


1-2-2020

Analyzing the Effect on Ligand Sensitivity for *Xenopus Tropicalis* MC1R, MC3R, MC4R, and MC5R as a Result of Coexpression with *Gallus Gallus* MRAP1 in Chinese Hamster Ovary Cells

Avery Niemann
University of Denver

Robert Dores
University of Denver - Advisor

Follow this and additional works at: <https://digitalcommons.du.edu/duurj>

 Part of the [Biochemistry, Biophysics, and Structural Biology Commons](#), [Biology Commons](#), [Cell Biology Commons](#), and the [Other Genetics and Genomics Commons](#)

Recommended Citation

Niemann, Avery and Dores, Robert (2020) "Analyzing the Effect on Ligand Sensitivity for *Xenopus Tropicalis* MC1R, MC3R, MC4R, and MC5R as a Result of Coexpression with *Gallus Gallus* MRAP1 in Chinese Hamster Ovary Cells," *DU Undergraduate Research Journal Archive*: Vol. 1 : Iss. 1 , Article 8. Available at: <https://digitalcommons.du.edu/duurj/vol1/iss1/8>

This Article is brought to you for free and open access by Digital Commons @ DU. It has been accepted for inclusion in DU Undergraduate Research Journal Archive by an authorized editor of Digital Commons @ DU. For more information, please contact jennifer.cox@du.edu, dig-commons@du.edu.

Analyzing the Effect on Ligand Sensitivity for *Xenopus Tropicalis* MC1R, MC3R, MC4R, and MC5R as a Result of Coexpression with *Gallus Gallus* MRAP1 in Chinese Hamster Ovary Cells

Abstract

Some melanocortin receptors and the accessory protein MRAP1 have been found to interact in novel ways when co-expressed in the same cells following stimulation with either ACTH (1-24) or α MSH. These interactions have been seen for mammalian, bird, and some bony fish melanocortin receptors. Such analysis has not been done yet on the amphibian melanocortin receptors, MC1R, MC3R, MC4R, and MC5R. To this end, this study was done on the effects regarding ligand sensitivity for ACTH (1-24) and α MSH, when the MC1R, MC3R, MC4R, and MC5R paralogs of the amphibian, *Xenopus tropicalis* were expressed in Chinese Hamster Ovary cells either in the presence of absence of *Gallus gallus* (c) MRAP1. Based on previous studies on human MC1R, MC3R, MC4R, and MC5R, the expectation was that the sensitivity to stimulation by α MSH would be lower for all the *X. tropicalis* receptors. However, for every *X. tropicalis* receptor tested, co-expression with cMRAP1 had no negative or positive effect on sensitivity to stimulation by xt α MSH. In a similar manner co-expression of xtMC1R, MC3R, or MC5R with cMRAP1 had no effect, positive or negative, on stimulation with xtACTH(1-24). However, co-expression of xtMC4R with cMRAP1 did lower sensitivity to stimulation by xtACTH (1-24) in a statistically significant manner. To date, this type of negative interaction between an MC4R ortholog and an MRAP1 ortholog has not been reported for other vertebrates.

Publication Statement

Copyright held by the author. User is responsible for all copyright compliance.

Analyzing the effect on ligand sensitivity for *Xenopus tropicalis* MC1R, MC3R, MC4R, and MC5R as a result of coexpression with *Gallus gallus* MRAP1 in Chinese Hamster ovary cells

Avery Niemann¹, Robert Dores²

¹Student Contributor, University of Denver

²Advisor, Department of Biology, University of Denver

Abstract

Some melanocortin receptors and the accessory protein MRAP1 have been found to interact in novel ways when co-expressed in the same cells following stimulation with either ACTH (1-24) or α MSH. These interactions have been seen for mammalian, bird, and some bony fish melanocortin receptors. Such analysis has not been done yet on the amphibian melanocortin receptors, MC1R, MC3R, MC4R, and MC5R. To this end, this study was done on the effects regarding ligand sensitivity for ACTH (1-24) and α MSH, when the MC1R, MC3R, MC4R, and MC5R paralogs of the amphibian, *Xenopus tropicalis* were expressed in Chinese Hamster Ovary cells either in the presence or absence of *Gallus gallus* (c) MRAP1. Based on previous studies on human MC1R, MC3R, MC4R, and MC5R, the expectation was that the sensitivity to stimulation by α MSH would be lower for all the *X. tropicalis* receptors. However, for every *X. tropicalis* receptor tested, co-expression with cMRAP1 had no negative or positive effect on sensitivity to stimulation by α MSH. In a similar manner co-expression of xtMC1R, MC3R, or MC5R with cMRAP1 had no effect, positive or negative, on stimulation with xtACTH(1-24). However, co-expression of xtMC4R with cMRAP1 did lower sensitivity to stimulation by xtACTH (1-24) in a statistically significant manner. To date, this type of negative interaction between an MC4R ortholog and an MRAP1 ortholog has not been reported for other vertebrates.

1 INTRODUCTION

Melanocortin receptors are a family of G-protein coupled receptors (GPCR) that are only present in chordates. There are 5 receptors: melanocortin-1 receptor (MC1R), melanocortin-2 receptor (MC2R), melanocortin-3 receptor (MC3R), melanocortin-4 receptor (MC4R), and melanocortin-5 receptor (MC5R)¹. Melanocortin receptors are activated by melanocortin related peptides such as adrenocorticotropin (ACTH), alpha-melanocyte stimulating hormone (α MSH), beta-melanocyte stimulating hormone (β MSH), and gamma-melanocyte stimulating hormone (γ MSH). These peptides are derived from the precursor, proopiomelanocortin (POMC) that is made in the pituitary². In mammals, the POMC gene is expressed in cells of the anterior pituitary. ACTH is a major melanocortin end product for these cells. However, the pituitary anterior lobe corticotrophs and the intermediate lobe melanotrophs that express POMC have α MSH, β MSH, and γ MSH as major melanocortin end products.

In mammals, MC1R is involved in influencing pig-

mentation. MC2R is involved in the production of glucocorticoids from cells of the adrenal cortex. The MC3R and MC4R influence metabolic rate and feeding behaviors in the central nervous system. MC5R regulates secretions by the exocrine glands. Melanocortin receptors have also been identified with similar functions in the major groups of jawed vertebrates (i.e. cartilaginous fishes, bony fishes, amphibians, reptiles, birds, and mammals)¹. This study focuses on the melanocortin receptors found in the genome of the amphibian *Xenopus tropicalis*. An analysis of the genome database of the *X. tropicalis* reveals all 5 melanocortin receptors are present in *X. tropicalis* genome³.

Previous studies on the *X. tropicalis* MC2R indicate that it is comparable with the mammalian ortholog MC2R. MC2R requires co-expression with melanocortin receptor accessory protein 1 (MRAP1) for functional expression⁴. Like the mammalian MC2R ortholog, *X. tropicalis* MC2R can be activated with ACTH, but not by an MSH-sized melanocortin peptide⁴. However, in order for functional expression of *X. tropicalis* MC2R in

Chinese Hamster cells (CHO), the receptor must be co-expressed with mammalian MRAP1⁴ or an MRAP ortholog from chicken (*Gallus gallus*)⁵. To date, an MRAP1 ortholog has not been detected in the genome database of *X. tropicalis*³.

The absence of an MRAP1 ortholog can be explained by an incomplete genome project or the use of another accessory protein⁶. There are very few studies on vertebrates. Our understanding of the absence of an MRAP1 ortholog can be furthered by examining the effects of MRAP1 on *X. tropicalis* melanocortin receptors. The purpose of this study is to observe how the co-expression with a tetrapod MRAP1 affects the ligand sensitivity of *X. tropicalis* MC1R, MC3R, MC4R, MC5R. xtMC2R was excluded since its activation has been shown to be dependent on the expression of MRAP1⁷. An earlier study done on human MC1R, MC3R, MC4R, and MC5R co-expression with MRAP1⁸ found that co-expression with MRAP1 lowered sensitivity to stimulation with [Nle4-D-Phe7]-alpha-melanocyte stimulating hormone, the synthetic analog for α MSH, for all 4 human receptors. However, this research did not examine the impacts of co-expression with ACTH. The operating assumptions of the research were that *X. tropicalis* MC1R, MC3R, MC4R, and MC5R (xtMC1R, xtMC3R, xtMC4R, and xtMC5R) when stimulated with a form of *X. tropicalis* α MSH, xt α MSH, in the presence of cMRAP1 would respond in a manner similar to what was seen in the human melanocortin study. In addition, it was assumed that co-expression with cMRAP1 would not affect receptors with stimulated with *X. tropicalis* ACTH (1-24).

2 METHODS

2.1 DNA Constructs

The cDNA sequences of xtMC1R, xtMC3R, xtMC4R, xtMC5R were obtained from *X. tropicalis* genome database³. The cDNA sequence of cMRAP1 was obtained from the *G. gallus* genome database³. Each cDNA was synthesized by GenScript (Piscataway, NJ) and inserted into a pcDNA3.1+ vector. The cAMP reporter gene construct CRE-Luciferase⁹ was provided by Dr. Patricia Hinkle (University of Rochester, NY).

2.2 Tissue Culture

Chinese Hamster Ovary (CHO) cells (ATCC, Manassas, VA) were grown in Kaighn's Modification of Ham's F12K media (ATCC) supplemented with 10% fetal bovine serum, 100unit/ml penicillin, 100 μ g/ml streptomycin, 100 μ g/ml normocin, and maintained in a humidified incubator with 95% air and 5% CO₂ at 37°C. When CHO cells reached 80% confluence, the cells were split into subcultures using 0.05% trypsin/0.53mM EDTA.

2.3 ACTH Analog Peptides

X. tropicalis (xt) ACTH (1-24) and *X. tropicalis* α MSH (α -melanocyte-stimulating hormone) were purchased from New England Peptides (Gardiner, MA). For the cAMP-reporter gene assay, xtACTH (1-24) was used to stimulate transfected cells at concentrations ranging from 10⁻¹² M to 10⁻⁶ M. xt α MSH was used at concentrations ranging from 10⁻¹²M to 10⁻⁶M.

2.4 cAMP-Reporter Gene Assay

For the cAMP-reporter gene assay⁴, 3.0 \times 10⁶ CHO cells were transiently co-transfected with a xtMCR cDNA construct either alone or with *G. gallus* (c)MRAP1 cDNA construct, and the CRE-Luciferase cDNA construct (2 μ g each) using the Amaxa Cell Line Nucleofector II system (Lonza, Portsmouth, NH) utilizing the Solution T transfection kit and program U-23 as recommended by the company. After a 10-minute post-transfection recovery period, cells were then seeded in a white 96-well plate at a final density of 1 \times 10⁵ cells/well. 48 hours after transfection, cells were stimulated with concentrations of xtACTH (1-24) or xt α MSH diluted in serum-free CHO media for 4 hours at 37°C. Following the incubation period, the stimulating media was removed, and the luciferase substrate reagent, Bright GLO (Promega, Madison, WI), was applied to the wells for a 5-minute incubation period at room temperature. Luminescence was immediately measured using a Bio-Tek Synergy HTX plate reader (Winooski, VT). To determine the background levels of cAMP production, a set of transfected CHO cells were stimulated with serum free CHO media for the 4-hour incubation period, and the average background luminescence reading for these control wells was subtracted from the ligand-stimulated luminescence readings. The dose response curves for the stimulated cells were analyzed using the Michaelis-Menton equation to obtain EC₅₀ values. All assays were done in triplicate. The data were plotted using the Kaleidograph software (www.synergy.com).

2.5 Statistical Analysis

Data points are expressed as the mean + standard error of the mean (n = 3). Statistical differences between the EC₅₀ value of the xtMCR positive control and the xtMCR co-expressed with cMRAP1 were evaluated using one-way ANOVA followed by Tukey's multi-comparison test using GraphPad Prism 2 (GraphPad Software Inc, La Jolla, CA, USA) for equal variance. Significance was set at P \leq 0.05.

3 RESULTS

The results from the expression of *X. tropicalis* MC1R, MC3R, MC4R, and MC5R are depicted in Figure 1. The receptors were expressed in either the presence or absence of cMRAP1. The cells were then stimulated with xtACTH (1-24) or xt α MSH to determine if cMRAP1 influences sensitivity of the melanocortin receptors for stimulation by these hormones. All EC_{50} values can also be found in Table 1, and all P Values from One-Way ANOVA Analysis can be found in 2.

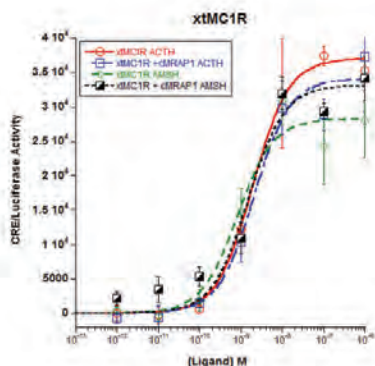


Figure 1. Expression of xtMC1R in the presence or absence of cMRAP1. As described in Methods, xtMC1R was expressed in CHO cells either in the presence or absence of cMRAP1, and transfected cells were stimulated in either xtACTH(1-24) or xt α MSH as described in METHODS. EC_{50} values appear in (Table 1), and the results of statistical analysis appear in (Table 2).

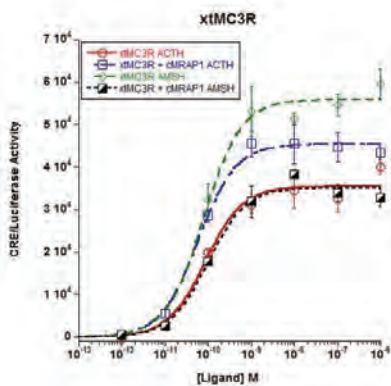


Figure 2. Expression of xtMC3R in the presence or absence of cMRAP1. As described in Methods, xtMC1R was expressed in CHO cells either in the presence or absence of cMRAP1, and transfected cells were stimulated in either xtACTH(1-24) or xt α MSH as described in METHODS. EC_{50} values appear in (Table 1), and the results of statistical analysis appear in (Table 2).

3.1 Coexpression of xtMC3R with cMRAP1

The cells expressed xtMC3R with or without cMRAP1 and were stimulated by xtACTH or xt α MSH (Figure 2). The EC_{50} values can be found in (Table 1). A One-Way ANOVA Analysis of variance did not find the results statistically significant for either hormone (Table 2).

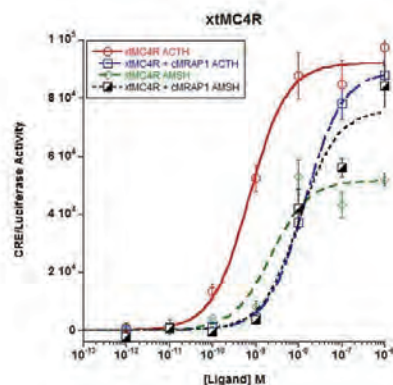


Figure 3. Expression of xtMC3R in the presence or absence of cMRAP1. As described in Methods, xtMC1R was expressed in CHO cells either in the presence or absence of cMRAP1, and transfected cells were stimulated in either xtACTH(1-24) or xt α MSH as described in METHODS. EC_{50} values appear in Table 1, and the results of statistical analysis appear in Table 2.

3.2 Coexpression of xtMC5R with cMRAP1

In Figure 4, the cells expressed xtMC5R with or without cMRAP1 and were stimulated by xtACTH or xt α MSH. The EC_{50} values can be found in Table 1. A One-Way ANOVA Analysis of variance did not find the results statistically significant for either hormone (Table 2).

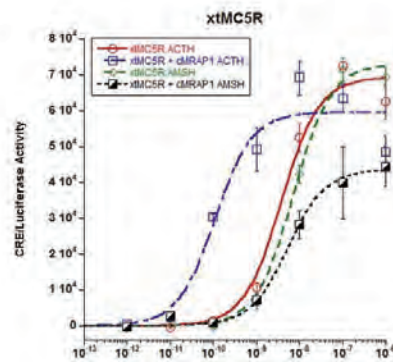


Figure 4. Expression of xtMC3R in the presence or absence of cMRAP1. As described in Methods, xtMC1R was expressed in CHO cells either in the presence or absence of cMRAP1, and transfected cells were stimulated in either xtACTH(1-24) or xt α MSH as described in METHODS. EC_{50} values appear in Table 1, and the results of statistical analysis appear in Table 2.

Table 1 EC_{50} values for xtMC1R, xtMC3R, xtMC4R, and xtMC5R with or without cMRAP1 and xtACTH or xt α MSH

Receptor	xtACTH or xt α MSH	Receptor with or without cMRAP1	EC_{50} Value (M)
xtMC1R	xtACTH	xtMC1R	$1.9 \times 10^{-9} \pm 2.6 \times 10^{-10}$
		xtMC1R+cMRAP1	$2.1 \times 10^{-9} \pm 7.0 \times 10^{-10}$
	xt α MSH	xtMC1R	$8.2 \times 10^{-10} \pm 3.3 \times 10^{-10}$
		xtMC1R+cMRAP1	$1.5 \times 10^{-9} \pm 6.7 \times 10^{-10}$
xtMC3R	xtACTH	xtMC1R	$8.6 \times 10^{-11} \pm 2.5 \times 10^{-11}$
		xtMC1R+cMRAP1	$5.7 \times 10^{-11} \pm 8.4 \times 10^{-12}$
	xt α MSH	xtMC1R	$7.4 \times 10^{-11} \pm 1.7 \times 10^{-11}$
		xtMC1R+cMRAP1	$9.8 \times 10^{-11} \pm 2.2 \times 10^{-11}$
xtMC4R	xtACTH	xtMC1R	$7.2 \times 10^{-10} \pm 1.3 \times 10^{-10}$
		xtMC1R+cMRAP1	$1.3 \times 10^{-8} \pm 5.9 \times 10^{-10}$
	xt α MSH	xtMC1R	$2.3 \times 10^{-9} \pm 4.4 \times 10^{-9}$
		xtMC1R+cMRAP1	$1.0 \times 10^{-8} \pm 4.4 \times 10^{-9}$
xtMC5R	xtACTH	xtMC1R	$3.6 \times 10^{-10} \pm 2.5 \times 10^{-10}$
		xtMC1R+cMRAP1	$9.6 \times 10^{-12} \pm 8.8 \times 10^{-12}$
	xt α MSH	xtMC1R	$2.8 \times 10^{-9} \pm 2.8 \times 10^{-9}$
		xtMC1R+cMRAP1	$4.0 \times 10^{-9} \pm 3.3 \times 10^{-9}$

Table 2 P Values from a One-Way ANOVA Analysis.

Receptor	Comparison	P Value
xtMC1R	MC1R vs MC1R+cMRAP1 stimulated with xtACTH(1-24)	0.85
	MC1R vs MC1R+cMRAP1 stimulated with xt α MSH	0.69
	MC1R alone stimulated with xtACTH(1-24) vs xt α MSH	0.99
xtMC3R	MC3R vs MC3R+cMRAP1 stimulated with xt α ACTH (1-24)	0.76
	MC3R vs MC3R+cMRAP1 stimulated with xt α MSH	0.47
	MC3R alone stimulated with xtACTH(1-24) vs xt α MSH	0.99
xtMC4R	MC4R vs MC4R+cMRAP1 stimulated with xtACTH(1-24)	0.006
	MC4R vs MC4R+cMRAP1 stimulated with xt α MSH	0.09
	MC4R alone stimulated with xtACTH(1-24) vs xt α MSH	0.94
xtMC5R	MC5R vs MC5R+cMRAP1 stimulated with xtACTH(1-24)	0.49
	MC5R vs MC5R+cMRAP1 stimulated with xt α MSH	0.99
	MC5R alone stimulated with xtACTH(1-24) vs xt α MSH	0.51

4 DISCUSSION

The purpose of this study was to see how co-expression with a tetrapod MRAP1 affects the ligand sensitivity of *X. tropicalis* MC1R, MC3R, MC4R, MC5R. An earlier study conducted on human MC1R, MC3R, MC4R, and MC5R co-expression with MRAP1⁸ demonstrated that co-expression with MRAP1 lowered sensitivity to stimulation with NDM-MSH for all 4 human receptors. However, this research did not examine the effect of co-expression following stimulation with ACTH. It was hypothesized that stimulation of *X. tropicalis* MC1R, MC3R, MC4R, and MC5R with a form of xt α MSH in the presence of cMRAP1 would result in a response similar to that of human melanocortin study. We further hypothesized that co-expression with cMRAP1 would not affect receptors with xtACTH (1-24).

When mouse MRAP1 is co-expressed with human MC5R in CHO cells, the receptor is glycosylated but remains concentrated at the endoplasmic reticulum in the cell⁷. This research was corroborated by several other studies and demonstrated that MC2R activation is dependent on the co-expression of MRAP1. When MRAP1 is not co-expressed, MC2R remains trapped in the endoplasmic reticulum^{7:10}. For this reason, MC2R was excluded from this study. However, unlike human MC5R, the activation of xtMC5R by ACTH (1-24) is not affected by co-expression with cMRAP1, and presumably there was no negative effect on the trafficking of xtMC5R to the plasma membrane (Figure 3).

In a different study, chicken melanocortin receptors (cMC2R, cMC3R, cMC4R, cMC5R) were transfected into CHO cells with chicken MRAP1 or MRAP2. α MSH was not used for stimulation due to the fact that avian pituitaries only have corticotropic cells and lack an intermediate lobe¹¹. cMC2R again required the co-expression of cMRAP1¹¹. cMC3R had no statistical change in sensitivity, while both MC4R and MC5R experienced an increase in sensitivity to ACTH with the co-expression of cMRAP1¹¹. Studies on the co-expression of MRAP1 with melanocortin receptors have been primarily limited to humans, chickens, and mice. Since other studies on vertebrates have been limited, this study was done on the amphibian, *X. tropicalis* as a MRAP1 ortholog has not been found in its genome.

Our research showed no statistically significant shift for MC1R, MC3R, or MC5R sensitivity to ACTH. However, a one-way analysis of variance found that the comparison of MC4R vs. MC4R + cMRAP1 stimulated with xtACTH (1-24) to be statistically significant with a P Value of 0.006. Previous research is limited to studies on the interaction between human or mouse MC4R and human or mouse MRAP2. In a study on the interaction between human MC4R and human MRAP2, it was found that human MRAP2 negatively affected trafficking of the receptor to the plasma membrane and its

activation with NDP-MSF⁸. Studies on mouse MC4R found that coexpression with MRAP2 increased sensitivity to mouse α MSH stimulation¹². Hence, it would appear that co-expression of xtMC4R and MRAP1 may be an interaction that is unique to amphibians.

5 ACKNOWLEDGEMENTS

This research was supported by the Long Research fund. I am grateful to Perry Davis for her assistance, and to Dr. Dores for his guidance in this project.

6 EDITOR'S NOTES

This work was adapted from a senior thesis and has been condensed for publication. Contact DUURJ staff for the full publication.

REFERENCES

- [1] Cone, R. D. Studies on the physiological functions of the melanocortin system (2006).
- [2] Sundström, G., Dreborg, S. & Larhammar, D. Concomitant duplications of opioid peptide and receptor genes before the origin of jawed vertebrates. *PLoS ONE* (2010).
- [3] Ensembl Genome Browser.
- [4] Liang, L., Reinick, C., Angleson, J. K. & Dores, R. M. Evolution of melanocortin receptors in cartilaginous fish: Melanocortin receptors and the stress axis in elasmobranchs (2013).
- [5] Davis, P., Franquemont, S., Liang, L., Angleson, J. K. & Dores, R. M. Evolution of the melanocortin-2 receptor in tetrapods: Studies on *Xenopus tropicalis* MC2R and *Anolis carolinensis* MC2R (2013).
- [6] Dores, R. M. Hypothesis and Theory: Revisiting Views on the Co-evolution of the Melanocortin Receptors and the Accessory Proteins, MRAP1 and MRAP2. *Frontiers in Endocrinology* (2016).
- [7] Sebag, J. A. & Hinkle, P. M. Opposite effects of the melanocortin-2 (MC2) receptor accessory protein MRAP on MC2 and MC5 receptor dimerization and trafficking. *Journal of Biological Chemistry* (2009).
- [8] Chan, L. F. *et al.* MRAP and MRAP2 are bidirectional regulators of the melanocortin receptor family. *Proceedings of the National Academy of Sciences of the United States of America* (2009).
- [9] Chepurny, O. G. & Holz, G. G. A novel cyclic adenosine monophosphate-responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein-coupled receptor drug discovery efforts. *Journal of Biomolecular Screening* (2007).

- [10] Barlock, T. K., Gehr, D. T. & Dores, R. M. Analysis of the pharmacological properties of chicken melanocortin-2 receptor (cMC2R) and chicken melanocortin-2 accessory protein 1 (cMRAP1). *General and Comparative Endocrinology* (2014).
- [11] 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)NH₂: Implications for the avian HPA axis and avian melanocortin circuits in the hypothalamus. *General and Comparative Endocrinology* (2018).
- [12] Asai, M. *et al.* Loss of function of the melanocortin 2 receptor accessory protein 2 is associated with mammalian obesity. *Science* (2013).