Molecular and pharmacological characterization of the melanocortin-2 receptor and its accessory proteins Mrap1 and Mrap2 in a Squalomorph shark, the Pacific spiny dogfish

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ABSTRACT

The hypothalamus-pituitary-adrenal/interrenal (HPA/I) axis is a conserved vertebrate neuroendocrine mechanism regulating the stress response. The penultimate step of the HPA/I axis is the exclusive activation of the melanocortin-2 receptor (Mc2r) by adrenocorticotropic hormone (ACTH), requiring an accessory protein, Mrap1 or Mrap2. Limited data for only three cartilaginous fishes support the hypothesis that Mc2r/Mrap1 function in bony vertebrates is a derived trait. Further, Mc2r/Mrap1 functional properties appear to contrast among cartilaginous fishes, (i.e., the holocephalans and elasmobranchs). This study sought to determine whether functional properties of Mc2r/Mrap1 are conserved across elasmobranchs and in contrast to holocephalans. The deduced amino acids of Pacific spiny dogfish (*Squalus suckleyi*; pd) pdMc2r, pdMrap1, and pdMrap2 were obtained from a *de novo* transcriptome of the interrenal gland. pdMc2r showed high primary sequence similarity with elasmobranch and holocephalan Mc2r except at extracellular domains 1 and 2, and transmembrane domain 5. pdMraps showed similarly high sequence similarity with holocephalan and other elasmobranch Mraps, with all cartilaginous fish Mrap1orthologs lacking an activation motif. cAMP reporter gene assays demonstrated that pdMc2r requires an Mrap for activation, and can be activated by stingray (sr) ACTH(1-24), srACTH(1-13)NH_2, and γ-melanocyte-stimulating hormone at physiological concentrations. However, pdMc2r was three orders of magnitude more sensitive to srACTH(1-24) than srACTH(1-13)NH_2. Further, pdMc2r was two orders of magnitude more sensitive to srACTH(1-24) when expressed with pdMrap1 than with pdMrap2. These data suggest that functional properties of pdMc2r/pdMrap1 reflect other elasmobranchs and contrast what is seen in holocephalans.

1. INTRODUCTION

The restoration of homeostasis following a stress event through the action of corticosteroids released *via* activation of the hypothalamus-pituitary-adrenal/interrenal (HPA/HPI) axis is a feature common to all vertebrates (Denver, 2009). To initiate this process, the hypophysiotropic factor, CRF (Deussing and Chen, 2018), activates corticotropic cells of the anterior pituitary (Sower, 2015; Trudeau and Somoza, 2020), which in turn synthesize the precursor protein, pro-opiomelanocortin (POMC; Nakanishi et al., 1979). Through the action of Prohormone Convertase 1/3, the 39-amino acid hormone, adrenocorticotropic hormone (ACTH), is excised from POMC in regulated secretory vesicles and released into the vascular system. ACTH then binds to the melanocortin-2 receptor (Mc2r) on either adrenal cortex cells (amniote tetrapods, Gallo-Payet and Battista, 2014) or interrenal cells (anamniote tetrapods, Davis et al., 2013; bony fishes, Takahashi et al., 2013; cartilaginous fishes, Liang et al., 2013). An understanding of the activation of Mc2r by cartilaginous fishes is the focus of the current study.

Mc2r is one of five G protein-coupled receptors in the melanocortin receptor gene family (Cone, 2006). There are several pharmacological features that unify bony vertebrate Mc2r orthologs. For these vertebrates, Mc2r requires co-expression with the accessory protein, Mrap1 (Metherell et al., 2005; Sebag and Hinkle, 2009, 2007) for trafficking to the plasma membrane and activation following an ACTH binding event (Dores et al., 2022). In addition, bony vertebrate Mc2r orthologs can only be activated by ACTH, but not by any of the melanocyte-stimulating hormone-sized ligands derived from POMC (i.e., αMSH, βMSH, γMSH; Dores and Chapa, 2021). The latter observation was initially surprising given that all melanocortin-related peptides (e.g., ACTH, αMSH, βMSH, γMSH) have the HFRW “message” motif (Schwyzer, 1977) that is required for the activation of all melanocortin receptors (Cone, 2006). However,

ACTH also has the K/RKRR "address" motif (Schwyzer, 1977), which is a requirement for the activation of all bony vertebrate Mc2r orthologs that have been studied (Dores et al., 2022; Dores and Chapa, 2021; Shaughnessy et al., 2022). Finally, the accessory protein, Mrap2 (Chan et al., 2009), the paralog of Mrap1, can facilitate the trafficking of, for example human (h) MC2R, to the plasma membrane. However, this accessory protein cannot facilitate the activation of hMC2R by ACTH when the receptor and accessory protein are co-expressed in mammalian cell lines (Chan et al., 2009; Sebag and Hinkle, 2009; Webb and Clark, 2010).

Some of the pharmacological properties observed for bony vertebrate Mc2r orthologs are apparent for cartilaginous fish Mc2r orthologs; however, there are some striking differences. Current studies on cartilaginous fish Mc2r orthologs have investigated the pharmacological properties for one species from subclass Holocephali, the elephant shark, *Callorhinchus milii* (Barney et al., 2019; Reinick et al., 2012), and two species from subclass Elasmobranchi, the stingray *Hemitrygon akajei* (Dores et al., 2018; Hoglin et al., 2020a, 2020b; Takahashi et al., 2016), and the whale shark, *Rhincodon typus* (Hoglin et al., 2020b). All cartilaginous fish Mc2r orthologs that have been studied can be activated by ACTH, but these orthologs can also be activated at physiological concentrations by the non-acetylated analog of αMSH (i.e., ACTH(1-13)NH_2; Dores and Chapa, 2021; Hoglin et al., 2020b). In addition, the elephant shark (es) Mc2r ortholog does not require co-expression with either esMrap1 or esMrap2 to facilitate trafficking to the plasma membrane. Hence, the activation of esMc2r is a Mrap-independent process (Barney et al., 2019). By contrast, the two elasmobranch Mc2r orthologs are dependent on co-expression with an Mrap1 ortholog for trafficking to the plasma membrane (Hoglin et al., 2020b), and the trafficking of whale shark (ws) Mc2r is facilitated by either co-expression with wsMrap1 or wsMrap2. Furthermore, once the elasmobranch Mc2r orthologs reach the plasma

membrane it does not appear that interaction with Mrap1 influences the sensitivity of the receptor to stimulation by ACTH (Hoglin et al., 2023).

Clearly cartilaginous fish Mc2r orthologs differ from bony vertebrate Mc2r orthologs in terms of ligand selectivity and the role that Mrap1 and Mrap2 play in the activation of the ortholog. In addition, among the cartilaginous fishes, there are apparent differences in the role that the Mraps play with respect to the interaction with Mc2r. The current studies on elasmobranch Mc2r orthologs have focused on one species of stingray (*H. akajei*) from Superorder Batoidea, and one species of Galean shark (*R. typus*) from Superorder Selachii. The objectives of this study were to evaluate the pharmacological properties of the Mc2r ortholog from a Squalomorph shark, *Squalus suckleyi*, the Pacific spiny dogfish (pd). To this end, the deduced amino acid sequences of pdMc2r, pdMrap1, and pdMrap2 were obtained from a transcriptome made from the interrenal gland of *S. suckleyi*. In a series of pharmacological studies, *pdmc2r* cDNA was transiently expressed in Chinese hamster ovary cells and a cAMP reporter gene assay was used to test whether: a) pdMc2r can be activated by ACTH in the absence of a Mrap; b) pdMc2r activation by ACTH requires pdMrap1, and the receptor can be activated with equal efficacy with pdMrap2; and c) pdMc2r can be activated by cartilaginous fish MSH-sized ligands with efficacy equal to stimulation with ACTH. Collectively, these analyses will clarify the role that the Mrap accessory proteins play in the activation of elasmobranch Mc2r orthologs.

2. MATERIALS AND METHODS

2.1. Ethical approval

Dogfish were collected under Fisheries and Oceans Canada permit XR-139 2021. All experimental procedures were approved by the Bamfield Marine Sciences Centre (BMSC) animal care committee and conducted as described in animal user protocol RS-21-03.

2.2. Animal collection and husbandry

Adult male dogfish (n = 4) were captured using rod-and-reel in Barkley Sound (British Columbia, Canada) during June and July 2021. Dogfish were transported to BMSC where they were maintained in a 155,000 L tank. The holding tank was continuously supplied with seawater (12 °C, 32 ppt) and dogfish were fed cut hake (*Merluccius merluccius*) every second day, ad libitum. Prior to tissue sampling, sharks were euthanized by emersion in an overdose of tricaine methanesulfonate (MS-222; > 0.2 g L-1; Syndel Labs, Vancouver, BC, Canada) followed by cervical dislocation.

2.3. Interrenal gland reference transcriptome

Interrenal glands were dissected from dogfish and stored in RNAlater at -20 °C. Total RNA was extracted using an Invitrogen PureLink RNA Mini Kit (Invitrogen, CA, USA) and resuspended in MilliQ water. Purity of total RNA was determined using a NanoDrop One (Invitrogen, CA, USA) and integrity was determined visually *via* gel electrophoresis on a 1% agarose 1x TAE gel stained with ethidium bromide. Total RNA (250 ng per dogfish) was submitted to the Centre d'Expertise et de Services Génome Québec for next-generation sequencing on an Illumina NovaSeq 6000 (paired-end 100 base pair reads). RNA integrity numbers (RIN) for all samples were 9.2 ± 0.4 (mean ± standard deviation), where a minimum acceptable RIN for sequencing is 6.5. mRNA stranded libraries were created with New England Biolabs NEBNext Dual adapters. On average, 85 million ± 3 million reads were sequenced.

Transcriptome assembly and annotation was conducted following Thorstensen et al., (2022). Quality control for raw reads was undertaken using FastQC version 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were then trimmed using Trimmomatic version 0.39 (Bolger et al., 2014), where reads under 36 base pairs were removed, leading and trailing base pairs with Phred scores under five were removed, and consecutive sets of four base pairs with mean quality scores under five were removed. The quality of the trimmed reads was then checked with FastQC. Next, trimmed reads from all four dogfish were assembled into a single reference transcriptome using Trinity version 2.12.0, using default parameters (Grabherr et al., 2011). Completeness of the transcriptome was quantified using BUSCO version 5.2.2 (Simão et al., 2015) against the vertebrate lineage (vertebrata_odb10). Transcriptome annotation as undertaken following the Trinotate pipeline (Bryant et al., 2017). Briefly, TransDecoder version 5.5.0 was used to detect the longest open reading frames for transcripts (https://github.com/TransDecoder/TransDecoder/wiki). Next blast+ version 2.12.0 was used to run the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) on transcripts and predicted peptides (Altschul et al., 1990). HMMER version 3.2.1 was run to identify protein families from predicted peptide sequences (Wheeler and Eddy, 2013). SignalP version 4.1f was run to identify signal proteins (Petersen et al., 2011). TMHMM version 2.0c was run to identify transmembrane helices of predicted peptides (Krogh et al., 2001). Finally, Trinotate version 3.2.2 was used to compile the resultant databases into a single annotation report, reporting only transcripts with E values below 0.001. The assembled and annotated interrenal gland transcriptome contained 485,425 unique transcripts representing 258,538 genes. The BUSCO completeness score was 94.6 %.

146 2.4. Mc2r, Mrap1, and Mrap2 sequences

147 Transcripts containing complete cDNA sequences for *pdmc2r, pdmrap1*, and *pdmrap2*
148 were located in the interrenal gland reference transcriptome. The nucleotide sequence for each
149 cDNA is presented in Supplementary Figure 1. Amino acid sequences for pdMc2r, pdMrap1, and
150 pdMrap2 were determined using the ExPASy translate tool (https://www.expasy.org/translate/).
151 The deduced amino acid sequences appear in Supplementary Figure 1.

152 The *pdmc2r, pdmrap1*, and *pdmrap2* cDNAs, and *wsmrap1* and bowfin (*Amia calva*; bf)
153 *bfmrap1* (Shaughnessy et al., 2022) cDNAs used in the cAMP reporter gene assay were
154 synthesized by GenScript (Piscataway, NJ). Each cDNA sequence was individually inserted into
155 a pcDNA3+ expression vector. The cAMP reporter gene construct CRE-Luciferase was provided
156 by Dr. Patricia Hinkle (University of Rochester, NY).

157 2.5. Melanocortin peptides

158 For the cAMP reporter gene assays, transfected cells were either stimulated with red
159 stingray (sr) ACTH(1-24), ACTH(1-13)NH_2 (Des-acetyl α-MSH), β-MSH, γ-MSH, or δ-MSH,
160 provided by Prof. A. Takahashi (Kitasato University, Japan). The melanocortin peptides were
161 used at concentrations from 10^{-13} M to 10^{-6} M. A comparison of the deduced amino acid
162 sequences for the stingray melanocortin peptides and dogfish melanocortin peptides is presented
163 in Supplementary Figure 2.

164 2.6. cAMP reporter gene assay

165 The cAMP reporter gene assay was conducted using Chinese hamster ovary (CHO) cells
166 (ATCC, Manassas, VA) grown in Kaighn's Modification of Ham's F12K media (ATCC) and
167 supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin,
168 100 μg mL^{-1} 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 CHO cells do not express endogenous melanocortin receptor genes (Noon et al., 2002; Sebag and Hinkle, 2007), or endogenous melanocortin receptor accessory protein genes (Reinick et al., 2012).

For the cAMP reporter gene assay, *pdmc2r* cDNA (10 nmol transfection^{-1}) was either expressed alone, or with *mrap* cDNA (30 nmol transfection^{-1}), and the *cre-luc* cDNA (83 nmol transfection^{-1}; cAMP CRE-Luciferase construct; Chepurny and Holz, 2007) in 3.0×10^6 CHO cells as described previously (Liang et al., 2011). The transient transfections were done using the Amaxa Cell Line Nucleofector II system (Lonza, Basel, Switzerland) using program U-23, and the transfection solution was Solution T (Lonza, Basel, Switzerland). The transfected cells were plated in a white 96-well plate (Costar 3917, Corning In., Kennebunk, ME) at a final density of 1.0×10^5 cells well^{-1}. After 48 hours, the transfected cells were stimulated with various concentrations (10^{-13} M to 10^{-6} M) of the stingray melanocortin peptides in serum-free CHO media. The stimulated cells were incubated for 4 h at 37°C. Following the incubation period, the stimulating media was removed, and 100 µl of luciferase substrate reagent (Bright GLO; Promega, Madison, WI) was aliquoted into each well. After a 5 min incubation period at room temperature, the luminescence from each well was immediately measured using a Bio-Tek Synergy HT plate reader (Winooski, VT). To determine the background levels of cAMP production, transfected CHO cells were stimulated with serum-free CHO media containing no melanocortin peptide for the 4 h incubation period, and the average background luminescence reading for each assay was subtracted from the luminescence readings of ligand-stimulated assays. All assays were performed in triplicate.

2.7. Statistical analyses

Dose response curves for each assay were fitted to the Michaelis-Menton equation to obtain half-maximal effective concentration (EC_{50}) and maximal response (V_{max}) values. Statistical analysis of the EC_{50} and V_{max} values for the dose response curves were performed using the extra-sum-of-squares *F*-test (Shaughnessy et al., 2022) in Prism 6 software (GraphPad Inc, La Jolla, CA, USA). Significance was set at $p < 0.05$. Unless otherwise noted, all data are presented as mean ± standard error of the mean with n = 3. Graphs were prepared in Prism.

3. RESULTS

3.1. Pacific spiny dogfish Mc2r

The deduced amino acid sequence of pdMc2r was aligned with esMc2r, srMc2r, and wsMc2r (Figure 1). While the N-terminal and C-terminal domains were variable in length it was possible to align the transmembrane domains and intracellular and extracellular domains by inserting a minimum number of gaps. The primary sequence identity for the four cartilaginous fish Mc2r orthologs was 36%. When both primary sequence identity (i.e., all residues the same at a given position) and primary sequence similarity (Stephenson and Freeland, 2013) were evaluated the percentage was 58%. When the analysis of primary sequence identity/similarity was done for just the elasmobranch Mc2r orthologs, the percentage was 63%. A comparison of the primary sequence identity/similarity for the various transmembrane domains (TM), intracellular domains (IC), and extracellular domains (EC) for the four cartilaginous fish Mc2r orthologs is presented in Figure 1. The various domains are rather well conserved (65% primary sequence identity/similarity or better) apart from EC1, EC2, and TM5 where the percent primary sequence identity was 33%, 0%, and 54%, respectively. When the comparison for these domains was limited to the elasmobranch sequences, the percent primary sequence identity was 50%, 25%, and 88%, respectively.

3.2. Pacific dogfish Mrap1 and Mrap2

The deduced amino acid sequence of pdMrap1 was aligned with the deduced amino acid sequences of esMrap1 and wsMrap1 (Figure 2). The primary sequence identity/similarity of the N-terminal domains of the three Mrap1 orthologs was 60%. The primary sequence identity/similarity of the transmembrane domain of the three orthologs was 63%. Currently, only a partial sequence of esMrap1 has been identified in the elephant shark genome; however, the primary sequence identity of the C-terminal domain of the two elasmobranch Mrap1 was 47%.

The deduced amino acid sequences of pdMrap2, esMrap2, and wsMrap2 are also presented in Figure 2. The N-terminal domains of the Mrap2 orthologs were aligned to the Mrap1 orthologs by first aligning the N-linked glycosylation site in each Mrap paralog and the conserved YEYY motif in each paralog, and the inserting gaps where it was appropriate. Using this approach, the remainder of the Mrap2 sequences (i.e., transmembrane domain and C-terminal domain) aligned with a minimum number of gaps inserted. Focusing on just the Mrap2 orthologs, the primary sequence identity/similarity of the N-terminal domain was 50%, whereas the primary sequence identity/similarity of the transmembrane domain and the C-terminal domain were 96% and 65%, respectively.

3.3. Pharmacological properties of pdMc2r

To determine whether pdMc2r is also an Mrap-independent Mc2r ortholog, pdMc2r was expressed alone in CHO cells and stimulated with srACTH(1-24). As shown in Figure 3A, pdMc2r was not activated at any of the concentrations of srACTH(1-24) tested. However, when pdMc2r was co-expressed with either pdMrap1 or pdMrap2 activation was achieved at physiologically relevant concentrations of the ligand (Figure 3A). While both dose response curves reached saturation with similar V_{max} values (Figure 3A), pdMc2r was nearly two orders of magnitude more sensitive to stimulation by srACTH(1-24) when co-expressed with pdMrap1 as

compared to when the receptor was co-expressed with pdMrap2 (Table 1). This difference in ligand sensitivity (i.e., EC_{50}) was statistically significant ($p < 0.001$; Table 1).

To evaluate whether pdMc2r could be activated by the non-acetylated form of αMSH, the receptor was co-expressed with either pdMrap1 or pdMrap2, and stimulated with srACTH(1-13)NH_2. As shown in Figure 3B, pdMc2r could be activated by srACTH(1-13)NH_2 when co-expressed with either Mrap paralog, and sensitivity to the ligand was enhanced nearly 10-fold when the receptor was co-expressed with pdMrap1 and compared to pdMrap2 (Table 1). Furthermore, co-expressing pdMc2r with pdMrap1 yielded a higher V_{max} relative to co-expression with pdMrap2 (Table 1). However, pdMc2r co-expressed with pdMrap1 was nearly three orders of magnitude more sensitive to stimulation by srACTH(1-24) as compared to srACTH(1-13)NH_2 (Table 1), and this difference in ligand sensitivity was statistically significant ($p < 0.001$).

Because pdMc2r could be stimulated with srACTH(1-13)NH_2, the selectivity for other MSH-related peptides was evaluated. As shown in Figure 3C, pdMc2r could be activated by γ-MSH, but the efficacy of this ligand was more than 10-fold lower than stimulation of the receptor with srACTH(1-13)NH_2; however, V_{max} values did not differ between these ligands (Table 1). The receptor only showed a weak response to β-MSH at a concentration of 10^{-6} M, and the receptor did not respond to stimulation by δ-MSH (Figure 3C).

To better understand the interaction between pdMc2r and pdMrap1 (i.e., trafficking only or trafficking and activation), pdMc2r was separately co-expressed with another elasmobranch Mrap1 ortholog (wsMrap1), or a bony vertebrate Mrap1 ortholog (bfMrap1). Co-expression of pdMc2r with pdMrap1 served as the positive control. The rationale for this experiment was that because Mrap1 orthologs have high primary sequence identity in their transmembrane domain,

and this domain facilitates trafficking of the receptor, then if the only role for Mrap1 is trafficking, the dose response curves should overlap (i.e., similar EC_{50} values). As shown in Figure 3D, the dose response curves for the receptor co-expressed with either pdMrap1 or wsMrap1 had similar EC_{50} values that were not statistically different, but with significantly different V_{max} (Table 1). However, when the receptor was co-expressed with bfMrap1, there was a decrease in ligand sensitivity of over two orders of magnitude, but an equivalent V_{max} (Table 1).

4. DISCUSSION

This study sought to characterize the pharmacological properties of the Mc2r ortholog of the Squalomorph shark, *S. suckleyi*, and the interactions of pdMc2r with its cognate Mrap paralogs, pdMrap1 and pdMrap2. With this study, representatives of all three major taxonomic groups of elasmobranchs (i.e., Squalomorphii, Galeomorphii, and Batoidea), have been analyzed and the pharmacological properties of elasmobranch Mc2r orthologs are strikingly similar from a functional perspective (Figure 4). In the case of the Pacific spiny dogfish, activation of pdMc2r expressed in CHO cells was only observed at physiologically relevant concentrations of srACTH(1-24) when the receptor was co-expressed with either pdMrap1 or pdMrap2. Similar results were observed for wsMc2r (Galeomorph; Hoglin et al., 2020b) and srMc2r (Batoid; Dores et al., 2018), and this enhancement in activation in the presence of an Mrap paralog has been attributed to increased trafficking of the Mc2r ortholog from the ER to the plasma membrane (Hoglin et al., 2020b). However, it appears that sensitivity to stimulation by srACTH(1-24) is enhanced nearly two orders of magnitude when pdMc2r is co-expressed with pdMrap1 as compared to pdMrap2. In addition, pdMc2r can be activated by either srACTH(1-24) or srACTH(1-13)NH_2 at physiologically relevant concentrations of the ligand; although,

pdMc2r is more sensitive to stimulation by srACTH(1-24) than srACTH(1-13)NH_2 by nearly three orders of magnitude when the receptor is co-expressed with pdMrap1. Both wsMc2r and srMc2r can also be active by srACTH(1-13)NH_2 and, like pdMc2r, the other elasmobranch Mc2r have a higher sensitivity for ACTH than for ACTH(1-13)NH_2 (Dores et al., 2018; Hoglin et al., 2020b).

A comparison of the pharmacological properties of the elasmobranch Mc2r orthologs with the Mc2r ortholog of the holocephalon, *C. milii* reveals some similarities among cartilaginous fish Mc2r orthologs, and some features unique to the elephant shark (Figure 4). For example, esMc2r can be activated by either ACTH or ACTH(1-13)NH_2; however, the two ligands are equipotent (Barney et al., 2019). In addition, esMc2r is Mrap independent (Reinick et al., 2012) and can move to the plasma membrane in the absence of interactions with either esMrap1 or esMrap2 (Barney et al., 2019). Given these observations, a closer inspection of the primary sequences of esMc2r and the elasmobranch Mc2rs was warranted.

The level of primary sequence conservation observed among the cartilaginous fish Mc2r orthologs is typical for vertebrate Mc2r orthologs (Dores, 2016; Wong and Dores, 2022), and the primary sequence identity/similarity of the four cartilaginous fish Mc2r orthologs was 58%. Most of the domains of the four elasmobranch Mc2r orthologs have at least 65% primary sequence identity/similarity with the exception of domains EC1, EC2, and TM5. The role of domain EC1 in melanocortin receptors is unclear. For example, substitution at this domain did not block the activation of human MC2R (Davis et al., 2022). However, domains EC2 and TM5 have been implicated in the activation and trafficking of human MC2R (Chen et al., 2007; Chung et al., 2008; Davis et al., 2022). Of note, the EC2 domain of cartilaginous fish Mc2r orthologs is small and not conserved. It would also appear that the TM5 domain of cartilaginous fish Mc2r

orthologs has a rather low primary sequence identity/similarity (i.e., 54%). However, when the comparison is limited to just the elasmobranch orthologs, the primary sequence identity/similarity is 88%. In addition, all three elasmobranch orthologs have a F residue in TM5 (i.e., wsMc2r – F^{202}; srMc2r – F^{216}; pdMc2r – F^{202}) that can be aligned. A F reside in human MC2R at a corresponding position in TM5 has been implicated in the trafficking of the human ortholog (Davis et al., 2022). Note that esMc2r has a L residue at this position. In addition, L^{187} in esMc2r is in a region of TM5 that differs substantially from the corresponding region in the elasmobranch Mc2r orthologs and could account for the differences in trafficking properties between esMc2r (i.e., no requirement for an interaction with an Mrap) and wsMc2r, srMc2r, and pdMc2r (a requirement for an interaction with an Mrap).

The trafficking of vertebrate Mc2r orthologs is dependent on interaction with the transmembrane domain of the Mraps (Hinkle and Sebag, 2009; Webb and Clark, 2010). The cartilaginous fish Mrap paralogs that have been detected demonstrate reasonably high sequence identity/similarity in the N-terminal and transmembrane domains. In addition, the transmembrane domain of cartilaginous fish Mraps have high primary sequence identity/similarity with the transmembrane domain of osteichthyan Mrap orthologs (Dores et al., 2022), which underscores the role of this domain in trafficking. The N-terminal domain of the cartilaginous Mraps has an N-linked glycosylation site and the YEYY motif, usually found in osteichthyan Mrap paralogs, but lacks the δDYδ (where δ represents hydrophobic amino acids) motif found in osteichthyan Mrap1 orthologs that is required for the activation of osteichthyan Mc2r orthologs following an ACTH binding event (Dores and Chapa, 2021; Hinkle and Sebag, 2009; Webb and Clark, 2010). Not surprisingly, co-expression of osteichthyan Mc2r orthologs

with cartilaginous fish Mrap1 orthologs does not result in activation of the osteichthyan receptors (Dores et al., 2022; Shaughnessy et al., 2022).

Cartilaginous fish Mrap2 orthologs also lack the δDYδ activation motif that is absent in the osteichthyan Mrap2 paralogs (Rouault et al., 2017). However, the cartilaginous fish Mrap2 orthologs have a remarkably high level of primary sequence conservation in their C-terminal domain. Similarly, osteichthyan vertebrate Mrap2 orthologs have considerable sequence identity/similarity in their C-terminal domain (Liang et al., 2011), which suggests that the C-terminal domain of Mrap2 is evolutionarily and functionally significant (Rouault et al., 2017). For instance, Mrap2 is hypothesized to play a role in regulating energy metabolism through its interactions with the Mc4r (Rouault et al., 2017), as has been demonstrated in zebrafish (*Danio rerio*; Sebag et al., 2013).

5. CONCLUSIONS

Phylogenetically ancient fishes are excellent models for studying the functional evolution of the HPA/I axis (Bouyoucos et al., 2021). As current research effort begins to describe a general role for Mraps in the functioning of elasmobranch Mc2r, future research effort that tests the relationship between elasmobranch Mc2r/Mrap structure and function is warranted. As the present study has accomplished regarding elasmobranchs, it will also be informative to more fully describe the function of the Mc2r/Mrap system in holocephalans, such as the small-eyed rabbitfish (*Hydrolagus affinis*), for which the appropriate genomic resources exist (Fonseca et al., 2020). Pharmacological studies of vertebrate Mc2rs have now (*c.* 2023) described functional properties of Mc2r/Mrap1 in representative gnathostomes spanning all major vertebrate taxa (Dores et al., 2022), except for the lobe-finned fishes (i.e., coelacanth and lungfishes). A 'final frontier' for understanding the functional evolution of the HPA/I axis and, specifically, the

evolution of Mcr/Mrap interactions will be the agnathans (i.e., hagfishes and lampreys; Haitina et al., 2007).

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AUTHOR CONTRIBUTIONS

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DECLARATION OF COMPETING INTEREST

No competing interests declared.

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FIGURE CAPTIONS

Fig. 1. Alignments of cartilaginous fish melanocortin-2 receptor (Mc2r). The deduced amino acids of Pacific spiny dogfish (*Squalus suckleyi*; pd) Mc2r, whale shark (*Rhincodon typus*; ws) Mc2r (accession number XP_020380838), elephant shark (*Callorhinchus milii*; es) Mc2r (FAA00704), and red stingray (*Hemitrygon akajei*; sr) Mc2r (LC108747) were aligned following the protocol outlined in Dores et al. (1996). The labeling of domains within the GPCRs was done using the DeepTMHMM tool (https://dtu.biolib.com/DeepTMHMM). Sequence identity was determined using the program BLOSUM (https://www.ncbi.nlm.nih.gov/Class/FieldGuide/BLOSUM62.txt). Positions with primary sequence identity are highlighted in dark blue. Positions with primary sequence similarity are highlighted in gray. Positions with primary identity/similarity for only the elasmobranch sequences are highlighted in light green. Regions with percent sequence identity/similarity below 65% are highlighted in red. Abbreviations: transmembrane domain, TM; intracellular domain, IC; extracellular domain, EC.

Fig. 2. Alignments of cartilaginous fish melanocortin-2 receptor accessory proteins, Mrap1 and Mrap2 orthologs. The deduced amino acid sequences Pacific spiny dogfish (*Squalus suckleyi*; pd) Mrap1, Mrap2, whale shark (*Rhincodon typus*; ws) Mrap1 (XP_020375601), Mrap2 (XP_020377388), and elephant shark (*Callorhinchus millii*; es) Mrap1 (XM_007903550.1), and Mrap2 (XP_007906624.1) were aligned following the protocol outlined in Dores et al. (1996). The labeling of domains within the Mraps was done using the DeepTMHMM tool (https://dtu.biolib.com/DeepTMHMM). The sequence identity/sequence similarity was determined using the program BLOSUM (https://www.ncbi.nlm.nih.gov/Class/FieldGuide/BLOSUM62.txt). For the Mrap1 sequences

positions with primary sequence identity are heighted in dark blue. Positions with primary sequence similarity are highlighted in yellow. The sequence of the C-terminal domain of esMrap1 is not complete. Residues in the C-terminal of pdMrap1 and wsMrap2 that are identical are highlighted in bold black.

Fig. 3. Pharmacological analysis of Pacific spiny dogfish (*Squalus suckleyi*; pd) melanocortin-2 receptor (Mc2r) using a cAMP reporter gene assay. A) To determine whether pdMc2r can be activated without co-expression with a Mc2r accessory protein (Mrap), pdMc2r was expressed alone in Chinese hamster ovary (CHO) cells and the transfected cells were stimulated with red stingray (*Hemitrygon akajei*; sr) adrenocorticotropic hormone (ACTH). In parallel, pdMc2r was co-expressed with either pdMrap1 or pdMrap2 and separately stimulated with srACTH(1-24). B) To determine whether pdMc2r could be activated by srACTH(1-13)NH_2, pdMc2r was either expressed alone, co-expressed with pdMrap1, or co-expressed with pdMrap2 and the respective transfected CHO cells were stimulated with srACTH(1-13)NH_2. C) To determine whether pdMc2r could be activated by other melanocyte-stimulating hormone (MSH)-related ligands, CHO cells were transfected with pdMc2r and pdMrap1 and stimulated with either srACTH(1-13)NH_2, β-MSH, γ-MSH, or δ-MSH. D) To test the efficacy of activation by other Mrap1 orthologs, pdMc2r was either co-expressed with pdMrap1, whale shark (*Rhincodon typus*; ws) Mrap1, or bowfin (*Amia calva*; bf) Mrap1, and the transfected CHO cells were stimulated with srACTH(1-24).

Fig. 4. Phylogeny of cartilaginous and bony fish melanocortin-2 receptor (Mc2r) and accessory protein 1 (Mrap1) function. Mc2r is portrayed in gray as a seven transmembrane domain protein. Mrap1 is portrayed in red by two peptides forming a homodimer in reverse orientation. Mrap1 drawn in contact with Mc2r denotes a dependence on Mrap1 for trafficking Mc2r to the plasma

membrane. Mrap1 drawn with a blue activation motif represents Mc2r that require Mrap1 for activation by ACTH. Elasmobranch pro-opiomelanocortin is drawn including color-coded adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormones. Melanocortin ligands that have been shown to activate Mc2r in different taxa are drawn above Mc2r and Mrap1. All species of cartilaginous fish that have been tested to date (c. 2023) are shown. Note that whale shark Mc2r and red stingray Mc2r have not been tested with β-, γ-, or δ-MSH; dogfish Mc2r was not activated by δ-MSH. References are denoted by superscript numbers: [1]Present study; [2]Reinick et al. 2012; [3]Takahashi et al. 2016; [4]Dores et al. 2018; [5]Barney et al. 2019; [6]Hoglin et al. 2020a; [7]Hoglin et al. 2020b. Branch lengths are not drawn to scale.

603 **TABLES**

604 **Table 1.** Half-maximal effective concentration (EC_{50}) and maximal response (V_{max}) values (means ± standard error of the mean) and statistical
605 comparisons for reporter gene assays of Pacific spiny dogfish (*Squalus suckleyi*) melanocortin-2 receptor (Mc2r) co-expressed with accessory
606 proteins (Mraps). Statistical comparisons were made with extra-sum-of-squares *F*-tests. Abbreviations: adrenocorticotropic hormone, ACTH;
607 bowfin, bf; Mc2r accessory protein 1, Mrap1; Mc2r accessory protein 2, Mrap2; melanocyte-stimulating hormone, MSH; Pacific spiny dogfish,
608 pd; stingray, sr; whale shark, ws

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Assay	Mrap	Ligand [range] (M)	EC_{50} (M)	V_{max}	Statistical comparisons (EC_{50})	Statistical comparisons (V_{max})
1 (Fig. 3A)	-	srACTH(1-24) $[10^{-13}$-$10^{-7}]$	NA	NA	pdMrap1 vs pdMrap2 $F_{1, 36} = 80.85$	pdMrap1 vs pdMrap2 $F_{1, 36} = 1.53$
	pdMrap1	srACTH(1-24) $[10^{-13}$-$10^{-7}]$	$3.3 \times 10^{-11} \pm 1.4 \times 10^{-11}$	$6.9 \times 10^{4} \pm 3.8 \times 10^{3}$	$p < 0.001$	$p = 0.225$
	pdMrap2	srACTH(1-24) $[10^{-13}$-$10^{-7}]$	$2.6 \times 10^{-9} \pm 7.9 \times 10^{-10}$	$7.5 \times 10^{4} \pm 1.6 \times 10^{3}$		
2 (Fig. 3B)	-	srACTH(1-13)NH_2 $[10^{-12}$-$10^{-6}]$	NA	NA	pdMrap1 vs pdMrap2 $F_{1, 36} = 63.78$	pdMrap1 vs pdMrap2 $F_{1, 36} = 8.33$
	pdMrap1	srACTH(1-13)NH_2 $[10^{-12}$-$10^{-6}]$	$3.9 \times 10^{-8} \pm 5.4 \times 10^{-9}$	$3.6 \times 10^{4} \pm 1.1 \times 10^{3}$	$p < 0.001$	$p = 0.007$
	pdMrap2	srACTH(1-13)NH_2 $[10^{-12}$-$10^{-6}]$	$1.8 \times 10^{-7} \pm 1.9 \times 10^{-8}$	$3.1 \times 10^{4} \pm 0.9 \times 10^{3}$		
3 (Fig. 3C)	pdMrap1	srACTH(1-13)NH_2 $[10^{-12}$-$10^{-6}]$	$2.5 \times 10^{-8} \pm 6.4 \times 10^{-9}$	$7.5 \times 10^{4} \pm 4.0 \times 10^{3}$	srACTH(1-13)NH_2 vs γ-MSH $F_{1, 36} = 57.35$	srACTH(1-13)NH_2 vs γ-MSH $F_{1, 36} = 0.27$
	pdMrap1	β-MSH $[10^{-12}$-$10^{-6}]$	NA	NA	$p < 0.001$	$p = 0.607$
	pdMrap1	γ-MSH $[10^{-12}$-$10^{-6}]$	$3.6 \times 10^{-7} \pm 5.5 \times 10^{-8}$	$8.0 \times 10^{4} \pm 4.1 \times 10^{3}$		
	pdMrap1	δ-MSH $[10^{-12}$-$10^{-6}]$	NA	NA		
4 (Fig. 3D)	pdMrap1	srACTH(1-24) $[10^{-13}$-$10^{-7}]$	$6.5 \times 10^{-11} \pm 2.3 \times 10^{-11}$	$1.6 \times 10^{4} \pm 0.8 \times 10^{3}$	All comparisons $F_{2, 54} = 39.74$	All comparisons $F_{2, 54} = 15.14$
	wsMrap1	srACTH(1-24) $[10^{-13}$-$10^{-7}]$	$1.6 \times 10^{-10} \pm 5.6 \times 10^{-11}$	$1.0 \times 10^{4} \pm 0.5 \times 10^{3}$	$p < 0.001$ pdMrap1 vs wsMrap1	$p < 0.001$ pdMrap1 vs wsMrap1
	bfMrap1	srACTH(1-24) $[10^{-13}$-$10^{-7}]$	$4.5 \times 10^{-9} \pm 1.7 \times 10^{-9}$	$1.9 \times 10^{4} \pm 1.6 \times 10^{3}$	$F_{1, 36} = 2.73$ $p = 0.107$	$F_{1, 36} = 15.14$ $p < 0.001$
					pdMrap1 vs bfMrap1 $F_{1, 36} = 59.70$ $p < 0.001$	pdMrap1 vs bfMrap1 $F_{1, 36} = 1.74$ $p = 0.196$
					wsMrap1 vs bfMrap1 $F_{1, 36} = 27.55$ $p < 0.001$	wsMrap1 vs bfMrap1 $F_{1, 36} = 28.00$ $p < 0.001$

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