were either co-transfected with a esMcr cDNA and a Cre/luciferase cAMP reporter cDNA, or co-transfected with a esMcr cDNA, esMrap1 cDNA, and a Cre/luciferase cAMP reporter cDNA as described in METHODS. After 48 h in culture the transfected cells were either stimulated with srACTH(1–24) at concentrations ranging from 10^{-12} M to 10^{-6} M or srDes-Acetyl-αMSH at concentrations ranging from 10^{-13} M to 10^{-7} M. All experiments were done in triplicate, and the data are presented as mean ± SEM. A) esMc2r transfected cells; B) esMc5r transfected cells; C) esMc3r transfected cells; D) esMc4r transfected cells. n = 3 for all data points. srDes-AcMSH = srDes-Acetyl-αMSH.

Table 1
Summary of ligand sensitivity studies: The EC_{50} values for the dose response curves presented in Figs. 3 and 4 are listed in this table, as well as the statistical analysis of each receptor expressed alone or co-expressed with either esMrap1 or esMrap2.

A. Elephant Shark MCRs co-expressed with Elephant Shark MRAP1

Receptor	Alone	+esMRAP1	p Value*
srACTH(1–24)			
MC2R	6.2×10^{-10} M ± 1.0×10^{-10}	3.7×10^{-11} M ± 5.4×10^{-12}	< 0.001
MC3R	3.4×10^{-10} M ± 4.4×10^{-11}	1.4×10^{-10} M ± 5.1×10^{-11}	0.91
MC4R	6.0×10^{-10} M ± 3.4×10^{-10}	5.6×10^{-11} M ± 3.2×10^{-11}	0.52
MC5R	3.0×10^{-09} M ± 1.2×10^{-09}	5.5×10^{-11} M ± 1.0×10^{-11}	< 0.001
srACTH(1–13)NH_2			
MC2R	8.7×10^{-11} M ± 1.9×10^{-11}	1.4×10^{-10} M + 3.5×10^{-11}	0.79
MC3R	4.2×10^{-10} M ± 6.1×10^{-11}	5.7×10^{-10} M ± 3.8×10^{-10}	0.90
MC4R	9.6×10^{-10} M ± 9.0×10^{-10}	2.4×10^{-10} M ± 8.0×10^{-11}	0.99
MC5R	1.6×10^{-09} M ± 4.4×10^{-10}	4.5×10^{-10} M ± 1.0×10^{-11}	0.17

B. Elephant Shark MCRs co-expressed with Elephant Shark MRAP2

Receptor	Alone	+esMRAP2	p Value*
srACTH(1–24)			
MC2R	7.4×10^{-10} M ± 1.7×10^{-10}	5.8×10^{-10} M ± 1.9×10^{-10}	0.33
MC3R	5.3×10^{-11} M ± 1.1×10^{-11}	6.1×10^{-11} M ± 1.5×10^{-11}	0.82
MC4R	6.0×10^{-10} M ± 3.4×10^{-10}	5.6×10^{-11} M ± 3.2×10^{-11}	0.83
MC5R	2.6×10^{-10} M ± 8.1×10^{-11}	1.4×10^{-10} M ± 5.0×10^{-11}	0.16
srACTH(1–13)NH_2			
MC2R	1.7×10^{-10} M ± 2.4×10^{-11}	1.2×10^{-10} M ± 1.5×10^{-11}	0.88
MC3R	5.5×10^{-11} M ± 2.3×10^{-11}	1.2×10^{-10} M ± 3.7×10^{-11}	< 0.001
MC4R	1.1×10^{-10} M ± 4.4×10^{-11}	1.1×10^{-10} M ± 3.4×10^{-11}	0.96
MC5R	5.0×10^{-10} M ± 1.0×10^{-10}	4.8×10^{-10} M ± 1.3×10^{-10}	0.98

* One-Way ANOVA followed by Tuckey's multi-comparison test.

However co-expression of esMc2r with esMrap1 increased sensitivity to stimulation by srACTH(1–24) over 10 fold (Fig. 3A and Table 1A) as compared to esMc2r expressed alone ($p < 0.0001$). Conversely, co-expression with esMrap1 had no effect on sensitivity to stimulation by srDes-Acetyl-αMSH (Fig. 3A and Table 1A; $p = 0.79$).

- When esMc5r was co-expressed with esMrap1, sensitivity to stimulation by srACTH(1–24) was more robust (Fig. 3B), and increased nearly two orders of magnitude (Fig. 3B and Table 1A; $p < 0.001$). The same effect was not seen when esMc5r was stimulated with srDes-Acetyl-αMSH. Co-expression with esMrap1 did not increase sensitivity for stimulation by Des-Acetyl-αMSH (Fig. 3B and Table 1; $p = 0.17$).
- Stimulation of esMc3r with either srACTH(1–24) or srDes-Acetyl-αMSH (Fig. 3C) was not affected in either a positive or negative manner by co-expression with esMrap1 (Fig. 3C and Table 1A). A similar lack of interaction was also observed for esMC4R. Once again, co-expression with esMRAP1 has neither a positive or negative effect on stimulation by either srACTH(1–124) or srDes-Acetyl-αMSH (Fig. 3D and Table 1A).

3.3. Expression of elephant shark Mcrs with elephant shark Mrap2

Since esMc2r, esMc3r, esMc4r, esMc5r as well as esMrap2 cDNAs were detected in brain and pituitary, an analysis was done to determine whether esMrap2 had any effect on ligand sensitivity when co-expressed with either esMc2r, esMc3r, esMc4r, or esMc5r. For example, as shown in Fig. 4A, when esMc2r was co-expressed with esMrap2 and the transfected cells were stimulated with srACTH(1–24), esMrap2 did not cause an increase in sensitivity (Table 1B; $p = 0.33$). Co-expression with esMrap2 also did not have an effect on ligand sensitivity following simulation with srDes-Acetyl-αMSH (Fig. 4A and Table 1B; $p = 0.88$). Co-expression of esMc5r and esMrap2 also did not appear to have an effect on ligand sensitivity for stimulation by either either srACTH (1–24) or srDes-Acetyl-αMSH (Fig. 4B and Table 1B).

Co-expression of esMc3r with esMrap2 did not have an effect on sensitivity for stimulation by srACTH(1–24) (Fig. 4C and Table 1B; $p = 0.82$). However, when esMc3r was stimulated with srDes-Acetyl-αMSH an effect on ligand sensitivity was observed (Fig. 4C and Table 1B). When co-expressed with esMrap2 there was a 10 fold drop in sensitivity to stimulation by srDes-Acetyl-αMSH ($p < 0.001$). In contrast to esMc3r, co-expression of esMc4r with esMrap2 did not have an effect on sensitivity to either ligand (Fig. 4D and Table 1B).

3.4. Evaluating the effect of esMrap1 on the trafficking of esMc2r and esMc5r

The trafficking of teleost and tetrapod Mc2r orthologs to the plasma membrane is dependent on interaction with Mrap1 (reviewed in Dores and Garcia (2015)). To determine whether the trafficking of esMc2r and esMc5r is influenced by co-expression with either esMrap1 or esMrap2, a cell surface ELISA assay was done (Fig. 5). In cells expressing esMc2r alone, the average absorbance at 405 nm was 0.51 ± 0.09 (n = 8), which is comparable to receptor levels at the surface of cells co-expressing esMc2r and esMrap1 (0.56 ± 0.11, n = 8) or esMrap2 (0.51 ± 0.09, n = 8) (Fig. 5A). We found that the effect of esMrap1 on the surface expression of esMc2r was not significant ($p = 0.59$). Similarly, we observed that receptor levels in cells expressing esMc5r alone (0.38 ± 0.11, n = 9) was comparable to receptor expression in cells co-transfected with esMc5r and esMrap1 (0.35 ± 0.09) or esMrap2

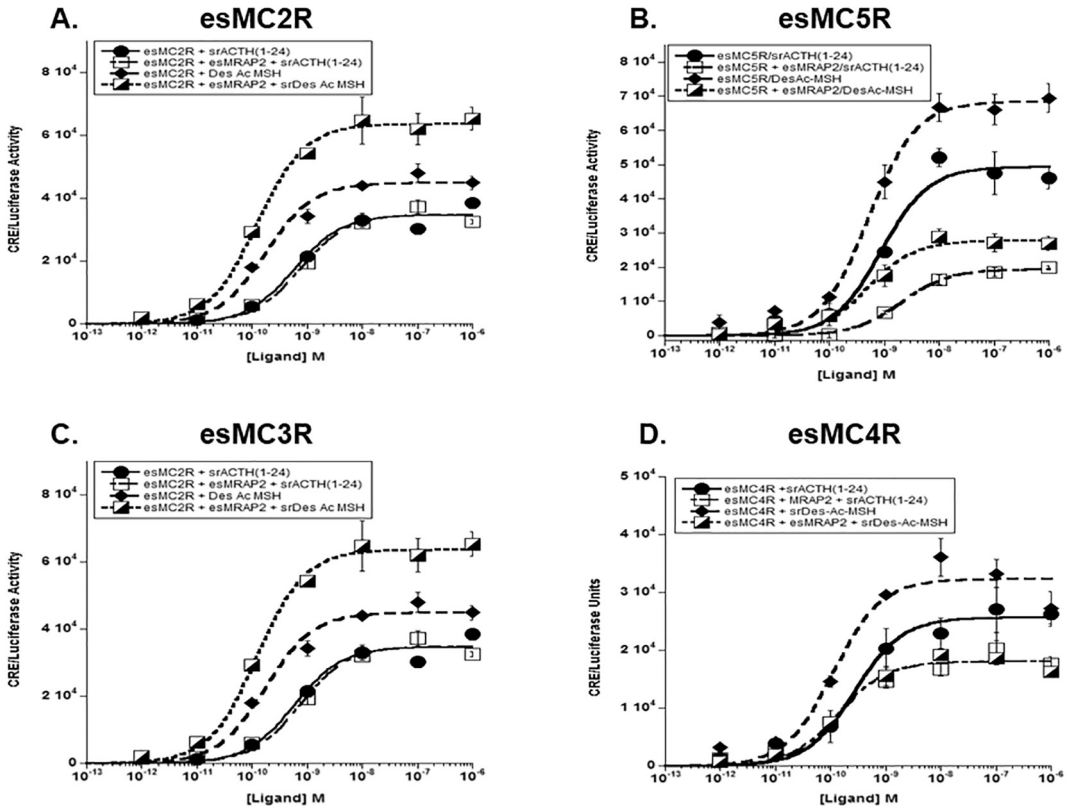

Fig. 4. Co-expression of esMc2r, esMc5r, esMc3r, and esMc4r with esMrap2. CHO cells were either co-transfected with a esMcr cDNA and a Cre/luciferase cAMP reporter cDNA, or co-transfected with a esMcr cDNA, esMrap2 cDNA, and a Cre/luciferase cAMP reporter cDNA as described in Methods. After 48 h in culture the transfected cells were either stimulated with srACTH(1–24) at concentrations ranging from 10^{-12} M to 10^{-6} M or srDes-Acetyl-αMSH at concentrations ranging from 10^{-12} M to 10^{-6} M. All experiments were done in triplicate, and the data are presented as mean ± SEM. A) esMc2r transfected cells; B) esMc5r transfected cells; C) esMc3r transfected cells; and D) esMc4r transfected cells. n = 3 for all data points. srDes-aca-MSH = srDes-Acetyl-αMSH.

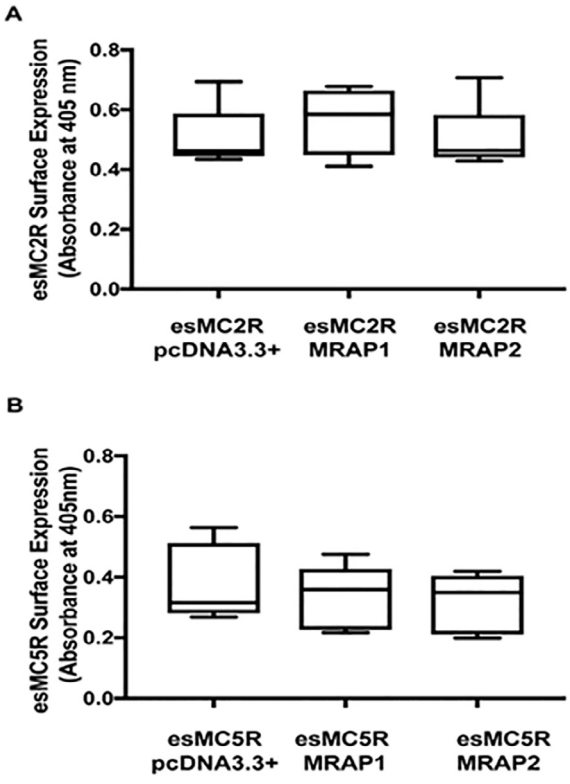

Fig. 5. Cell Surface ELISA. Cos 7 cells were co-transfected with a esMcr cDNA and either pcDNA3.3+ vector plasmid, esMrap1 or esMrap2 cDNA. After 48 h, transfected cells were fixed and labeled with primary antibody specific for the epitope fused to each esMcr, and the amount of antibody bound was quantified by measuring absorbance at 405 nm. All experiments were performed in triplicate, and data are presented as mean ± SD. A) V5-esMc2R transfected cells labeled with anti-V5 antibody; B) HA-esMc5r transfected cells labeled with anti-HA antibody.

(0.32 ± 0.09) (Fig. 5B). Co-expression of Mrap1 with esMc5r did not significantly affect receptor surface expression ($p = 0.71$). These findings suggest that Mrap1 enhancement of esMc2r and esMc5r signaling is not due to an increase in receptor surface expression.

3.5. Imaging of esMC2R/esMRAP1 and esMC5R/esMRAP1

Immunofluorescence confocal microscopy was used to confirm that esMc2r and esMc5r localize to the plasma membrane with esMrap1. Cells transfected with esMc2r (V5 epitope tagged) and either esMrap1 (Flag epitope tagged) or esMrap2 (Flag epitope tagged) were fixed and labeled with V5-epitope and FLAG-epitope primary antibodies, respectively. In permeabilized cells, esMc2r and esMrap1 localize to both the plasma membrane and to perinuclear regions consistent with endoplasmic reticulum (Fig. 6A). Under non-permeabilizing conditions, only proteins trafficking to the plasma membrane of the cell are labeled with antibody. We observe that esMc2r and esMrap1 co-localize at the plasma membrane in non-permeabilized cells (Fig. 6A). In contrast, esMrap2 appears to localize primarily to structures consistent with the ER in permeabilized cells, and is not labeled by antibody under non-permeabilizing conditions (Fig. 6B).

Similarly, cells expressing esMc5r V5 epitope tagged and either esMrap1 or esMrap2 were co-labeled and imaged using fluorescence confocal microscopy. In permeabilized cells, esMc5r co-localizes with esMrap1 at the plasma membrane and at perinuclear regions (Fig. 7a). Under non-permeabilizing conditions, esMc5r is observed at the plasma membrane in regions containing esMrap1 (Fig. 7A). Consistent with our previous observations, esMrap2 is localized primarily to ER, and is not expressed at the cell surface (Fig. 7B).

As a control, we examined the expression of esMc2r and esMc5r in cells co-transfected with pcDNA3.3+ vector (no co-expression with MRAP1). Both esMc2r and esMc5r are expressed at the cell surface (Supplemental Fig. 1), demonstrating that neither esMc2r or esMc5r require co-expression with an exogenous Mrap for sorting of these receptors to the plasma membrane. The esMc2r results are consistent with our previous study on esMc2r trafficking in CHO cells (Reinick et al., 2012).

4. Discussion

The divergence of the ancestral cartilaginous fishes and the ancestral bony fishes from a common ancestral gnathostome lineage (Brazeau and Friedman, 2015) set the stage for the parallel evolution of the melanocortin receptors and the Mrap accessory proteins in extant cartilaginous fishes (i.e., holocephalans and elasmobranchs) and extant bony vertebrates (i.e., ray-finned fishes, lobe-finned fishes, and tetrapods; Dores, 2016). For the bony vertebrates, studies on representative ray-finned fishes, an amphibian, a reptile, a bird, and a mammal present a very uniform relationship (Metherell et al., 2005; Liang et al., 2011; Davis et al., 2013; Barlock et al., 2014; Liang et al., 2015). These Mc2r orthologs form a heterodimer complex with an Mrap1 ortholog to facilitate trafficking of the Mc2r/Mrap1 heterodimer from the ER to the plasma membrane. In addition, the heterodimer places the Mc2r ortholog in the proper conformation to allow for an ACTH binding event. The later event involves two motifs in the primary sequence of ACTH, the $H^6F^7R^8W^9$ motif, and the $K/R^{15}K^{16}R^{17}R^{18}$ motif (Schwyzer, 1977) binding to two respective sites (the HFRW binding site and the proposed KKRR binding site) on the bony vertebrate Mc2r ortholog (Dores, 2018). In addition, MSH-sized ligands which uniformly lack the R/KKRR motif cannot activate bony vertebrate Mc2r orthologs.

As noted in the Introduction, cartilaginous fish Mc2r orthologs from the elephant shark (Reinick et al., 2012) and the red stingray (Takahashi et al., 2016) can be activated by either ACTH(1–24) or Des-Acetyl-αMSH, and these Mc2r orthologs could be functionally expressed in CHO cells without co-expression with an exogenous Mrap1 ortholog. As a result both Mc2r orthologs were designated as being Mrap1 independent (Dores et al., 2016). However, the recent detection of an Mrap1 gene in the genome of the elephant shark (Dores, 2016), required a re-evaluation of the possible role of Mrap1 with respect to the pharmacology of elephant Mc2r, and served as the rationale for the current study.

4.1. Observations on interrenal tissue and implications for the HPI axis

In the current study RT-PCR analysis of various elephant shark tissues for melanocortin receptor mRNAs or melanocortin receptor accessory protein mRNAs revealed a tissue distribution pattern, not unlike the tissue distribution pattern observed for teleosts and tetrapods (Klovins et al., 2004; Cone, 2006; Agulleiro et al., 2010; Hinkle and Sebag, 2009), and similar to the tissue distribution pattern for the red stingray (Takahashi et al., 2016) with a few notable exceptions and additions.

Of particular interest for this study was whether melanocortin receptors and accessory proteins are present in elephant shark interrenal tissue. The RT-PCR analysis revealed mRNAs for esMc2r, esMc5r, and to a lesser extent, esMc3r. The same melanocortin receptor mRNAs were also present in red stingray interrenal tissue (Takahashi et al., 2016). The presence of multiple melanocortin receptors associated with glucocorticoid-secreting cells has also been observed for the chicken (*Gallus gallus*) adrenal gland, where all five melanocortin receptor mRNAs were detected (Thomas et al., 2017).

However, of greater interest was the observation that in the elephant shark interrenal tissue esMrap1 mRNA was clearly present (Fig. 1), while esMRAP2 mRNA was below the level of detection. These

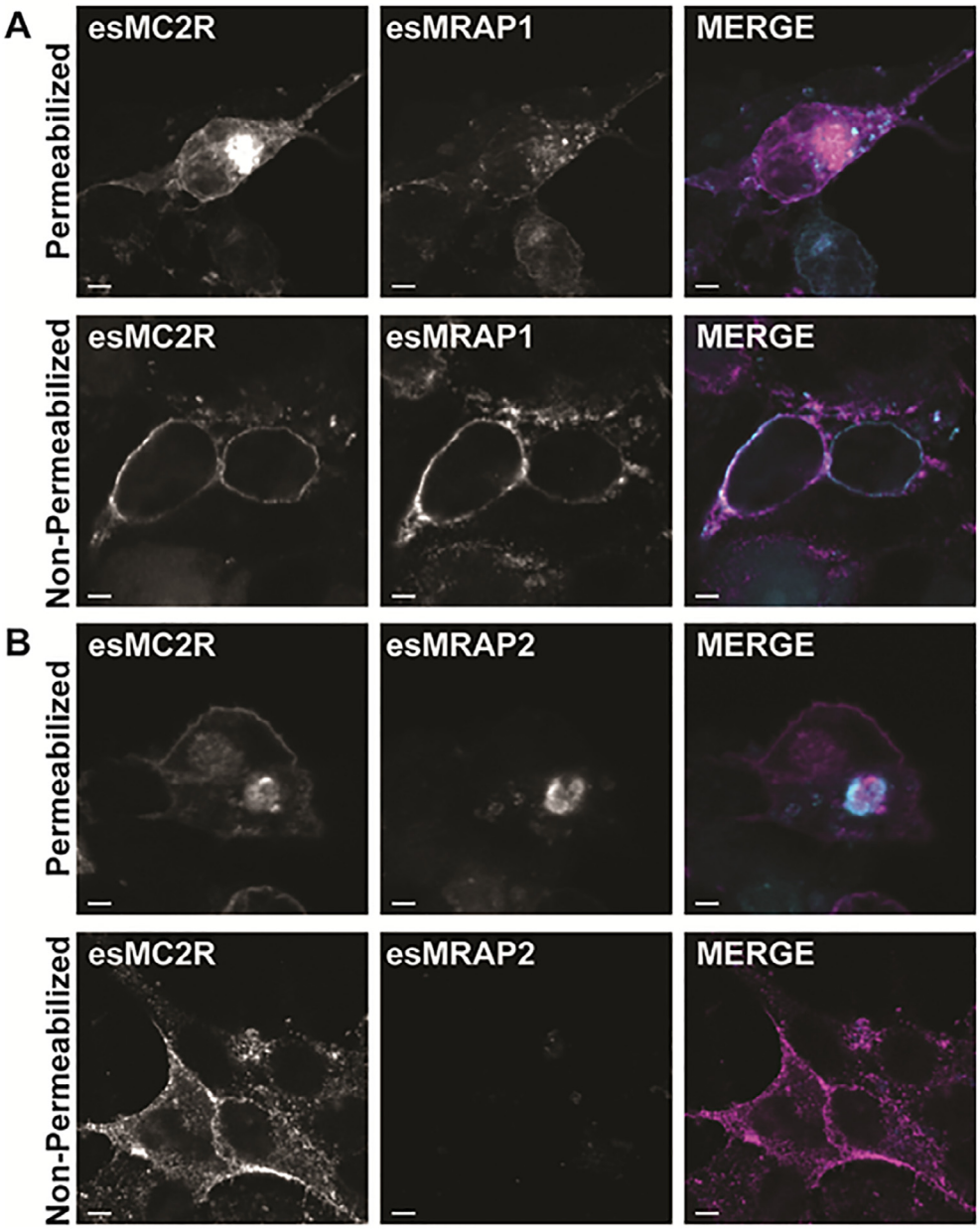

Fig. 6. Imaging of esMC2R & esMRAP1 or esMRAP2. Cos 7 cells were grown on glass coverslips and co-transfected with esMc2r cDNA and either plasmids containing Mrap1, Mrap2, or pcDNA3.3 + vector plasmid. After 48 h, transfected cells were fixed and either incubated with 100% methanol (permeabilized) or washed with 1x PBS (non-permeabilized) prior to labeling with anti-V5 and anti-Flag antibodies. Images are representative of three independent experiments. A) V5-esMc2r (magenta) co-transfected with FLAG-esMrap1 (cyan); B) V5-esMc2r (magenta) co-transfected with FLAG-esMrap2 (cyan). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observations are consistent with observations made for a teleost interrenal tissue (Agulleiro et al., 2010), an avian adrenal cortex tissue (Thomas et al., 2017), and mammalian adrenal cortex tissue (for review see Gallo-Payet and Batista (2014)).

Since bony vertebrate glucocorticoid-synthesizing cells require Mrap1 for the activation of the respective Mc2r ortholog, a pharmacological analysis was done to evaluate whether co-expression of esMrap1 with esMc2r, esMc5r, esMc3r, or esMc4r would affect the ligand sensitivity of these receptors for stimulation by either srACTH (1–24) or srDes-Acetyl-MSH (Fig. 3 and Table 1A). While sensitivity to stimulation by srACTH(1–24) was statistically increased for esMc2r (over 10 fold) and esMc5r (over 100 fold) when these receptors were co-expressed in CHO cells with esMrap1, co-expression with esMrap1 had no apparent effect on ligand sensitivity for either esMc3r or esMc4r.

In support of the pharmacological experiments, imaging analysis provided evidence for heterodimer formation between esMc2r and esMrap1, and esMc5r and esMrap1, respectively (Figs. 6 and 7, respectively). Hence, it would appear that the apparent heterodimer formation may be affecting the three dimensional structure of esMc2r and esMc5r to make these receptors more accommodating for docking with ACTH(1–24). However, a somewhat surprising outcome of this analysis was that sensitivity to Des-Acetyl-αMSH was not affected in either a positive or negative manner by co-expression with esMrap1 for either esMc2r or esMc5r (Fig. 3A and B). At this stage it may be reasonable to assume that both esMc2r and esMc5r have a single binding site for melanocortin ligands (i.e. the HFRW binding site); whereas, teleost and tetrapod Mc2r orthologs require two binding sites to facilitate interaction with ACTH (for review see Dores, 2018). That said, it is possible that formation of an esMc2r/Mrap1 heterodimer and an esMc5r/Mrap1 heterodimer may expose a site on these receptors that facilitates enhanced interaction between the heterodimer and ACTH (1–24), but has no apparent effect on interaction with srDes-Acetyl-αMSH. To begin to resolve these issues, an understanding of the contact site(s) between these melanocortin receptors and esMRAP1 is required.

A final issue with respect to the interactions between esMc2r, esMc5r and esMrap1 in elephant shark interrenal cells is whether formation of the heterodimer has any effect on the trafficking of the receptor to the plasma membrane. As seen in Fig. 5, a cell surface ELISA

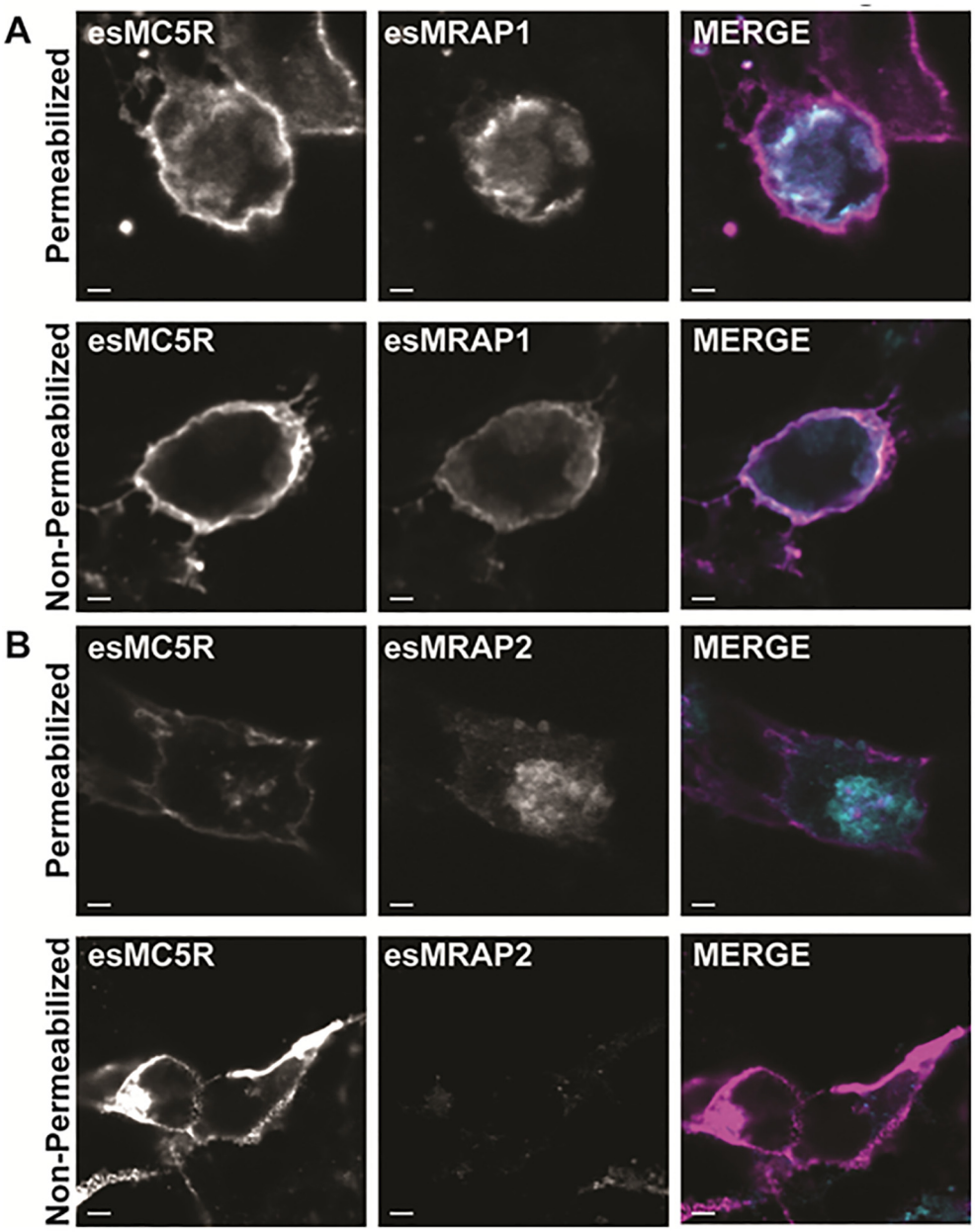

Fig. 7. Imaging of esMc5r & esMrap1 or esMrap2. Cos 7 cells were grown on glass coverslips and co-transfected with esMc5r cDNA and either plasmids containing Mrap1 or Mrap2. After 48 h, transfected cells were fixed and either incubated with 100% methanol (permeabilized) or washed with 1x PBS (non-permeabilized) prior to labeling with anti-HA and anti-Flag antibodies. Images are representative of three independent experiments. A) HA-esMc2r (magenta) co-transfected with FLAG-esMrap1 (cyan); B) HA-esMc5r (magenta) co-transfected with FLAG-esMrap2 (cyan); C) HA-esMc5r co-transfected with pcDNA3.3+ vector plasmid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analysis did not indicate an additive effect on either esMc2r or esMc5r trafficking as a result of co-expression with esMrap1. This observation highlights a major difference between the Mrap1 interaction for elephant shark Mc2r as compared to the interaction for bony vertebrate Mc2r orthologs. For the latter Mc2r orthologs, formation of the Mc2r/Mrap1 heterodimer is required for efficient trafficking of the Mc2r ortholog to plasma membrane (Hinkle and Sebag, 2009; Webb and Clark, 2010).

Collectively, these observations on the elephant shark interrenal tissue, coupled with the presence of a Pomc gene in the elephant shark genome, and the detection of a Crf-like gene in the genome of the elephant shark (Nock et al., 2011) are consistent with the conclusion that the elephant shark has a hypothalamus/pituitary/interrenal axis. Whether this putative HPI axis is involved in osmoregulation (Evans et al., 2010), chronic stress regulation (Geisleicher, 2004), or both functions has not been determined. In addition, since esMc2r can be activated by either ACTH(1–24) or Des-Acetyl-αMSH, there is the possibility that for the elephant shark, a distinct hypothalamus/anterior pituitary/interrenal circuit (ACTH-driven) and a distinct hypothalamus/intermediate pituitary/interrenal circuit (Des-Acetyl-αMSH/βMSH/γMSH/δMSH driven) may be present.

4.2. Observations on melanocortin receptors and Mraps in the CNS

Another focal point for melanocortin receptor/melanocortin receptor accessory protein interaction has been the central nervous system of vertebrates. In mammals, Mrap2 is highly expressed in the CNS (Chan et al., 2009), and there are very clear roles for neurons in the hypothalamus that express Mc3r and Mc4r in energy mobilization and feeding behavior (Cone 2006; Renquist et al., 2011; Begriche et al., 2013). For example, studies on a teleost (Sebag et al., 2017; Agulleiro et al., 2013) and a mammal (Asai et al., 2013) indicate that interaction with Mrap2 has effects on Mc4r ligand sensitivity and selectivity. RT-PCR analysis of the hypothalamus of the red stingray detected mRNAs for Mc3r, Mc4r, and Mc5r, but attempts to identify a srMrap2 mRNA were negative (Takahashi et al., 2016).

For elephant shark, a different mRNA pattern was detected. As shown in Fig. 2A and B, esMrap1 and esMrap2 mRNAs were detected,

as well as mRNAs for esMc2r, esMc3r, esMc4r, and esMc5r in the hypothalamus. In the remainder of the elephant shark brain, esMrap1, esMrap2, esMc4r mRNA was clearly detected, however, the levels of esMc2r, esMc3r, and esMc5r mRNA were just barely detected, or below the level of detection (Fig. 2A and B).

The presence of esMrap1 mRNA in the CNS was not expected. However, as noted co-expression of either esMc3r or esMc4r with esMrap1 in CHO cells had no apparent effect on selectivity or sensitivity following stimulation with either srACTH(1–24) or srDes-Acetyl-αMSH (Fig. 4C and D). However, co-expression of esMc3r with esMrap2 in CHO cells while having no effect on sensitivity following stimulation with srACTH(1–24), did result in approximately a 10 fold drop in sensitivity to stimulation by srDes-Acetyl-αMSH (Fig. 4C). This type of an effect was also observed when human Mc3r was co-expressed with human Mrap2 in CHO cells (Chan et al., 2009). By comparison, co-expression with esMrap1 had no effect, either positive or negative, on the ligand sensitivity of esMc4r.

At present it is not clear whether melanocortin receptors in the hypothalamus of the elephant shark are playing a role in energy mobilization and feeding behavior, nor is it known whether either Mrap1 or Mrap2 are expressed in the same neurons with melanocortin receptors in the elephant shark CNS. That said, the presence of Mrap2 in the CNS may involve other protein/protein interactions. For example in the mouse CNS, Mrap2 was observed to interact with the Ghrelin-GHSR1 receptor (Srisai et al., 2017) and the Prokineticin Receptor-1 (Chaly et al., 2016).

4.3. Melanocortin receptors and MRAPs in other tissues

RT-PCR analysis also revealed the presence of melanocortin-related and MRAP-related mRNAs in gill, atrium (heart), ventricle (heart), spleen, kidney, ovary, uterus, testes, and muscle. At present, the physiological significance of these observations is not apparent. However, the detection of melanocortin-related and Mrap-related mRNAs in the pituitary may be of interest. In the female pituitary (Fig. 2A), Mrap1, Mrap2, Mc2r, Mc4r, and Mc5r mRNAs were very clearly present. In the male pituitary, Mrap1, Mc2r, Mc4r, and Mc5r mRNAs were also detected. At present it is not known which pituitary cell types express these receptor genes. However, these observations do raise the possibility that putative Pomc-neurons may terminate at the median eminence of the elephant shark, and melanocortin peptides may function as hypophysiotropic factors. This possibility is also not unprecedented. A recent study has detected a melanocortin hypophysiotropic network in the anterior pituitary of the zebrafish (Zhang et al., 2012).

4.4. Conclusions

The outcomes of the current study indicate that esMrap1 facilitates the functionality of esMc2r by increasing the sensitivity of the receptor to stimulation by ACTH(1–24), but does not appear to be required for the trafficking of esMC2R to the plasma membrane. In addition, interaction with esMrap1 did not make esMc2r exclusively selective for ACTH(1–24) as compared to Des-Acetyl-αMSH. These features are in sharp contrast to teleost and tetrapod Mc2r/Mrap1 heterodimers which are exclusively selective for activation by ACTH(1–24), and cannot be activated by any of the MSH-sized melanocortin peptides (Mountjoy et al., 1992; Dores and Garcia, 2015). In addition the teleost and tetrapod Mc2r/Mrap1 heterodimer is required for trafficking to the plasma membrane. These novel features of the esMc2r/Mrap1 heterodimer may have implications with respect to the regulation of the HPI axis by the elephant shark pituitary gland.

In addition, formation of an esMc5r/MRAP1 heterodimer also increased sensitivity of the receptor for stimulation by ACTH(1–24). Since esMc5r and esMc2r mRNAs were both detected in the elephant shark interrenal tissue, both receptors may be playing a role in regulating the physiology of these glucocorticoid-producing cells. This possibility may reflect the close evolutionary relationship between the Mc2r and the Mc5r paralogs since these two receptors appear to have originated as a result of a local gene duplication event (for review see Dores (2013)). Additional studies are needed to determine whether interaction with Mrap1 is a feature common for other cartilaginous fish Mc5r orthologs. In this regard, studies on the interaction between Mrap1 and Mc5r in teleosts and tetrapods are limited. For example, co-expression of *Gallus gallus* Mc5r with *Gallus gallus* Mrap1 in CHO cells increased sensitivity of the chicken receptor for stimulation by ACTH(1–24) (Thomas et al., 2017). However, co-expression of human Mc5r and mouse Mrap1 in CHO cells decreased the trafficking of human Mc5r to the plasma membrane (Sebag and Hinkle, 2009). Clearly additional studies are needed on other bony vertebrate Mc5r orthologs before any generalizations can be made on the interaction between Mrap1 and Mc5r in bony vertebrates.

Finally, esMRAP2 did not have any effect on the sensitivity of either esMC2R or esMC5R to stimulation by either ACTH or Des-Acetyl-αMSH (Fig. 4), and did not have an effect on the trafficking of either receptor (Fig. 5). In addition, in the imaging analysis (Figs. 6 and 7), esMRAP2 does not appear to traffic to the plasma membrane with either esMc2r or esMc5r. Perhaps, during the parallel evolution of melanocortin receptor networks in cartilaginous fishes and bony vertebrates, a novel role for Mrap2 orthologs may have emerged that does not involve melanocortin receptors to any significant extent.

Acknowledgements

This research was supported by the Long Research Fund (R.M.D.; University of Denver), a Grant-in-Aid for Scientific Research (B) and (C) (S.H.; the Japan Society for the Promotion of Science), and the Hofstra University Faculty Research and Development Grant (M.R.D, Hofstra University). We wish to thank Ms. Brianne Hoglin for her careful reading of the manuscript and helpful suggestions.

Conflict of interests

The authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.11.009.

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