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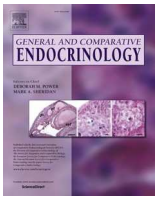

Trends in the evolution of the elasmobranch melanocortin-2 receptor: Insights from structure/function studies on the activation of whale shark Mc2r

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ABSTRACT

To understand the mechanism for activation of the melanocortin-2 receptor (Mc2r) of the elasmobranch, *Rhincodon typus* (whale shark; ws), *wsmc2r* was co-expressed with *wsmrap1* in CHO cells, and the transfected cells were stimulated with alanine-substituted analogs of ACTH(1–24) at the "message" motif ($H^6F^7R^8W^9$) and the "address" motif ($K^{15}K^{16}R^{17}R^{18}P^{19}$). Complete alanine substitution of the $H^6F^7R^8W^9$ motif blocked activation, whereas single alanine substitution at this motif indicated the following hierarchy of position importance for activation: $W^9 > R^8$, and substitution at F^7 and H^6 had no effect on activation. The same analysis was done on a representative bony vertebrate Mc2r ortholog (*Amia calva*; bowfin; bf) and the order of position importance for activation was $W^9 > R^8 = F^7$, (alanine substitution at H^6 was negligible). Complete alanine substitution at the $K^{15}K^{16}R^{17}R^{18}P^{19}$ motif resulted in distinct outcomes for wsMc2r and bfMc2r. For bfMc2r, this analog blocked activation-an outcome typical for bony vertebrate Mc2r orthologs. For wsMc2r, this analog resulted in a shift in sensitivity to stimulation of the analog as compared to ACTH(1–24) by two orders of magnitude, but the dose response curve did reach saturation. To evaluate whether the EC2 domain of wsMc2r plays a role in activation, a chimeric wsMc2r was made in which the EC2 domain was replaced with the EC2 domain from a melanocortin receptor that does not interact with Mrap1 (i.e., *Xenopus tropicalis* Mc1r). This substitution did not negatively impact the activation of the chimeric receptor. In addition, alanine substitution at a putative activation motif in the N-terminal of wsMrap1 did not affect the sensitivity of wsMc2r to stimulation by ACTH(1–24). Collectively, these observations suggest that wsMc2r may only have a HFRW binding site for melanocortin-related ligand which would explain how wsMc2r could be activated by either ACTH or MSH-sized ligands.

1. Introduction

The functionality of the hypothalamus/pituitary/adrenal-interrenal (HPA/HPI) axis in vertebrates is dependent on the activation of the melancortin-2 receptor (Mc2r) located on glucocorticoid-producing cells (Denver, 2009; Romero and Gormally, 2019; Bouyoucos et al., 2021). However pharmacological studies indicate that there is a dichotomy in the ligand selectivity properties of elasmobranch cartilaginous fish Mc2r orthologs and bony vertebrate Mc2r orthologs (Dores and Chapa, 2021). Studies on bony vertebrate Mc2r orthologs indicate that these receptors form a heterodimer with the accessory protein, Mrap1, to facilitate trafficking of the receptor from the ER to the plasma membrane (Hinkle and Sebag, 2009; Webb and Clark, 2010). Once at the plasma membrane, the receptor can only be activated by the pituitary hormone ACTH as a result of interaction with Mrap1 (Cone, 2006; Hinkle and Sebag, 2009; Webb and Clark, 2010; Dores and Garcia, 2015; Dores and Chapa, 2021; Davis et al., 2022). However, pharmacological studies on two elasmobranch Mc2r orthologs (i.e., red stingray and whale shark) indicate that while these orthologs interact with Mrap1 to also facilitate trafficking from the ER to the plasma membrane, once at the plasma membrane these receptors can be activated by either ACTH or MSH-sized peptides and the role that Mrap1 plays in this activation is not as yet resolved (Dores et al., 2018; Hoglin et al., 2020). The latter issue is the subject of this study.

On first inspection this difference in ligand selectivity between elasmobranch Mc2r orthologs and bony vertebrate Mc2r orthologs is

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surprising since all melanocortin peptides (i.e., ACTH and the MSH-sized ligands; Supplementary Fig. 1) have the "message sequence" HFRW (Schwyzer, 1977), and all melanocortin receptors (i.e., Mc1r, Mc2r, Mc3r, Mc4r, Mc5r) are activated by ligands that have this motif (Cone, 2006; Dores and Chapa, 2021). However, ACTH also has the tetrabasic motif KKRR (Supplementary Fig. 1) that is present in all vertebrate ACTH sequences (Dores and Lecaude, 2005). Elimination of this "address" motif (Schwyzer, 1977) or alanine-substitution of the tetra-basic motif results in analogs of ACTH that block the activation of mammalian (Schwyzer, 1977; Liang et al., 2013), avian (Barlock et al. 2014), reptilian (Davis et al. 2013), amphibian (Davis et al. 2013), and neopterygian fish (Liang et al. 2015; Wolverton et al., 2019) Mc2r orthologs.

These observations can be rectified by assuming that bony vertebrate Mc2r orthologs have two binding sites for ACTH, the "message" binding site for the HFRW motif that is present on all melanocortin receptors, and the "address" binding site for the tetra-basic motif of ACTH that is unique to Mc2r orthologs. An operating assumption is that the HFRW binding site on bony vertebrate Mc2R orthologs is closed prior to a binding event. That assumption would explain why α-MSH-related ligands, which lack the tetrabasic motif, cannot activate bony vertebrate Mc2R orthologs (Schwyzer, 1977; Cone, 2006; Dores and Chapa, 2021). Support for this proposed mechnaism comes from analyses that have utilized alanine-substituted analogs of ACTH(1–24) at the "message" motif and the "address" motif (Supplementary Fig. 2; for review see Dores and Chapa 2021).

With respect to the proposed "address" binding site of hMC2R, studies by Chen et al. (2007) and Chung et al, 2008) have implicated Extracellular Domain 2 (EC2). In addition, chimeric receptor studies on hMC2R have proposed that EC2, interacting with the N-terminal domain of Mrap1, may create this binding site (Fridmanis et al., 2010, 2014). In this regard, an earlier study had shown that alanine substitution at residues $L^{18}D^{19}Y^{20}I^{21}$ (i.e., δDYδ motif or "activation" motif) in the N-terminal domain of mouse (m) Mrap1 (Fig. 3C) completely blocked activation of hMC2R but had no effect on the trafficking of the receptor to the plasma membrane (Sebag and Hinkle, 2009). Subsequently, Malek et al. (2015) used a novel chimeric receptor paradigm to show that the N-terminal domain of the Mrap1 homodimer that faces the extracellular space interacts with an extracellular domain on hMC2R to facilitate activation. Following up on the latter observation, Davis et al. (2022) observed that replacing EC2 of hMC2R with a corresponding EC2 domain from a melanocortin receptor that does not require Mrap1 for activation (i.e., *Xenopus tropicalis* Mc1r) interfered with the activation of hMC2R. This study also observed that alanine replacement of residues in TM4 and TM5 of hMC2R interfered with trafficking of the receptor to the plasma membrane. Collectively these various studies all point to EC2 and the δDYδ motif in the N-terminal of Mrap1 as playing a role in the activation of hMC2R presumably by creating a binding site for the "address" motif in ACTH.

Given these observations for bony vertebrate Mc2r orthologs, the following study was done to gain an understanding of the ACTH activation mechanism for the Mc2r ortholog of the whale shark (ws; *Rhincodon typus*, order Orectolobiformes, subclass Elasmobranchii, class Chondricthyes). A previous study has shown that wsMc2r interacts with wsMrap1 to facilitate trafficking of the receptor to the plasma membrane (Hoglin et al., 2020). In addition, the wsMc2r/wsMrap1 heterodimer could be activated by either ACTH(1–24) or des-acetyl-ACTH(1–13) amide with 10-fold higher sensitivity to ACTH(1–24) as compared to the non-acetylated form of α-MSH (Hoglin et al., 2020).

The first objective of this study was to evaluate how alanine-substituted analogs of ACTH(1–24) affect the activation of wsMc2r. For this part of the study, a parallel analysis was done on a representative bony vertebrate Mc2r ortholog, the bowfin (bf) Mc2r (*Amia calva*), a neopterygian ray-finned fish, to show how a typical bony vertebrate Mc2r ortholog responds to stimulation by these ACTH(1–24) analogs.

The second objective of this study was to evaluate whether the EC2 domain of wsMc2r plays a role in the activation process. To test this hypothesis, a chimeric receptor paradigm was used based on the study by Davis et al. (2022).

The third objective of this study was to determine what role the N-terminal domain of wsMrap1 plays in the activation of wsMc2r. As shown in Fig. 3A, wsMrap1 lacks a δDYδ activation motif that is found in bony vertebrate Mrap1 orthologs (Dores et al., 2022), but instead has the motif, ELDI (Fig. 3C). This experiment tested the hypothesis that the ELDI motif is the "activation" motif for wsMrap1, and alanine substitution at the $E^{32}L^{33}D^{34}I^{35}$ motif of wsMrap1 would decrease sensitivity of wsMc2r to activation following stimulation of wsMc2r with a cartilaginous fish ACTH(1–24) analog. Collectively, these three experiments would provide new insights into the activation mechanism for an elasmobranch Mc2r ortholog.

2. Materials and methods

2.1. cDNA sequences

All melanocortin-related cDNAs used in this project were synthesized by GenScript (Piscataway, NJ) and were individually inserted into a pcDNA3.1 + expression vector. The cDNAs used included wsMc2r (XM_020525249.1), wsMrap1 (XM_020520012.1), bfMc2r (LOC: 24677757-24678686), bfMrap1 (AMCT00016091), the chimeric receptor wsMc2R/EC2 xtMc1r (see Supplementary Fig. 4), and wsMrap1 $A^{28}A^{29}A^{30}A^{31}$. Dr. Patricia Hinkle (University of Rochester, NY retired) provided cAMP reporter gene construct CRE-Luciferase (Chepurny and Holz, 2007).

2.2. Melanocortin peptides

For the cAMP reporter gene assays presented in RESULTS Section 3a, Chinese hamster ovary (CHO) cells were transiently transfected with *wsmc2r* and *wsmrap1* and stimulated with human adrenocorticotropic hormone [hACTH(1–24)] (Sigma-Aldrich Inc.; St. Louis, MO) or the alanine-substituted analogs of hACTH(1–24) (Supplementary Fig. 2) (New England Peptide; Gardiner, MA). The alanine-substituted analogs of hACTH(1–24) and hACTH(1–24) were diluted in Serum-Free CHO media and used at concentrations from 10^{-12}M to 10^{-6}M. The rationale for using hACTH(1–24) analogs in the cAMP reporter gene assays for wsMc2r was the following. The deduced amino acid sequence of wsACTH (1–24) (SYSMEHFRWGKPMGRKRRPIKVYP; accession number: XP_020377873) differs from the primary sequence of hACTH(1–24) (SYSMEHFRWGKPVGKKRRPVKVYP; accession number CAA00890) at three neutral positions. In addition, wsACTH(1–24) has the same amino acid sequence as stingray (sr) ACTH(1–24) (accession number: ANN89221). As indicated in Supplementary Fig. 3, stimulation with hACTH(1–24) resulted in an EC_{50} value of $4.4 \times 10^{-10} \pm 2.0 \times 10^{-10}$ which was not significantly different from stimulation with srACTH (1–24) which had an EC_{50} value of $8.6 \times 10^{-10} \pm 2.1 \times 10^{-10}$ (Student's t-Test; $p = 0.11$).

For the cAMP reporter gene assays presented in RESULTS Sections 3b and 3c, CHO cells transiently transfected with *wsmc2r* and *wsmrap1* were stimulated with stingray (sr) ACTH(1–24). The cAMP/reporter gene assays using *bfmc2r/bfmrap1* transiently transfected CHO cells (RESULTS Section 3.a) were stimulated with human (h) ACTH(1–24).

2.3. wsMc2r/EC2 xtMc1r chimeric receptor design

To evaluate the potential role of the EC2 domain of wsMc2r in the activation of the receptor following stimulation with hACTH(1–24), a chimeric receptor was made as described in Davis et al. (2022). In brief, the EC2 domain of wsMc2r was replace with the corresponding EC2 domain of *Xenopus tropicalis* (xt) Mc1r, a melanocortin receptor that does not require interaction with an Mrap1 ortholog for activation or trafficking (Davis et al., 2022; see Supplementary Fig. 4 for the nucleotide

sequence of the *wsMc2r/ec2 xtmc1r* chimeric receptor). The activation of the wsMc2r/EC2 xtMc1r chimeric receptor was evaluated in the cAMP reporter gene assay. The chimeric receptor was co-expressed with *wsmrap1* and the transfected cells were stimulated with srACTH(1–24) (RESULTS Section 3.b).

2.4. Tissue culture Protocol/cAMP reporter gene assay

The cAMP reporter gene assay was done in CHO cells (ATCC, Manassas, VA) grown in Kaighn's Modification of Ham's F12K media (ATCC) and supplemented with 10% fetal bovine serum, 100unit/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml normocin. The CHO cells were maintained in a humidified incubator with 95% air and 5% CO_2 at 37 °C. This cell line was selected because the CHO cells do not express endogenous *mcr* genes (Noon et al., 2002; Sebag and Hinkle, 2007), or endogenous *mrap* genes (Reinick et al., 2012).

Receptor cDNAs (10 nmole/transfection) were transiently transfected into CHO cells and co-expressed with a species specific *mrap1* cDNA (30 nmole/transfection) and the *cre-luciferase* reporter gene cDNA (85 nmole/transfection; Chepurny and Holz 2007) as described by Liang et al. (2011). The transfected cells were maintained at 37 °C in an CO_2 incubator. The transient transfections were done using the Solution T kit (Lonza, Portsmouth, NH) and the Amaxa Cell Line Nucleofector II system (Lonza, Portsmouth, NH). The transfected CHO cells were seeded in a white flat-bottom 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 ACTH(1–24) analogs in serum-free CHO-media at concentrations ranging from 10^{-12} M to 10^{-6} M.

Following a 4-hour incubation at 37 °C, the stimulating solutions were removed and a luciferase substrate reagent (BrightGLO; Promega, WI) was added to each well as described in Liang et. al (2011). Cells incubated with standard ACTH(1–24) were included with each experimental group as a control. A Bio-TEK Synergy HTX plate reader (Agilent Technologies, Santa Clara, CA) measured the luminescence generated after a five-minute incubation period at room temperature. Transfected CHO cells incubated with serum-free media, but no ACTH, were analyzed along with each experimental group to determine basal cAMP levels. Luminescence readings were corrected by subtracting the basal cAMP readings (serum-free media/no ligand) for each transfection dose response curve.

2.5. Statistical analysis

The data for each dose response curve were fitted to the Michaelis-Menten equation to obtain EC_{50} values using Kaleidograph software (https://www.synergy.com). Data points are expressed as the mean ± SEM (n = 3). The data were analyzed using either a One-way ANOVA Turkey multi-comparison test using GraphPad Prism 2 software (GraphPad Inc, LaJolla, CA, USA), or the Students t-Test using significance set at $P \leq 0.05$.

3. Results

3.1. Activation of wsMc2r using Alanine-substituted analogs of hACTH (1–24)

To determine whether the activation of wsMc2r requires both the "message' motif and the "address" motif of hACTH(1–24), *wsmc2r/wsmrap1* transfected CHO cells were stimulated with the hACTH(1–24) alanine-substituted analogs listed in Supplementary Fig. 2. Schwyzer (1977) had identified the "address" motif as residues $K^{15}K^{16}R^{17}R^{18}$. However, Costa et al (2004) observed that amino acid substitution at P^{19} also lowered the activation of hMC2R. As a result the alanine-substituted analogs of the "address" motif targeted residues $K^{15}K^{16}R^{17}R^{18}P^{19}$ (Supplementary Fig. 2). The affects of the various hACTH(1–24) analogs on the activation of wsMc2r (Fig. 1A, B, and C) was compared to the activation of a representative bony vertebrate Mc2r ortholog from the neopterygian fish, *Amia calva* (i.e., bowfin; bf) as shown in Fig. 1D, E, and F.

The results of stimulating wsMc2r with alanine-substituted analogs in the HFRW motif of hACTH(1–24) are presented in Fig. 1A, Fig. 1B, and Table 1. As expected, alanine substitution at all four positions in the HFRW motif (HFRW/AAAA analog) completely blocked activation of wsMc2r (Fig. 1B and Table 1). However, evaluation of single-alanine analogs of the HFRW motif revealed that some positions in the motif are essential for activation, and other positions have little effect on the activation process. For wsMc2r activation, residues H^6 and F^7 fit into the latter category. The EC_{50} values for the H^6/A analog (AFRW) or the F^7/A analog (HARW) were not statistically different from the EC_{50} for hACTH (1–24) (Table 1). However, alanine substitution at R^8 (HFAW analog) resulted in an analog that was nearly two orders of magnitude less potent at stimulating wsMc2r as compared to wsMc2r stimulated with hACTH(1–24) (Fig. 1A & Table 1). Finally, alanine substitution at W^9 (HFRA analog) resulted in a dose response curve that was nearly three orders of magnitude less potent at stimulating wsMc2r as compared to stimulation with hACTH(1–24) (Fig. 1B & Table 1). Hence, the hierarchy of residues in the HFRW motif required for the activation of wsMc2r is $W > R \gg\gg H = F$, with residues H^6 and F^7 playing a negligible role in the activation of this receptor.

The analysis of the effect of alanine-substituted analogs in the "address" motif hACTH(1–24) on the activation of wsMc2r is presented in Fig. 1C. Partial alanine substitution (i.e., AARRP analog or KKAAA analog) resulted in dose response curves that were roughly 10-fold less potent than hACTH(1–24), but neither analog yielded a statistically significant negative effect on ligand sensitivity when their EC_{50} values were compared to hACTH(1–24) (Table 1). Complete substitution of alanines in the KKRRP motif (KKRRP/AAAAA analog) did produce a statistically significant shift in EC_{50} value (Table 1) that was roughly two orders of magnitude higher that the EC_{50} value for hACTH(1–24). However, the KKRRP/AAAA dose response curve did reach saturation. The relevance of this effect will become apparent from the ACTH(1–24) analog analysis of bfMc2r.

The results of stimulating bfMc2r with alanine-substituted analogs in the HFRW motif of hACTH(1–24) are presented in Fig. 1D, Fig. 1E, and Table 1. As expected, alanine substitution at all four positions in the HFRW motif (HFRW/AAAA analog) completely blocked activation of wsMc2r (Fig. 1E and Table 1). Single alanine substitution at H^6 (AFRW analog) did not have a statistically significant effect on the activation of bfMc2r as compared to stimulation with hACTH(1–24) (Fig. 1D) (Table 1). However, single alanine substitution at F^7 (HARW analog) and R^8 (HFAW analog) both resulted in two orders of magnitude shift in EC_{50} value relative to the EC_{50} value for hACTH(1–24) and these shifts were statistically significant (Fig. 1D & Table 1). Finally alanine substitution at W^9 (HFRA analog) resulted in a dose response curve with minimal stimulation of bfMc2r apparent only at the 10^{-6} M dose of the analog (Fig. 1E). It appears that the hierarchy of residues in the HFRW motif required for the activation of bfMc2r is $W > R = F > H$, with residue H^6 playing a negligible role in the activation of this receptor.

Stimulating bfMc2r with alanine-substituted analogs of the "address" motif is presented in Fig. 1F. Alanine substitution at positions K^{15}-K^{16} (AARRP analog) yielded a dose response curve with an EC_{50} value that was 10-fold higher than the EC_{50} value for hACTH(1–24) and that EC_{50} value was not statistically difference from the positive control (Fig. 1E and Table 1). However, alanine substitution at positions $R^{17}R^{18}P^{19}$ (KKAAA analog) resulted in a dose response curve with an EC_{50} value that was two orders of magnitude higher than the EC_{50} value for hACTH (1–24) and this EC_{50} value was statistically different from the positive control (Table 1). Alanine substitution at all five positions in the "address" motif (KKRRP/AAAAA analog) resulted in a dose response curve with no activation at physiologically relevant concentrations of the analog (i.e., 10^{-11}M to 10^{-8}M) (Fig. 1F).

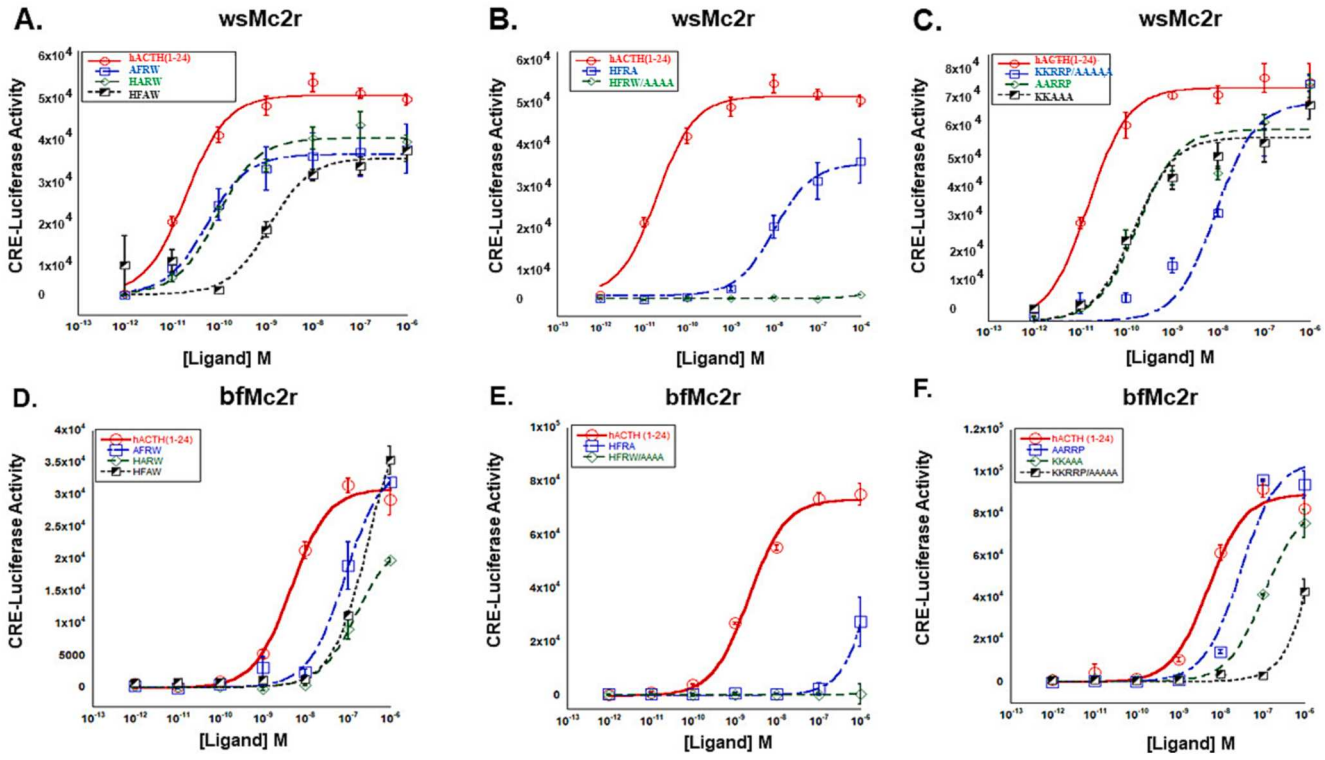

Fig. 1. Stimulation of wsMc2r and bfMc2r with alanine-substituted analogs of hACTH(1–24). CHO cells were co-transfected with a receptor cDNA, species specific mrap1 cDNA and a cre/luciferase cAMP reporter cDNA as described in Methods. After 48 h in culture the transfected cells were stimulated with either wild-type hACTH(1–24) or alanine-substituted analogs of hACTH(1–24) (see Supplementary Fig. 2) at concentrations varying from 10^{-12} M to 10^{-6} M. Data are presented as mean ± SEM and n = 3. The EC_{50} values for all the dose response curves are presented in Table 1. The transfections presented in Fig. 1A and 1B were done in the same experiment and the dose response curve for hACTH(1–24) is shown in both panels for ease in viewing. A) Stimulation of wsMc2r with either hACTH(1–24), the HFRW/AAAA analog, or the AFRW analog. B) Stimulation of wsMc2r with either hACTH(1–24), the HARW analog, the HFAW analog, or the HFRA analog. C) Stimulation of wsMc2r with hACTH(1–24), the AARRRP analog, the KKAAA analog or the KKRRP/AAAAA analog. D) Stimulation of bfMc2r with hACTH(1–24), the AFRW analog, the HARW analog, or the HFAW analog. E) Stimulation of bfMc2r with hACTH(1–24), the HFRA analog or the HFRW/AAAA analog. F) Stimulation of bfMc2r with hACTH(1–24), the AARRRP analog, the KKAAA analog or the KKRRP/AAAAA analog.

Table 1
Summary of Fig. 1.

wsMc2r + wsMrap1			
Stimulation Panel	with	EC_{50} Value (M)	p*
1A	hACTH(1–24)	$2.0x10^{-11} \pm 3.6x10^{-12}$	
	AFRW analog	$5.5x10^{-11} \pm 7.6x10^{-12}$	0.99
	HARW analog	$9.1x10^{-11} \pm 2.1x10^{-11}$	0.99
	HFAW analog	$1.1x10^{-09} \pm 7.1x10^{-10}$	0.05
1B	HFRA analog	$1.0x10^{-08} \pm 1.5x10^{-09}$	<0.001
	HFRW/AAAA	n.d	
1C	hACTH(1–24)	$1.5x10^{-11} + 1.9x10^{-12}$	
	KKRRP/AAAAA	$8.0x10^{-09} + 4.3X10^{-09}$	<0.001
	AARRP analog	$1.8x10^{-10} + 1.1x10^{-10}$	0.99
	KKAAA analog	$1.5x10^{-10} + 6.2x10^{-11}$	0.99
bfMc2R + bfMrap1			
Stimulation			
Panel	with	EC_{50} Value (M)	p*
1D	ACTH(1–24)	$3.0x10^{-09} \pm 8.2x10^{-09}$	
	AFRW analog	$4.6x10^{-08} + 4.0x10^{-07}$	0.28
	HARW analog	$1.2x10^{-07} \pm 3.0x10^{-07}$	0.03
	HFAW analog	$2.7x10^{-07} \pm 5.2x10^{-07}$	0.008
1E	ACTH(1–24)	$2.1x10^{-09} + 3.8x10^{-10}$	
	HFRA analog	n.d.	
	HFRW/AAAA	n.d	
1F	hACTH(1–24)	$4.9x10^{-09} \pm 9.6x10^{-10}$	
	KKRRP/AAAAA	n.d.	
	AARRP analog	$2.9x10^{-08} \pm 8.2x10^{-09}$	0.38
	KKAAA analog	$1.1x10^{-07} \pm 1.8x10^{-08}$	0.0016

3.2. Chimeric receptor analysis of the EC2 domain of wsMc2r

Since several studies have pointed to a role for the EC2 (Extracellular Loop 2) domain in the activation of human (h) MC2R (Chen et al., 2007, Chung et al., 2008; Fridmantis et al., 2010; Fridmantis et al., 2014; Davis et al., 2022), we used the chimeric receptor paradigm designed by Davis et al. (2022 see MATERIALS & METHODS) to test the hypothesis that exchanging the EC2 domain of wsMc2r with the EC2 domain of the Mc1r of the amphibian *Xenopus tropicalis* (Fig. 2A) would interfere with the activation of the chimeric receptor, wsMc2r/EC2 xtMc1r. As shown in Fig. 2B, when stimulated with srACTH(1–24), the wsMc2r/EC2 xtMc1r chimeric receptor dose response curve had an EC_{50} value (9.4×10^{-10} M $\pm 1.7 \times 10^{-10}$ M) that was nearly identical to the EC_{50} value for wild-type wsMC2R (2.7×10^{-10} M $\pm 4.5 \times 10^{-11}$ M) (p = 0.93; Student's t-Test).

3.3. Does wsMrap1 have an activation motif?

As shown in Fig. 3A, bony vertebrate Mrap1 orthologs have the δDYδ activation motif in their N-terminal domain (Dores et al., 2022). The corresponding amino acid sequence in wsMrap1 is ELDI. This motif does not fit the bony vertebrate consensus sequence for an activation motif but may represent an activation motif unique to cartilaginous fishes. To test that hypothesis, the $E^{32}L^{33}D^{34}I^{35}$ motif was replaced with alanines, and *wsmc2r* cDNA was co-expressed with the *wsmrap1* alanine mutant as shown in Fig. 3 B. As a control, *wsmc2r* was co-expressed with the wild-type *wsmrap1* cDNA construct. Alanine substitution had no effect on the sensitivity of the receptor to stimulation by srACTH(1–24) (p = 0.32; Students t-Test; see figure legend for EC_{50} values). However, a

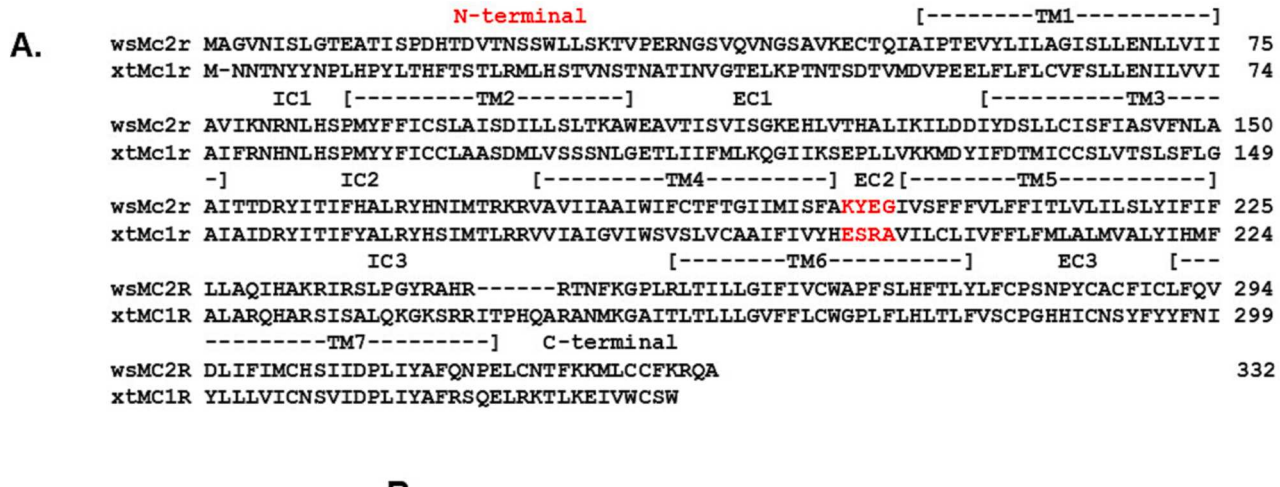

Fig. 2. Testing the activation of a wsMc2r/EC2 xtMc1r chimeric receptor. A) The amino acid sequences of wsMc2r and xtMc1r were aligned and the residues in Extracellular Domain (EC2) are highlighted in red. As described in METHODS, the EC2 domain of wsMc2r was replaced with the EC2 domain of xtMc1r. The nucleotide sequence of the wsmc2r/ec2 xtmc1r chimeric receptor cDNA is presented in Supplementary Fig. 3. B) The dose response curves for the wild-type wsMc2r and the chimeric receptor, wsMc2r/EC2 xtMc1r, both co-expressed with wsMrap1 are compared following stimulation with srACTH(1–24) as described in METHOD. The data are presented as mean ± S.E.M and n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

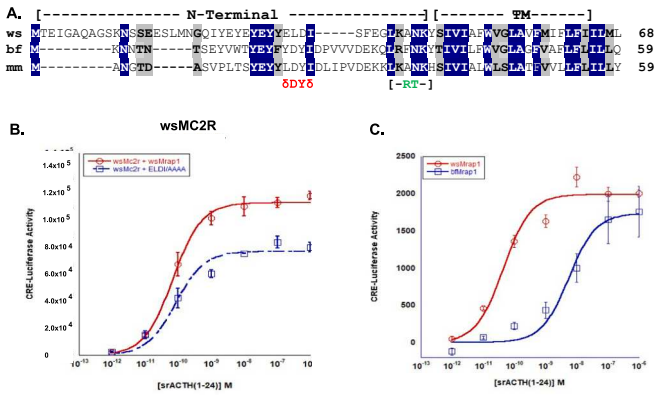

Fig. 3. Evaluation of an alanine-substituted Analog of wsMrap1. A) The N-terminal and transmembrane domains of whale shark (wsMrap1), bowfin (bfMrap1) and mouse (mmMrap1) were aligned and primary sequence identity (highlighted blue) and primary sequence similarity (highlighted gray) were determined using BLOSUM (https://www.ncbi.nlm.nih.gov/Class/FieldGuide/BLOSUM62.txt). Abbreviations: **δDYδ** – consensus activation motif for a bony vertebrate Mrap1 ortholog. RT – reverse topology motif. TM – transmembrane domain. B) A comparison of the dose response curves for *wsmc2r* co-expressed with *wsmrap1* and *wsmc2r* co-expressed with *bfmrap1* following stimulation with srACTH(1–24). The data are presented as mean ± S.E.M and n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

comparison of Vmax values indicated that activation of the *wsmc2r* cDNA was lowered by 32% when the receptor was co-expressed with *wsmrap1e*^{32}*l*^{33}*d*^{34}*i*^{35}*/aaaa* as compared to co-expression with wild-type *wsmrap1* (p = 0.001; Student's t-Test; see figure legend for Vmax values).

Fig. 3B suggests that the N-terminal domain of wsMrap1 may play a role in activation of wsMc2r. As a way to test this hypothesis, *wsmc2r* was co-expressed with either *wsmrap1* or *bfmrap1*. The rationale for this experiment is that trafficking is the primary function of an elasmobranch Mrap1 ortholog (Hoglin et al., 2020). Since the transmembrane domains of wsMrap1 and bfMrap1 have 74% primary sequence identity/similarity (Fig. 3A), we tested the hypothesis that co-expression of wsMc2r with either Mrap1 ortholog should result in very similar dose response curves. However, as shown in Fig. 3C, a comparison of EC_{50} values for the wsMrap1 dose response curve ($4.4x10^{-11}$M ± $1.6x10^{-11}$) and the EC_{50} value for the bfMrap1 dose response curve ($5.5x10^{09}$M + $2.0x10^{-09}$) resulted in dose response curves with a nearly two orders of magnitude shift in sensitivity to stimulation with srACTH(1–24) that was statistically different (p = 0.03; Students t-Test).

4. Discussion

Studies on the activation of cartilaginous fish Mc2r orthologs have been done on the elephant shark, *Callorhinchus milii* (subclass Holocephali; order Chimaeriformes; Barney et al., 2019), the red stingray, *Dasyatis akajei* (subclass Elasmobranchii; order Rajiformes; Dores et al., 2018), and the whale shark, *Rhincodon typus* (subclass Elasmobranchii,

order Orectolobiformes; Hoglin et al., 2020 and the current study). An alignment of these cartilaginous fish Mc2r orthologs is presented in Fig. 4. The primary sequence identity/similarity for these orthologs is 58%, and while there is only 15% primary sequence identity/similarity in the N-terminal domain, the other domains have much higher primary sequence conservation (see legend for Fig. 4). In addition, the cartilaginous fish Mc2r orthologs have the critical residues (marked with a star) near TM2, TM3, and in TM6 that are present in all vertebrate Mcrs which are associated with the HFRW binding pocket (Mulholland et al., 2005; Dores et al., 2022).

The unifying feature of these cartilaginous fish Mc2rs is that all three orthologs can be activated by ACTH or the non-acetylated form of αMSH at physiological concentrations of each ligand. This reality is in sharp contrast to the ligand selective properties of bony vertebrate Mc2r orthologs which are exclusively selective for ACTH (Dores and Chapa, 2021; Shaughnessy et al., 2022, Dores et al., 2022). While all vertebrate Mcrs apparently have a common binding site for the "message" motif (HFRW) present in ACTH and the MSH-sized ligands (Pogosheva et al. 2005; Baron et al., 2009. Dores, 2009; Dores and Chapa, 2021), MSH-sized ligands lack the "address" motif, KKRRP (See Supplementary Fig. 1) which is a requirement for the activation of bony vertebrate Mc2r orthologs (Schwyzer, 1977; Dores and Chapa, 2021). A way to screen for whether a Mc2r ortholog may require the "address" motif of ACTH for activation is to test the efficacy of alanine-substituted analogs of ACTH (Supplementary Fig. 2). As shown in Fig. 1F, alanine-substitution of the KKRRP motif of ACTH(1–24) blocked activation of bfMc2r at all physiologically relevant concentrations of the ligand. This response is typical for a bony vertebrate Mc2r ortholog (Dores and Chapa, 2021). When the same screening was done on elephant shark (es) Mc2r, alanine-substitution at the KKRRP motif had no negative statistical effect on the activation of esMc2r (Hoglin et al., 2019). Since esMc2r could be effectively activated by ACTH(1–13)amide, the non-acetylated form of αMSH (Barney et al., 2019), it appears that esMc2r has one binding site for ACTH or the MSH-sized ligands, the HFRW binding site, and no "address" motif binding site. In addition, the HFRW binding site must be open prior to the ligand binding event to accept either ACTH or MSH-sized ligands. By contrast, bony vertebrate Mc2r orthologs, of which the bowfin ortholog is a typical example, are postulated to have a "message" binding site and an "address" binding site for ACTH. In addition, the "message" site appears to be closed prior to a binding event; hence MSH-sized ligands are excluded.

The elephant shark is a holocephalan cartilaginous fish, and currently the only representative from that subclass that has been analyzed. The objective of this study was to evaluate how an elasmobranch Mc2r ortholog (i.e., whale shark) would respond to stimulation with the alanine substituted analogs of ACTH. Previous studies had shown that wsMc2r could be activated at physiologically relevant concentrations by either ACTH or ACTH(1–13)amide (Hoglin et al., 2020), hence it would be reasonable to assume that the HFRW binding site on wsMc2r is always open. Stimulation of wsMc2r with single-alanine analogs in the HFRW motif of ACTH (Fig. 1A&1B; Table 1) yielded dose response curves that paralleled the observations for bfMc2r (Fig. 1D&1E; Table 1) and other bony vertebrate Mc2r orthologs (Dores and Chapa, 2021). Alanine substitution at W^9 essentially blocks activation of the receptor. Alanine substitution at R^8 also had a negative effect on activation of both wsMc2r and bfMc2r (Fig. 1A & 1E; Table 1). However, alanine substitution at H^6 had no statistical effect on either wsMc2r or bfMc2r activation (Fig. 1A, 1D; Table 1). While alanine substitution at F^7 had no statistical effect on the activation of wsMc2r (Fig. 1A; Table 1), this residue appears to play some role in the activation of bfMc2r (Fig. 1D; Table 1).

Based on the HFRW analog study, wsMc2r could operate in a manner similar to esMc2r. However, stimulation of wsMc2r with the KKRRP/AAAAA analog of ACTH resulted in a dose response curve (Fig. 1C) that was distinct from esMc2r (Hoglin et al, 2019) or bfMc2r (Fig. 1F). Unlike esMc2r where the analog had no negative effect on activation (Hoglin et al., 2019) or bfMc2r where the analog essentially blocked activation (Fig. 1F), stimulation of wsMc2r with the KKRRP/AAAAA analog of ACTH lowered the sensitivity of wsMc2r to stimulation by the analog by nearly 2 orders of magnitude relative to the positive control, an intermediate effect (Table1) that did not completely block activation and the dose response curve did reach saturation. This "intermediate" outcome was also observed for stingray Mc2r (Hoglin et al., 2019). In that study stingray Mc2r was co-expressed with a heterologous Mrap1 (i.e., esMrap1) which initially raised concerns that the "intermediate" dose response curve may have been confounded by the heterologous Mrap1. In the current study wsMc2r was co-expressed with wsMrap1 to alleviate this issue. Hence, the "intermediate" response to KKRRP/AAAAA analog of ACTH may be typical for elasmobranch Mc2r orthologs. An analysis of the pharmacological properties of a Mc2r orthologs from another order of the elasmobranchs is warranted to validate this generalization.

The preceding observations could be interpreted as an indication that, like bony vertebrate Mc2r orthologs, activation of the wsMc2r by ACTH may involve the "address" motif interacting with Extracellular Loop 2 and the N-terminal of Mrap1 as appears to be the case for bony vertebrate Mc2r orthologs (Davis et al., 2022; Dores et al., 2022). Alternatively, the presence of five alanines in the ACTH(1–24)KKRRP/AAAAA analog may have distorted the conformation of the analog, and that outcome led to the shift in EC_{50} value observed in Fig. 1C. To test the former hypothesis, a chimeric receptor was constructed in which the TM2 domain of wsMc2r was replaced with the EC2 domain of a Mcr (xtMc1r) that does not interact with Mrap1 (Fig. 2A and Supplementary Fig. 3). As indicated in Fig. 2B that manipulation had no negative effect on activation. To determine whether the N-terminal domain of wsMrap1 has a role in activation the four amino acid motif ELDI that can be aligned to the δDYδ activation motif of bowfin and mouse Mrap1s (Fig. 3A) was replaced with alanines. The operating assumption for this experiment was that the ELDI motif in wsMrap1 is a novel activation motif. However, this manipulation has no effect on EC_{50} values (Fig. 3B). None the less, the Vmax value for the *wsmc2r/wsmrap1eldi/aaaa* dose response curve was statistically lower than the dose response curve for *wsmc2r/wsmrap1* (Fig. 3B). Since wsMrap1 forms a heterodimer with wsMc2r, perhaps alteration of the N-terminal domain of wsMrap1 has some effect on the conformation of wsMc2r which affects activation. To explore this possibility further, wsMc2r was co-expressed with either wsMrap1 or bfMrap1 (Fig. 3C). The rationale for this experiment was that the N-terminals of the two Mrap1 orthologs are different in length and primary sequence identify/similarity (29%), while the TMs of the two Mraps have high primary sequence identity/similarity (74%). If the only role of Mrap1 is to facilitate the trafficking of an elasmobranch Mc2r ortholog to the plasma membrane, then the dose response curves for the two transfections should overlap. As can be seen in Fig. 3C, wsMc2r co-expressed with bfMrap1 was nearly two orders of magnitude less sensitive to stimulation by ACTH as compared to the receptor co-expressed with wsMrap1. Perhaps the N-terminal of wsMrap1 does affect the conformation of wsMc2r, and that interaction facilitates interaction with ACTH.

In any event, it would appear that wsMc2r, and perhaps all elasmobranch Mc2r orthologs, have a single binding site for melanocortin ligands, the HFRW binding site. The same conclusion has been made for esMc2r (Hoglin et al., 2019). However, looking at the alignment of the cartilaginous fish Mc2r orthologs in Fig. 4, it is not apparent why esMc2r does not require interaction with Mrap1 for trafficking, and both srMc2r and wsMc2r do require interaction with Mrap1 to facilitate trafficking. A clue to differences in interaction with Mrap1 for elasmobranch Mc2r orthologs and holocephalan Mc2r orthologs may be apparent from an analysis of the TM4/EC2/TM5/IC3 domain of just the elasmobranch Mc2r orthologs (Fig. 5). This region of the elasmobranch Mc2r has a much higher primary sequence identity/similarity than the corresponding region of esMc2r. In this regard, 3-dimensonal modeling of these cartilaginous Mc2r sequence with a focus on TM4/EC2/TM5/IC3 domain may be informative.

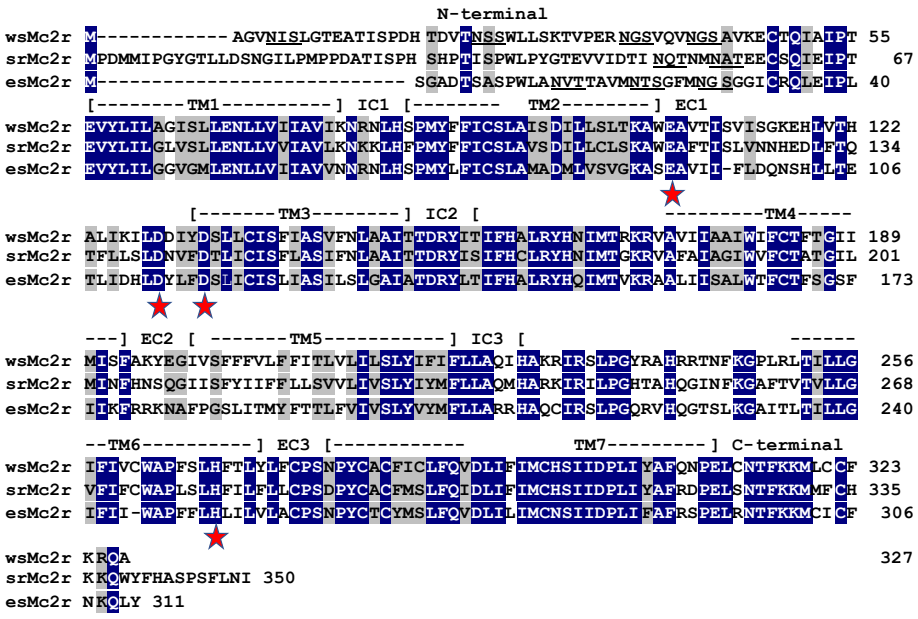

Fig. 4. Amino Acid sequence Alignment of Cartilaginous Fish Mc2r Orthologs. The deduced amino acid sequences of whale shark Mc2r (ws; accession number: XM_020525249.1), stingray (sr; accession number: BAU98230) and elephant shark (es; accession number: AAVX01069419.1) were aligned by inserting the minimal number of gaps, and primary sequence identity (highlighted in dark blue) and primary sequence similarity (highlighted in gray) were determined using BLOSUM https://www.ncbi.nlm.nih.gov/Class/FieldGuide/BLOSUM62.txt). The primary sequence identity/similarity of each domain is: N-terminal (15%), TM1 (91%), IC1 (71%), TM2 (91%), EC1 (42%), TM3 (84%), IC2 (95%), TM4 (68%), EC2 (33%), TM5 (61%), IC3 (46%), TM6 (61%), EC3 (93%), TM7 (88%), and C-terminal (37%). Abbreviations: TM -transmembrane domain; IC – intracellular loop; EC (extracellular loop). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

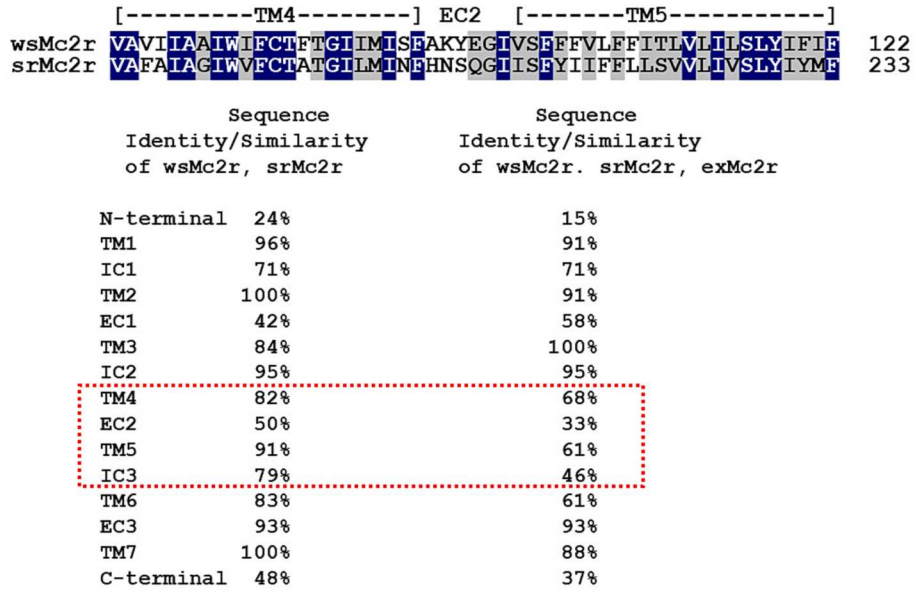

Fig. 5. Alignment of the TM4/EC2/TM5/IC3 domains of two Elasmobranch Mc2r Orthologs. The TM4/EC2/TM5/IC3 of wsMc2r and srMc2r were analyzed for primary sequence identity and similarity as described in the legend to Fig. 4. Positions that are identical are highlighted in dark blue and positions that are similar are highlighted in gray. A comparison of the percent sequence identity/similarity for just wsMc2r and srMc2r, and wsMc2r, srMC2r, and esMc2r (see Fig. 4) is presented. The TM4/EC2/TM5/IC3 precent sequence identify/similarity are highlighted with the dashed red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2023.114278.

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