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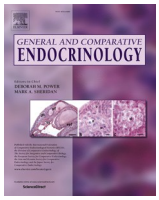

Research paper

Promiscuous melanocortin receptors in hagfish indicate that receptor function preceded peptide specialization☆

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ABSTRACT

Hagfishes are representatives of the phylogenetically important, early-branching vertebrate lineage Agnatha, and many endocrine signaling systems in this group remain poorly understood. In this study, we provide the first molecular and functional characterization of melanocortin receptors (Mcrs) and their accessory protein (Mrap) in hagfishes. Using genomic and transcriptomic resources from inshore hagfish (*Eptatretus burgeri*), Pacific hagfish (*Eptatretus stoutii*), and Atlantic hagfish (*Myxine glutinosa*), we identified genes encoding two hagfish melanocortin receptors, Mcar and Mcbr, and a single Mrap. When expressed in mammalian cells, both receptors responded to human ACTH(1–24) and α-MSH with similar affinities. Co-expression with Mrap reduced maximal activity of Mcbr but not Mcar and only modestly modulated the ligand sensitivity of either receptor. Gene expression analyses revealed that *mcbr* and *mrap* are prominently expressed in the slime gland, a tissue that also transcriptionally expressed the steroidogenic enzymes *star* and *cyp11a1*, whereas *mcar* is most prominently expressed in the brain. These findings suggest that hagfish Mcrs retain broad ligand responsiveness and relative Mrap-independence, consistent with a hypothesized ancestral mode of melanocortin signaling. Although no genes encoding known melanocortin prohormones have yet been identified in hagfish genomes, the presence of functional receptors and tissue-specific expression patterns suggest these genes may have physiological roles and that an as-yet-unidentified ligand may exist in hagfish. Together, these results provide new insight into the organization and evolution of the vertebrate melanocortin system and highlight hagfish as a key model for reconstructing the functional evolution of this essential endocrine signaling pathway.

1. Introduction

The hypothalamus-pituitary-interrenal (HPI) axis in fishes and non-amniote tetrapods and the homologous hypothalamus–pituitary–adrenal (HPA) axis in amniote tetrapods are conserved neuroendocrine systems that regulate stress responses, metabolism, and various aspects of physiological homeostasis in vertebrates (Denver, 2009). In this axis, psychological or environmental stressors stimulate the hypothalamus to release corticotropin-releasing hormone (Crh), which in turn promotes secretion of adrenocorticotropic hormone (Acth) from the anterior pituitary. Acth acts on the interrenal or adrenal cortex to stimulate the biosynthesis and release of corticosteroids such as cortisol or corticosterone (Best et al., 2024; Mommsen et al., 1999).

A particular focus of the present work is the melanocortin receptors (Mcrs), a subfamily of Class A G protein-coupled receptors (GPCRs) that includes five paralogous subtypes in gnathostomes (Mc1r–Mc5r), each with distinct tissue distributions and functional roles (Cone, 2006). These receptors are activated by peptides derived from the proopiomelanocortin (Pomc) precursor, including Acth and various melanocyte-stimulating hormones such as α-Msh, the cleavage product produced by the first 13 residues of Acth. Although melanocortins are the canonical ligands, some mammalian melanocortin receptors can also be activated by non-melanocortin peptides such as defensins, linking melanocortin signaling components to immune system processes (Candille et al.,

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2007). Among the five subtypes of melanocortin receptors, Mc2r is uniquely selective for Acth (Mountjoy et al., 1992) and initiates activation of cellular signaling pathways that result in corticosteroid biosynthesis in the interrenal and adrenal (Best et al., 2024). Importantly, Mc2r function in osteichthyans (i.e., bony vertebrates) is strictly dependent on the melanocortin 2 receptor accessory protein (Mrap1), which is necessary for trafficking of the receptor to the plasma membrane and for facilitating Acth-induced signaling (Agulleiro et al., 2013; Dores and Chapa, 2021; Hinkle and Sebag, 2009; Sebag and Hinkle, 2009; Webb and Clark, 2010). A related paralog, Mrap2, has modulatory roles on Mc4r and other Mcrs, but does not substitute for Mrap1 in supporting Mc2r function (Chan et al., 2009; Dores et al., 2014; Soletto et al., 2019).

Recent comparative studies indicate that many features of the Mcr system in osteichthyans are derived traits that evolved throughout the radiation of gnathostomes (Dores and Chapa, 2021). Like in tetrapods and teleost fishes, the Mc2rs of even the most basal osteichthyans, including the sarcopterygian fishes (i.e., lobed-fin fishes) and non-teleost actinopterygians (i.e., ray-finned fishes), exhibit strict Acth-specificity and obligate Mrap1-dependence (Dores et al., 2022; Shaughnessy et al., 2022, 2023a,b). However, in elasmobranchs, Mc2r can be activated by both Acth and Msh peptides and interacts variably with either Mrap1 or Mrap2 (Bouyoucos et al., 2023; Dores et al., 2018; Hoglin et al., 2022, 2023). In the holocephalan elephant shark (*Callorhinchus milii*), Mc2r activation is Mrap-independent (Reinick et al., 2012).

The melanocortin system of the agnathans (i.e., jawless vertebrates), represented by the extant lampreys and hagfishes, is remarkably dissimilar to that of gnathostomes. Agnathans diverged from the lineage leading to gnathostomes over 500 million years ago (Chang et al., 2014, 2014; Gess et al., 2006; Miyashita et al., 2019). In lamprey, the melanocortin system has been previously characterized and exhibits several features distinct from gnathostomes. Notably, lampreys lack a single *pomc* gene and instead express genes for two functionally distinct prohormones: *proopiocortin* (*poc*), which encodes an Acth-like peptide, and *promelanotropin* (*pom*), which encodes two Msh-like peptides (Takahashi et al., 2006). The two identified lamprey Mcrs, Mcar and Mcbr, are activated by both Acth- and Msh-like peptides, with Mcar showing greater sensitivity to the Msh peptides (Haitina et al., 2007; Shaughnessy et al., 2025). Moreover, while lamprey Mcrs do not require Mrap for activation, co-expression with Mrap does modulate receptor activity, including suppression of maximal responses and alteration of EC_{50} values for certain ligands (Shaughnessy et al., 2025; Zhu et al., 2019). These findings support the view that the highly specialized Mc2r of osteichthyans is a derived evolutionary innovation among vertebrates.

Despite these insights from lamprey, the melanocortin signaling system from the other extant agnathan lineage, the hagfishes, has remained entirely uncharacterized. Prior to the recent release of the genome assembly of the inshore hagfish (*Eptatretus burgeri*) (Yu et al., 2024), no genes encoding melanocortin receptors, Mraps, or Pomc-like melanocortin prohormones had been identified in hagfishes. These absences have allowed a major gap to persist in attempts to resolve the origin and early evolution of the melanocortin signaling pathway in vertebrates. Here, in the wake of the recent release of the *E. burgeri* genome, we have identified genes encoding two melanocortin receptors and a melanocortin receptor accessory protein, which appear to be orthologous to the Mcar, Mcbr, and Mrap of lampreys, respectively. Intriguingly, still no orthologs of Pomc, Poc, or Pom have yet been described (Best et al., 2024; Bouyoucos et al., 2021), raising the possibility that hagfishes have either lost this gene or utilize an entirely distinct mechanism for melanocortin peptide production and signaling.

Here, we provide the first molecular and functional analysis of melanocortin receptors in hagfish. We analyzed the primary protein structures of the *E. burgeri* Mcar, Mcbr, and Mrap with respect to other vertebrate Mcrs and Mraps. Using a system of heterologous expression in mammalian cells, we examined the pharmacology of hagfish Mcrs against human ACTH(1–24) and α-MSH peptides, two ligands known to activate lamprey Mcrs (Haitina et al., 2007), as well as their modulation by hagfish Mrap and their association with G proteins. In parallel, to provide preliminary insight into potential physiological roles of these signaling components, we examined the tissue-specific gene expression of melanocortin receptors and steroidogenic enzymes in the Pacific hagfish (*Eptatretus stoutii*). Through these experiments, we aimed to examine whether the unique properties of the lamprey Mcrs and Mrap relative to their gnathostome counterparts are conserved across agnathans or reflective of lamprey-specific specialization. Finally, we address the 'elephant in the room': how to explain the presence and function of the hagfish Mcrs and Mrap considering the apparent absence of a *pomc*-like gene in the hagfish genome to encode melanocortin ligands.

2. MATERIALS & METHODS

2.1. Sequences and molecular phylogenetic analyses

Nucleotide and corresponding primary amino acid sequences for hagfish *mcar*, *mcbr*, and *mrap* were obtained from the newly updated genome assembly (GCA_900186335.3) for *E. burgeri* deposited to the National Center for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm.nih.gov). The amino acid sequences for hagfish Mcar, Mcbr, and Mrap were aligned with corresponding sequences from sea lamprey (*Petromyzon marinus*) and/or elephant shark (*Callorhinchus milii*) using a method of conserving the alignment of functional amino acid motifs (Dores et al., 1996). Transmembrane (TM) domains were predicted using the DeepTMHMM tool (https://www.bioinformatics.dtu.dk).

To further confirm the identity of the hagfish melanocortin receptors relative to the lamprey counterparts, we analyzed the syntenic relationships of the genomic regions flanking the hagfish *mcar* and *mcbr* loci. Included in these analyses were two hagfish species, inshore hagfish and Atlantic hagfish (*Myxine glutinosa*; GenBank: GCA_040869285.1), and three lamprey species, sea lamprey (GCA_048934315.1), far eastern brook lamprey (*Lethenteron reissneri*; GCA_015708825.1), and European river lamprey (*Lampetra fluviatilis*; GCA_964198595.1). The NCBI Genome Data Viewer was used to identify neighboring genes to the putative melanocortin receptor loci; the inshore hagfish assembly (Eburgeri_3.2) accessed through Ensembl (https://www.ensembl.org) was used to identify neighboring genes to the putative melanocortin receptor loci in this species. Gene annotation files containing gene identifiers and chromosomal or contig coordinates were downloaded and organized manually. Raw gene tables were cleaned to remove duplicate entries and non-coding RNAs. Predicted genes that lacked annotation in the reference assemblies were manually annotated by identifying open reading frames and primary protein structure with NCBI ORFfinder and confirming putative gene identity based on sequence similarity using consensus results from NCBI BLAST analyses.

For multiple sequence alignment and phylogenetic analyses, primary amino acid sequences of agnathan Mcar, Mcbr, and Mrap and gnathostome Mc1r, Mc2r, Mc3r, Mc4r, Mc5r, Mrap1, and Mrap2 were obtained from NCBI GenBank (see accession numbers in **Supplementary File 1**). Multiple sequence alignment was performed via the MUSCLE alignment tool and phylogeny was inferred in MEGA12 software (Tamura et al., 2021) using Maximum Likelihood with the Jones-Taylor-Thornton substitution model, incorporating a discrete Gamma distribution, allowing for invariant sites, and using partial deletion to remove sites with less than 95% coverage. Branch support was assessed with 1,000 bootstrap replicates.

2.2. Pharmacological analyses

The nucleotide sequences for inshore hagfish *mcar*, *mcbr*, and *mrap* were individually inserted into pcDNA3 + expression vectors (GenScript; Piscataway, NJ), along with a cAMP reporter gene construct,

which was comprised of a *firefly luciferase* gene promoted by a cAMP-response element (*cre-luciferase*) (Chepurny and Holz, 2007). In one experiment, we also compared the receptor-mediated luciferase activity using a cAMP-responsive reporter (CRE-luciferase) to that using a Ca^{2+}-responsive reporter (Nuclear Factor of Activated T-cells, NFAT-luciferase). Human ACTH and α-MSH (ACTH(1–24) and α-MSH, respectively) were commercially obtained (Sigma-Aldrich Inc., St. Louis, MO).

Chinese hamster ovary (CHO) cells (ATCC, Manassas, VA) were cultured in Kaighn's modification of Ham's F12 media (ATCC) made complete by supplementing with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL normacin. Cells were maintained in a humidified incubator with 95% air and 5% CO_2 at 37 °C. CHO cells were selected for this project because this cell line does not express endogenous melanocortin receptors (Noon et al., 2002; Sebag and Hinkle, 2007) or Mraps (Reinick et al., 2012).

For the reporter gene assay, cDNA constructs of hagfish *mcar* and *mcbr* were either expressed alone or with hagfish *mrap*, and all transfected cells were co-expressed in CHO cells with the *cre-luciferase* reporter gene. Transfections were performed using the Amaxa Nucleofector 2b System (Lonza, Switzerland) with the Solution T transfection kit (Lonza Bioscience, Rockland, ME). Approximately 2 μg of cDNA per 1×10^5 cells was used for each transfection, with a 1:3 M ratio of Mcr:Mrap. The transfected cells were plated in an opaque 96-well plate (Costar 3917, Corning Inc., Kennebunk, ME) at a final density of 1×10^5 cells per well. After 48 h post-transfection incubation, the transfected CHO cells were stimulated with a range of concentrations of melanocortin ligands (from 10^{-12} M to 10^{-6} M) made up in serum-free CHO media, along with the inclusion of a ligand-free control. Each concentration of ligand (and the ligand-free control) was replicated in triplicate. Following a 4 h incubation period, the stimulating media was removed and a luciferase substrate reagent (BrightGLO, Promega, Madison, WI) was added following the manufacturer protocols. After a 5 min incubation with the luciferase substrate, luminescence from each well was quantified using a BioTek Synergy HT plate reader (Winooski, VT). To account for any background levels of cAMP production, the average luminescence reading for the ligand-free control wells for each assay was subtracted from the luminescence readings for the ligand-containing wells.

2.3. Gene expression analyses

Pacific hagfish (45 ± 2 cm; 103 ± 16 g) were collected via trapping from Barkley Sound, Bamfield, BC, Canada in July 2023 as permitted by a license from the Department of Fisheries and Oceans (Permit No. XR 264 2023). All procedures were approved in advance by the Bamfield Marine Science Centre (BMSC) Animal Care Committee (Protocol No. RS-22–10) and carried out in accordance with the guidelines of the Canadian Council for Animal Care. Hagfish were kept in 5,000 L flow-through holding tanks under natural conditions (~30–35 ‰, ~12–14 °C; natural photoperiod) with a low-translucence cover to darken the tanks. After 2 weeks acclimation to holding conditions, five hagfish ($n = 5$) were euthanized using an overdose of tricaine methanesulfonate (MS-222; 5 g L^{-1}) and sampled for tissues: brain, pituitary, gill, heart, liver, three regions (1, anterior-most; 2, middle; 3, posterior-most) of the kidney and hindgut, gonad, muscle, skin, and slime gland. Samples were briefly equilibrated to RNAlater at room temperature, then flash-frozen in liquid nitrogen, then stored in −80 °C prior to RNA extraction.

Protocols for RNA extraction, cDNA synthesis, and quantitative PCR closely followed previously described methods (Shaughnessy et al., 2025). Frozen tissue was processed for extraction of total RNA using the TRIzol method (Molecular Research Center Inc., Cincinnati, OH, USA), following the manufacturer protocols. First-strand cDNA synthesis was performed using a commercial reverse transcriptase kit (Applied Biosystems Inc.) following the manufacturer protocols. Quantitative PCR analyses were conducted in 10 μL reactions, each containing 4 ng of cDNA, 150 nM of both forward and reverse primers, and 2X SYBRselect master mix (Applied Biosystems Inc.). The reaction cycle was carried out using a QuantStudio™ 3 Real-Time PCR System with the following protocol: 2 min at 50 °C, 2 min at 95 °C for holding and activation, followed by 40 cycles of 15 sec at 95 °C, 1 min at 60 °C, and 30 sec at 72 °C. After cycling, a thermal ramp from 60 to 95 °C was carried out as a melt curve analysis to confirm the presence of a single product in each reaction.

Relative mRNA abundance was calculated using the $2^{-\Delta CT}$ method, with elongation factor 1 alpha (*ef1a*) serving as reference genes (Pfaffl, 2001). The details of the PCR primers, reactions, and products for the assays for *ef1a*, *mcar*, *mcbr*, *mrap*, *star*, and *cyp11a1* assays are presented in **Supplementary File 2**.

2.4. Statistical analysis

The dose–response curves for receptor activation were fitted with a three-parameter polynomial model by plotting the concentration of the ligand (log-normalized) against luminescence reading. Values for half-maximal effective concentration (EC_{50}) and maximal response (R_{max}) were obtained from the fitted curves and compared between groups using the extra-sum-of-squares F test, with a significance level of $\alpha = 0.05$. All statistical analyses and figure preparation were performed in Prism 9 software (GraphPad Inc., La Jolla, CA). Results are presented as mean ± standard error (n values are indicated in figure legends).

3. Results

3.1. Sequences and molecular phylogenetic analyses

The hagfish Mcar was 49% identical to the sea lamprey Mcar and contained seven predicted TM domains that aligned closely with the predicted TM domains of the sea lamprey Mcar (Fig. 1**A**). Outside of minor insertions in the amino and carboxyl terminal ends, only one other insertion was needed, a three-residue insertion in intracellular loop 3 (IC3). Several residues associated with melanocortin receptor activation were conserved in hagfish Mcar, including E^{94} in TM2, D^{117} and D^{121} near or in TM3, and W^{259}, F^{261} and H^{263} in TM6 (Baron et al., 2009). The D-R-Y motif (E^{141}-R^{142}-Y^{143}) was present in Intracellular Loop 2 (IC2), a feature required for phosphorylation (Gallo-Payet and Battista, 2014). Also present in the hagfish Mcar were the conserved cysteines in the N-terminal domain (C^{35}) and Extracellular Loop 3 (EC3) (C^{278} or C^{280}), which are involved in forming a disulfide bridge to draw the N-terminal region into proximity with TM7 and supporting the barrel-like receptor configuration characteristic of all melanocortin receptors (Yang et al., 2011). The deduced amino acid sequence of the putative hagfish Mcbr shared sequence identity of 55% with the sea lamprey Mcbr (Fig. 1**B**) and exhibited similar hallmark features of melanocortin receptors, as described above for Mcar.

Comparative synteny analysis of agnathan *mcar* and *mcbr* confirmed the identity of the hagfish receptors in relation to the lamprey receptors. There was strong internal synteny around the *mcar* and *mcbr* loci within the agnathan lineages and weaker synteny around these loci between agnathan lineages (Fig. 2**A-B**). Within hagfishes, the *mcar* locus shared identity and order among at least 10 of the nearest neighboring genes; the *mcbr* locus, although less conserved, still showed moderate synteny, sharing identity and order among the 5 nearest neighboring genes. Within lampreys, both the *mcar* and *mcbr* loci shared identity and order among at least 20 of the nearest neighboring genes. For the *mcar* locus, only weak synteny was observed between hagfishes and lampreys; only a single gene out of ~ 20 neighboring genes around the hagfish and lamprey *mcar* loci shared identity (Fig. 2**A**). For the *mcbr* locus, moderate synteny was observed between hagfishes and lampreys; 5 genes out of ~ 20 neighboring genes around the hagfish and lamprey *mcbr* loci shared identity with some rearrangement in order (Fig. 2**B**).

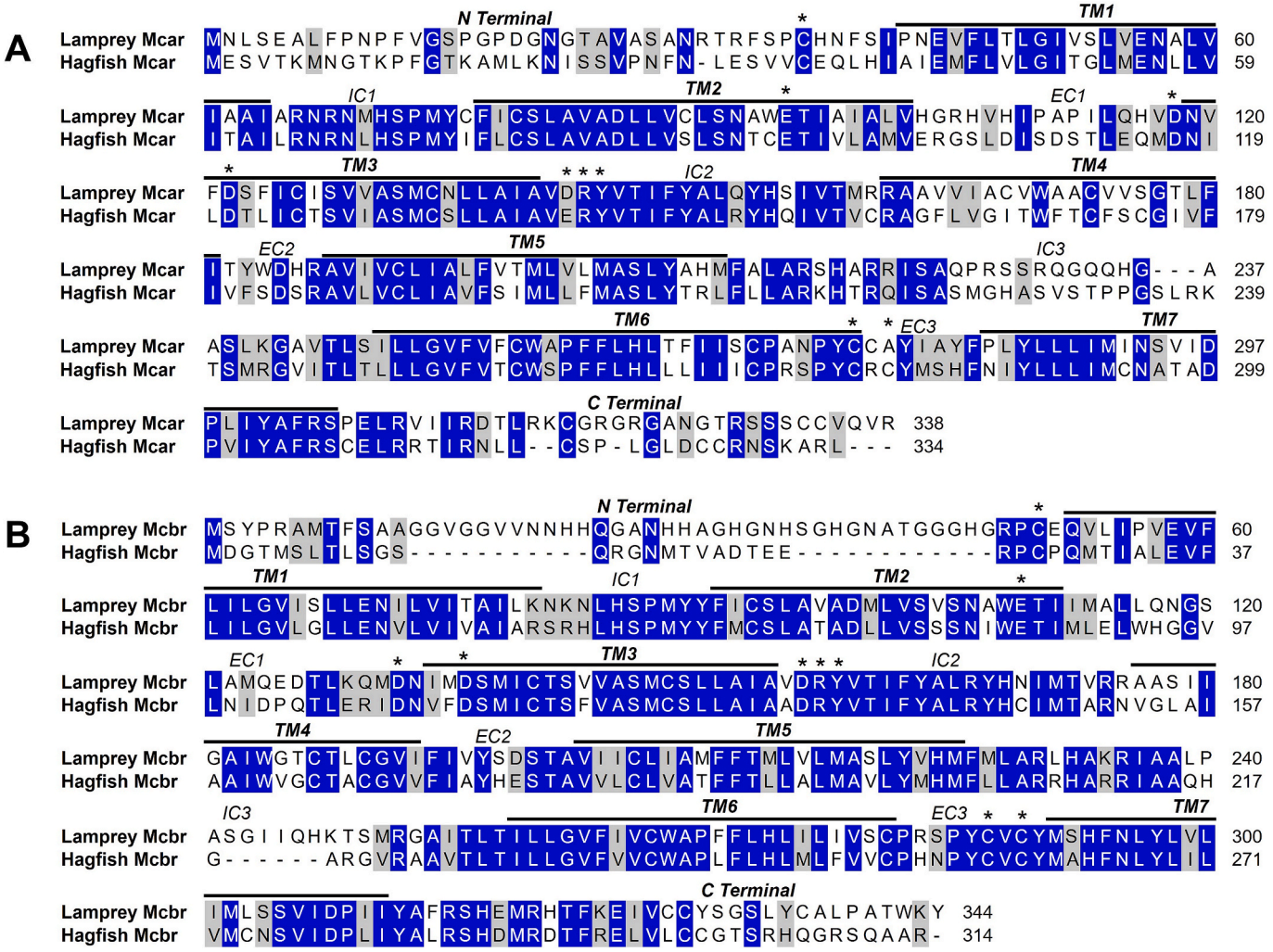

Fig. 1. Primary sequence alignment of agnathan melanocortin receptors. The deduced amino acid sequences of Mcar (A) and Mcbr (B) from inshore hagfish (*Eptatretus burgeri*) and sea lamprey (*Petromyzon marinus*) are aligned. Identical residues are highlighted in blue; similar sequences are indicated in grey. Asterisks indicate functionally important residues discussed in text. See text for sequence accession numbers. Abbreviations: EC, extracellular loop; IC, intracellular loop; TM, transmembrane domain.

The deduced amino acid sequences of the hagfish Mcars and Mcbrs shared a sequence identity with each other of approximately 52% (Fig. 3; **Supplementary File 3**). The hagfish Mcars shared a 50% sequence identity with lamprey Mcars, a 58% identity with lamprey Mcbrs, and a mean sequence identity of 49% with all other gnathostome Mcrs. The hagfish Mcbrs shared a 54% sequence identity with lamprey Mcars, a 61% identity with lamprey Mcbrs, and a mean sequence identity of 51% with all other gnathostome Mcrs. Generally, both the Mcars and Mcbrs of agnathans shared lower sequence identities (35–50%) with gnathostome Mc1rs and Mc2rs and higher sequence identities (50–65%) with gnathostome Mc3rs, Mc4rs, and Mc5rs (Fig. 3; **Supplementary File 3**).

As expected, in our phylogenetic analysis of vertebrate melanocortin receptors, gnathostome Mc1r, Mc2r, Mc3r, Mc4r, and Mc5r all formed distinct clades with strong branch support (>95% bootstrap support) (Fig. 4). Mc2r (and Mc1r to a lesser extent) exhibited large within-clade divergence, whereas Mc3r, Mc4r, and Mc5r exhibited very limited within-clade divergence, reflective of the different rates of sequence divergence among the melanocortin receptor genes (Shaughnessy et al., 2023a; Wong and Dores, 2022). In contrast, lamprey and hagfish Mcar and Mcbr sequences placed near the base of the radiation of melanocortin receptors, and the precise arrangement of these relationships was not well supported (<50% bootstrap support) (Fig. 4).

The deduced amino acid sequence of the hagfish Mrap was aligned with the sea lamprey Mrap and lungfish Mrap1 and Mrap2. Due to amino acid repeats and insertions in the N-terminal domain of the sea lamprey Mrap, alignment required gap insertions in the corresponding region of the hagfish and lungfish sequences (Fig. 5). Conserved motifs typical of Mrap proteins (Hinkle and Sebag, 2009) were also present in the hagfish Mrap, including the N-linked glycosylation site (N^{18}-X^{19}-T/S^{20}) and the Y^{25}-E^{26}-Y^{27}-Y^{28} motif in the N terminal domain, the reverse topology (RT) motif, and the single TM domain. The hagfish Mrap, like the lamprey Mrap and the gnathostome Mrap2s, is missing the δ-D-Y- δ activation motif observed in gnathastome Mrap1s directly following the Y-E-Y-Y motif in the N terminal domain. Numerous point mutations were observed in the RT and TM motifs of the hagfish Mrap relative to the lamprey and lungfish sequences. That the lamprey and lungfish sequences demonstrate high sequence conservation within these RT and TM domains indicates the hagfish Mrap uniquely exhibits divergence in these functionally important regions of Mrap.

In our phylogenic analysis of vertebrate Mraps, gnathostome Mrap1s and Mrap2s formed distinct clades (Fig. 6). The lamprey Mraps grouped together and were not clearly within either Mrap clade, radiating from a position basal to both gnathostome Mrap clades. The hagfish Mraps also grouped together, but radiating from a position between the chondrichthyan and osteichthyans Mrap1.

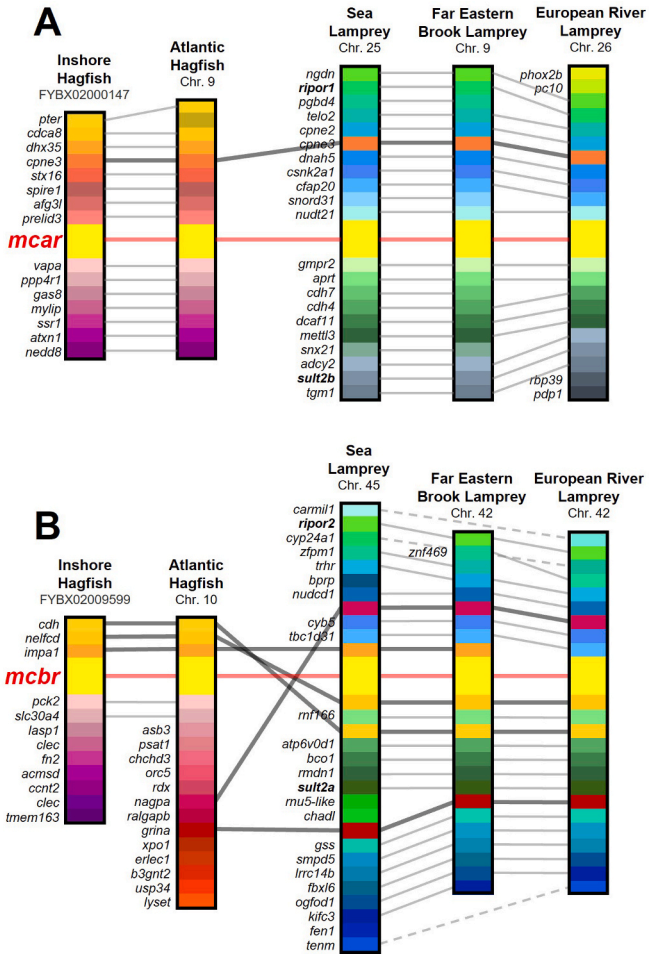

Fig. 2. Conserved chromosomal synteny surrounding the agnathan melanocortin receptors. Syntenic relationships flanking the hagfish *mcar* (A) and *mcbr* (B) loci are shown in comparison with corresponding chromosomal regions from lampreys. Colored blocks represent orthologous genes. The order of blocks reflects genome coordinates along each chromosome or scaffold; the size and distances between blocks is not drawn to scale. Thin lines connect putative orthologs within hagfishes or lampreys; thick lines connect putative orthologs between hagfishes and lampreys. Gene blocks are not drawn to scale. Bolded gene names denote synteny between *mcar* and *mcbr* loci.

3.2. Pharmacological analyses

Given the absence of an established *pomc* gene in the hagfish genome (Best et al., 2024) and the known efficacy of human ACTH(1–24) and α-MSH in activating lamprey Mcrs (Haitina et al., 2007; Zhu et al., 2019), the hagfish Mcar and Mcbr were tested with the human peptides (Fig. 7**A-B;** Table 1). The hagfish Mcar was also activated by both peptides, yielding mean Log(EC_{50}) values of −8.187 M and −9.197 M ($F_{1,36}$ = 6.080; $P < 0.05$), respectively (Fig. 7**A**). The mean R_{max} values for each were 2,016 and 1,572 RLU ($F_{1,36} = 4.108$; $P = 0.050$), respectively. Similarly, the hagfish Mcbr was activated in a dose-dependent and saturating manner by both ligands, with mean Log(EC_{50}) values of −8.657 M for ACTH(1–24) and −8.724 M for α-MSH ($F_{1,36} = 0.136$; $P = 0.715$) (Fig. 7**B**). The mean R_{max} values for each were 21,800 and 19,691 relative light units (RLU) ($F_{1,36} = 1.995$; $P = 0.166$), respectively.

To assess whether the hagfish Mrap affects receptor pharmacology, hagfish Mcar and Mcbr were co-expressed with the hagfish Mrap and stimulated with α-MSH (Fig. 7**C-D;** Table 1). For the hagfish Mcar, co-expression with the hagfish Mrap resulted in a mean Log(EC_{50}) of −7.185 M, compared to −7.585 M when expressed alone ($F_{1,36} = 8.526$; $P < 0.05$) (Fig. 7**C**). The mean R_{max} values for each were 23,776 and 20,284 RLU ($F_{1,36} = 6.395$; $P < 0.05$), respectively. For the hagfish Mcbr, co-expression with the hagfish Mrap resulted in a mean Log(EC_{50}) of −8.280 M, compared to −8.693 M when expressed alone ($F_{1,36} = 5.856$; $P < 0.05$) (Fig. 7**D**). The mean R_{max} values for each were 113,673 and 69,468 RLU ($F_{1,36} = 68.02$; $P < 0.001$), respectively.

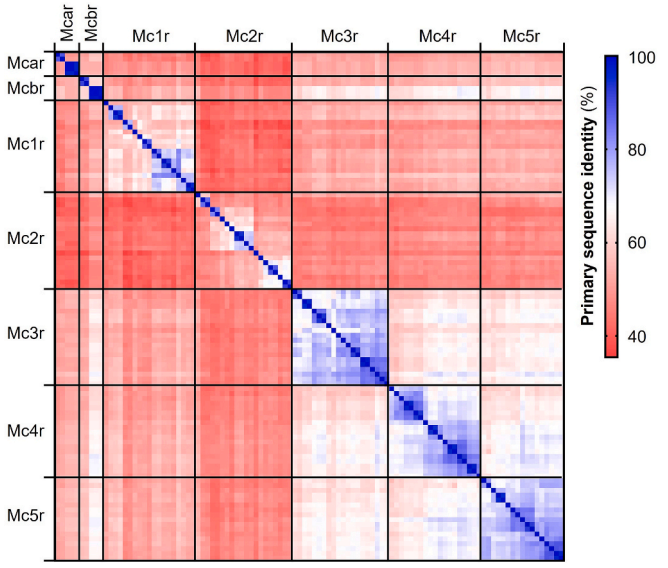

Fig. 3. Sequence identities among vertebrate melanocortin receptors. The percentage identities of the primary amino acid sequences of a wide selection of vertebrate Mcrs are presented as a heat map matrix, with darker blue shading indicating higher sequence identities and darker red shading indicating lower sequence identities. For Mcar and Mcbr, 2 hagfish and 3 lamprey species are included for each. Accession numbers available in Supplementary File 1.

Lastly, we sought to examine the G-protein associations of the hagfish Mcar and Mcbr. To do this, we conducted side-by-side assays for each receptor stimulated with α-MSH, using different reporter genes, either the cAMP-responsive reporter (CRE-luciferase) that had been used in all previous assays or a Ca^{2+}-responsive reporter (NFAT-luciferase). For the hagfish Mcar, stimulation from α-MSH was reported by both CRE-luciferase and NFAT-luciferase, with mean Log(EC_{50}) values of −9.253 M and −9.936, respectively, not differing significantly between the two reporters ($F_{1,36} = 0.301$; $P = 0.585$) (Fig. 8**A;** Table 1). However, the R_{max} values did differ significantly between the two reporters, with CRE-luciferase yielding 64,217 RLU and NFAT-luciferase yielding 5,737 RLU ($F_{1,36} = 4.169$; $P < 0.05$). For the hagfish Mcbr, stimulation by α-MSH was reported by CRE-luciferase as a mean Log(EC_{50}) of −8.867 M with a mean R_{max} of 60,958 RLU (Fig. 8**B;** Table 1). No NFAT-luciferase reporting was observed from α-MSH stimulation of Mcbr, so no curve fitting or comparison analyses could be performed.

3.3. Gene expression analyses

In our tissue profile, expression of *mcar* was strongly tissue-dependent ($F_{14,60} = 4.980$; $P < 0.001$), with the most prominent expression observed in the brain, which was > 20-fold higher than in any other tissue (Fig. 9**B**). Expression of *mcbr* was also strongly tissue-dependent (one-way ANOVA: $F_{14,60} = 5.856$; $P < 0.001$) with the most prominent expression observed in the slime gland, which was 2- to > 10-fold higher than in any other tissue (Fig. 9**A**). Like *mcbr*, *mrap* was also strongly differentially expressed across tissues ($F_{14,60} = 2.290$; $P < 0.05$), with the most prominent expression observed in the slime gland (Fig. 9**C**). Expression of *star* ($F_{14,60} = 1.014$; $P = 0.452$) and *cyp11a1* ($F_{14,60} = 1.648$; $P = 0.092$) exhibited less robust tissue-specific expression, although some pairwise differences were observed (Fig. 9**D-E**). For *star*, notable expression was detected in the gonad, slime gland, gill, and skin. For *cyp11a1*, notable expression was detected in the gonad and

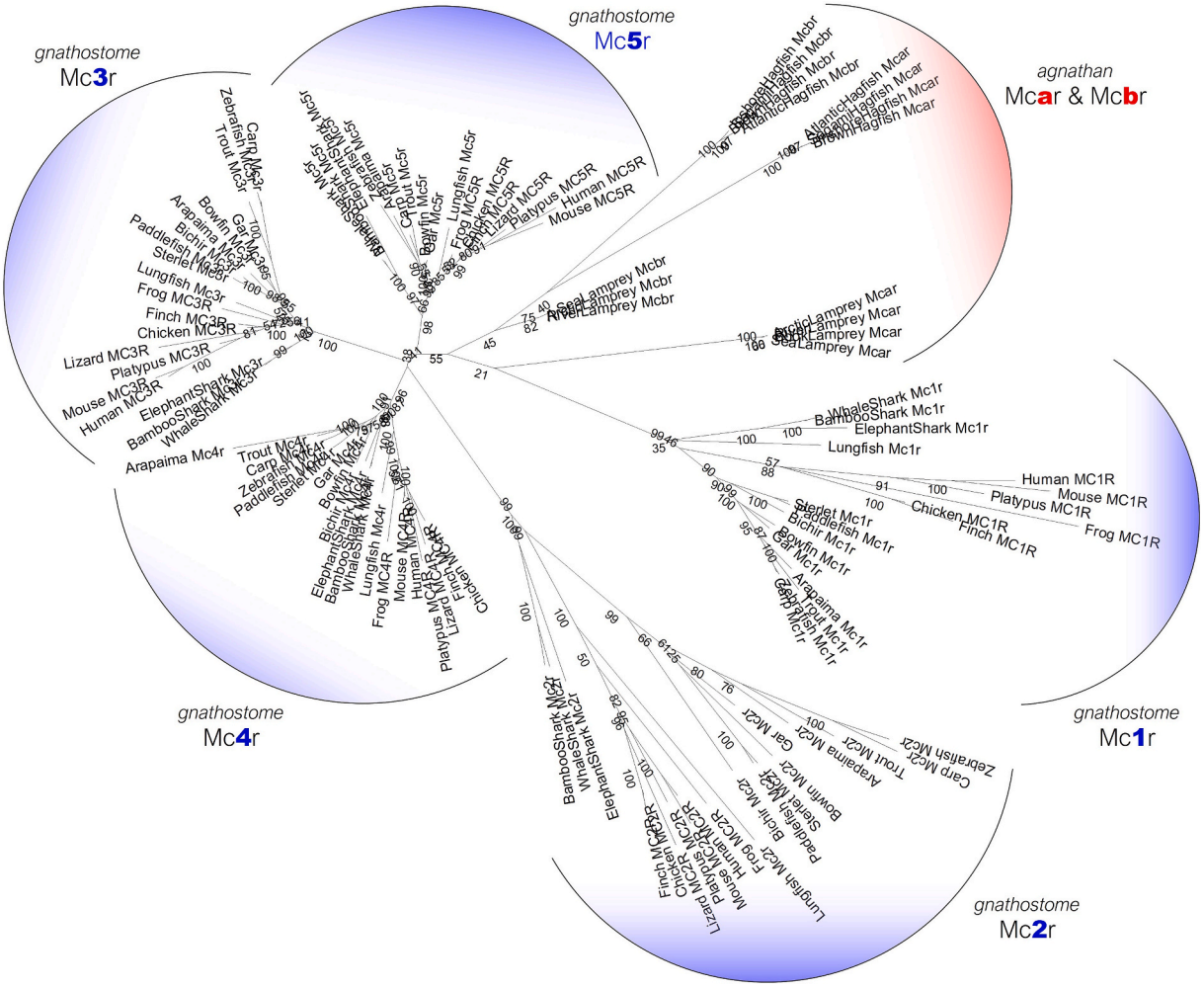

Fig. 4. Molecular phylogeny of vertebrate melanocortin receptors. Molecular phylogeny of deduced amino acid sequences of agnathan Mcars and Mcbrs inferred among other vertebrate melanocortin receptors (Mc1r, Mc2r, Mc3r, Mc4r, and Mc5r). Numbers at nodes indicate bootstrap support (1,000 replicates). See text for sequence accession numbers.

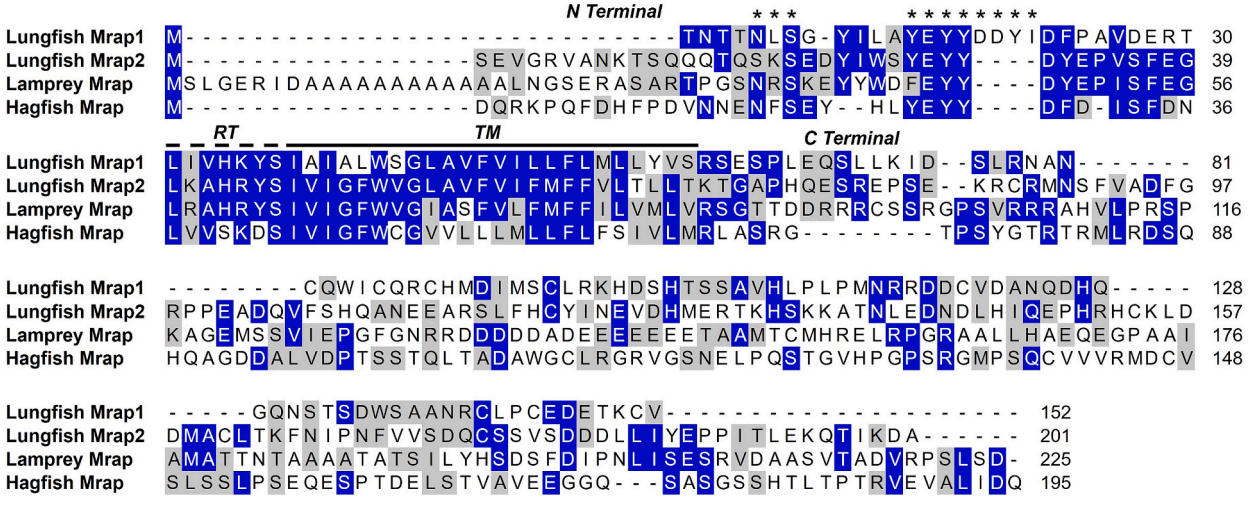

Fig. 5. Primary sequence alignment of agnathan melanocortin receptor accessory proteins. The deduced amino acid sequences of Mrap from inshore hagfish (*Eptatretus burgeri*) and sea lamprey (*Petromyzon marinus*) are aligned, along with Mrap1 and Mrap2 from the West African lungfish (*Protopterus annectens*). Residues that are identical among at least two of the four sequences are highlighted in blue; residues that are similar among at least two of the four sequences are highlighted in grey. Asterisks indicate functionally important residues discussed in text. See Table SI for sequence accession numbers. Abbreviations: RT, reverse topology motif; TM, transmembrane domain.

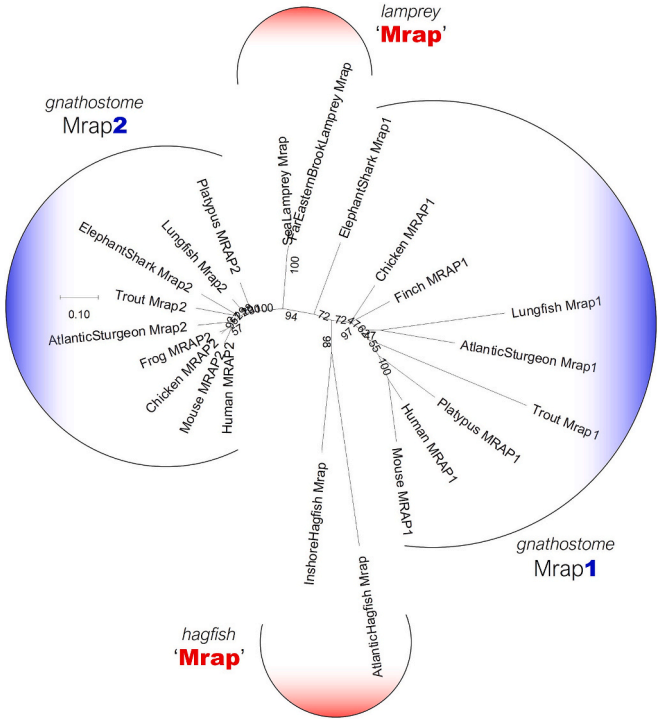

Fig. 6. Molecular phylogeny of vertebrate melanocortin receptor accessory proteins. Molecular phylogeny of deduced amino acid sequences of agnathan Mraps are presented among other vertebrate melanocortin receptor accessory proteins (Mrap1 and Mrap2). Numbers at nodes indicate bootstrap values (1,000 replicates). See Table SI for sequence accession numbers.

slime gland. In our tissue profile, there was no difference in *ef1a* expression across tissues ($F_{14,60} = 1.516$; $P = 0.262$) and no pairwise differences were detected (Fig. 9**F**).

4. Discussion

4.1. Origin of melanocortin receptors and observations on hagfish

Although melanocortin receptor genes have been detected in the genomes of chordates, these genes have not been detected in other deuterostomes or any of the protostome lineages (Västermark and Schiöth, 2011). The operating assumption for the origin of melanocortin receptors has been that the ancestral gene for melanocortin receptors could have arisen in an ancestral protochordate lineage and duplicated after the first round (1R) of whole-genome duplication (WGD) in stem vertebrates (Dores, 2013; Västermark and Schiöth, 2011). The extant protochordate lineages are limited to the cephalochordates and the urochordates, and Class A GPCRs have been detected in the genomes of both groups (Ji et al., 2024). These orphan GPCRs exhibit the D/$E^{127}R^{128}$ Y^{129} (residue numbers corresponding to human MC2R) phosphorylation motif in IC2 and appear to act through a cAMP/PKA pathway (Ji et al., 2024). However, none of these orphan receptors in protochordates could be activated by any melanocortin peptides (Ji et al., 2024). One interpretation for this lack of activation of the Mcr-like homologues of protochordates by melanocortin peptides could be that this is example of secondary loss of functionality as a result of lineage-specific sequence divergence. However, it is important to consider that no *pomc*-like gene has been found in the representative protochordate lineages. An alternative interpretation for this lack of function in the protochordate Mcr-like homologues is that these extant protochordate lineages never possessed a functional melanocortin receptor or a melanocortin peptide. In this scenario, the true ancestral genes for the vertebrate melanocortin system may have been present in a now-extinct protochordate or pre-1R ancestral vertebrate lineage. Although it may not be possible to resolve these two competing interpretations, it is apparent that lampreys, an extant post-1R agnathan lineage, express the two putative melanocortin receptor genes (Haitina et al., 2007; Shaughnessy et al., 2025; Zhu et al., 2019) that have been hypothesized to have originated after 1R (Dores, 2013; Västermark and Schiöth, 2011).

This present study provides further context into the operation of melanocortin signaling in post-1R agnathans, suggesting that functional melanocortin receptors may be present in hagfish and warranting renewed investigation into what their endogenous ligand(s) might be.

4.2. Functional divergence of agnathan and gnathostome Mcrs

In contrast to the highly specialized Mc2r of bony vertebrates, the hagfish Mcar and Mcbr exhibited more generalized activation profiles consistent with what has been observed in lamprey Mcrs (Haitina et al., 2007; Shaughnessy et al., 2025). However, unlike the lamprey Mcar, which shows a pronounced preference for Msh-sized peptides over Acth, the hagfish receptors showed no such bias. Another important difference is that the hagfish Mcbr exhibits a far greater sensitivity to melanocortin ligands than the lamprey Mcbr. These differences suggest that functional divergence of Mcr ligand selectivity and sensitivity may have occurred independently among the agnathan lineages. Despite their shared ancestry, the hagfish and lamprey receptors exhibit only modest primary sequence identity (~50–55%), which may underlie the functional differences between the hagfish and lamprey Mcrs we now observe.

Although our assays were designed to evaluate ligand-dependent activation, we did observe some baseline luciferase activity in receptor-transfected cells, suggesting that hagfish Mcrs may exhibit low-level constitutive signaling, as has been reported for some other vertebrate Mcrs (Ji et al., 2022). However, we did not conduct our experiments with receptor-free controls, which would be required to quantify constitutive activity more precisely.

Our present findings with hagfish receptors support the hypothesis that the ligand specificity and Mrap-dependence of Mc2r in osteichthyans represent derived characteristics, rather than ancestral features of the melanocortin signaling system. In this regard, the hagfish and lamprey systems may provide a useful proxy for understanding the ancestral state of Mcr function in early vertebrates.

4.3. The role of Mrap in hagfish

In osteichthyans, Mrap1 contains an N-terminal δ-D-Y-δ motif, which is required for ACTH-induced activation (Sebag and Hinkle, 2009; Webb and Clark, 2010). The osteichthyan Mrap2 lacks this activation motif and thus has little effect on Mc2r function. The lamprey Mrap also lacks this motif, which it is why it has been referred to as a lamprey 'Mrap2' (Zhu et al., 2019). Given that lampreys have only the single Mrap and its relatedness to either gnathostome Mrap1 or Mrap2 has not yet been definitively resolved, the lamprey Mrap has also been referred to as 'Mrap-prime' (Dores et al., 2022) or simply just 'Mrap' (Shaughnessy et al., 2025). We have continued to use the latter, 'Mrap', as the terminology for the hagfish ortholog, and retained distinct 'Mrap1' and 'Mrap2' nomenclature for gnathostome Mraps.

The transmembrane and reverse topology domains of the hagfish Mrap exhibit several point mutations relative to the lamprey Mrap, whereas these same regions in the lamprey Mrap share a strong identity to the gnathostome Mraps. It is unclear whether these structural differences between the hagfish and lamprey Mrap explain the modest differences in how the Mraps modulate the Mcrs in each species. Our present analyses of the hagfish Mrap-Mcr relationship generally reflect the findings of Mrap-independence in lamprey Mcrs and support the idea that the strict Mrap-dependence observed in Mc2r osteichthyans may be a derived feature that evolved alongside the development of Acth-specificity. In contrast, the agnathan Mrap may play a subtler

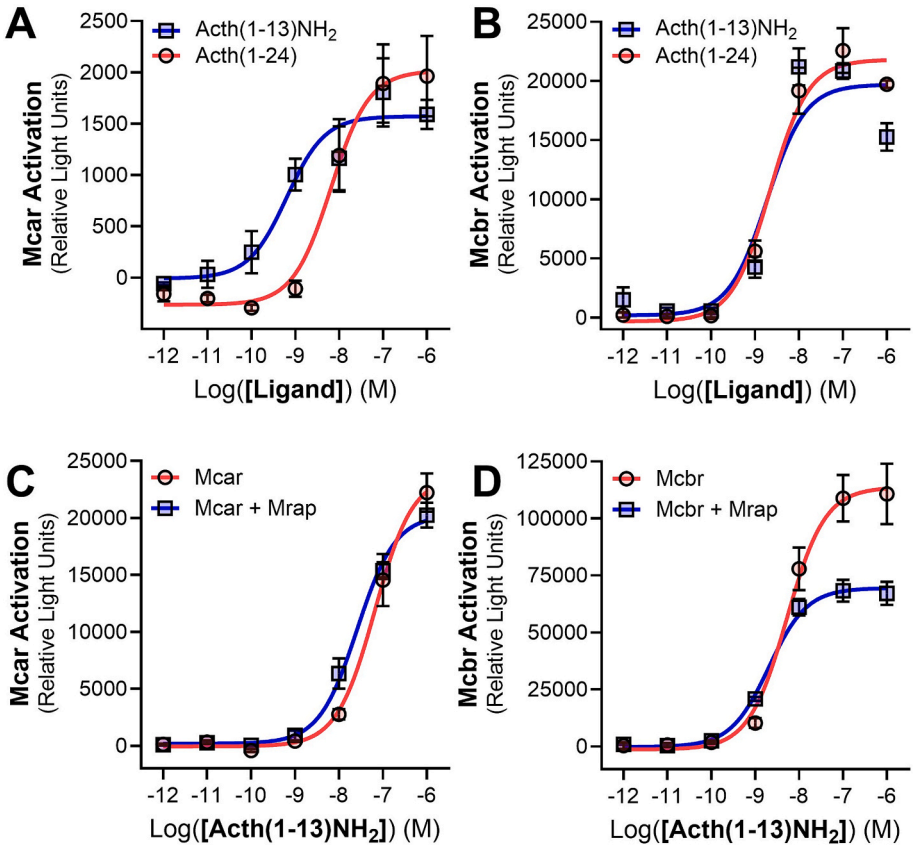

Fig. 7. Pharmacology of hagfish melanocortin receptors. Inshore hagfish (*Eptatretus burgeri*) Mcar (A, C) or Mcbr (B, D) were expressed in CHO cells without (A, B) or with (C, D) co-expression of inshore hagfish Mrap then stimulated by human melanocortin ligands, ACTH(1–24) and/or ACTH(1–13)NH_2 (α-MSH). In all panels, data reflect cAMP-responsive luciferase activity (presented as relative light units). Lines represent the fitted dose–response curve (unconstrained three-parameter polynomial). Data points are presented as mean ± standard error ($n = 3$). See Table I for curve fitting analyses.

Table 1
Curve fitting analyses of pharmacological experiments with hagfish melanocortin receptors. Inshore hagfish (*Eptatretus burgeri*) Mcar or Mcbr were co-expressed with or without hagfish Mrap and cAMP- (CRE-Luc) or Ca^{2+}-responsive (NFAT-Luc) luciferase reporters, then stimulated by human melanocortin peptides.

Figure	Transfection	Reporter	Ligand	Log(EC_{50}) (M)	Student's *t*-Test	R_{max} (RLU)	Student's *t*-Test
6A	Mcar	CRE-Luc	ACTH(1–24)	−8.187 (−8.617, −7.757)	$P < 0.05$	2,016 (1,637, 2,394)	$P = 0.050$
6A	Mcar	CRE-Luc	ACTH(1–13)NH_2	−9.197 (−9.758, −8.637)		1,572 (1,298, 1,846)	
6B	Mcbr	CRE-Luc	ACTH(1–24)	−8.657 (−8.895, −8.418)	$P = 0.715$	21,800 (20,038, 23,562)	$P = 0.166$
6B	Mcbr	CRE-Luc	ACTH(1–13)NH_2	−8.724 (−9.144, −8.305)		19,691 (16,969, 22,412)	
6C	Mcar	CRE-Luc	ACTH(1–13)NH_2	−7.185 (−7.39, −6.98)	P < 0.05	23,766 (21,190, 26,341)	P < 0.05
6C	Mcar + Mrap	CRE-Luc	ACTH(1–13)NH_2	−7.585 (−7.752, −7.418)		20,284 (18,813, 21,755)	
6D	Mcbr	CRE-Luc	ACTH(1–13)NH_2	−8.28 (−8.538, −8.021)	P < 0.05	113,673 (102,639, 124,708)	P < 0.001
6D	Mcbr + Mrap	CRE-Luc	ACTH(1–13)NH_2	−8.693 (−8.872, −8.514)		69,468 (65,302, 73,634)	
7A	Mcar	CRE-Luc	ACTH(1–13)NH_2	−9.253 (−9.644, −8.861)	P = 0.585	64,217 (56,609, 71,826)	P < 0.05
7A	Mcar	NFAT-Luc	ACTH(1–13)NH_2	−9.936 (−11, −8.873)		5,737 (3,028, 8,446)	
7B	Mcbr	CRE-Luc	ACTH(1–13)NH_2	−8.867 (−9.211, −8.524)	—	60,958 (53,621, 68,294)	—
7B	Mcbr	NFAT-Luc	ACTH(1–13)NH_2	—		—	

Data are presented as mean (95% CI; low, high) and compared using the Extra Sum-of-Squares F Test (DFn = 1; DFd = 36). Abbreviations: RLU, relative light units.

modulatory role on the Mcrs, and this role may be to attenuate trafficking rather than promote it. It is also possible that agnathan Mrap may have functions outside the melanocortin signaling pathway altogether, which has been observed for Mrap2 (Rouault et al., 2020, 2017). Given limited regulatory interaction with agnathan Mcrs, it seems likely (although further studies are needed to clarify this) that the agnathan Mrap has an ancestral function that is still not fully resolved, and that the role of Mrap1 in aiding chaperoning and activation of Mc2r in gnathostomes reflects a neofunctionalization of Mrap.

4.4. Gene expression patterns and physiological implications

The expression of *star* and *cyp11a1* in the gonads is not surprising, given that this is a site of production of sex steroids (Nozaki, 2013). Interestingly, we also observed expressions of *star* and *cyp11a1* in the slime gland, a specialized organ that produces rapid and voluminous mucosal secretions as a defensive maneuver to avoid predation (Fudge et al., 2015). However, transcriptional detection of these steroidogenic enzymes in the slime gland does not necessarily indicate steroidogenesis occurs in this tissue. Given that no corticosteroid has been definitively identified in hagfish, it is possible that the enzymes detected here contribute to other steroidogenic or metabolic processes unrelated to

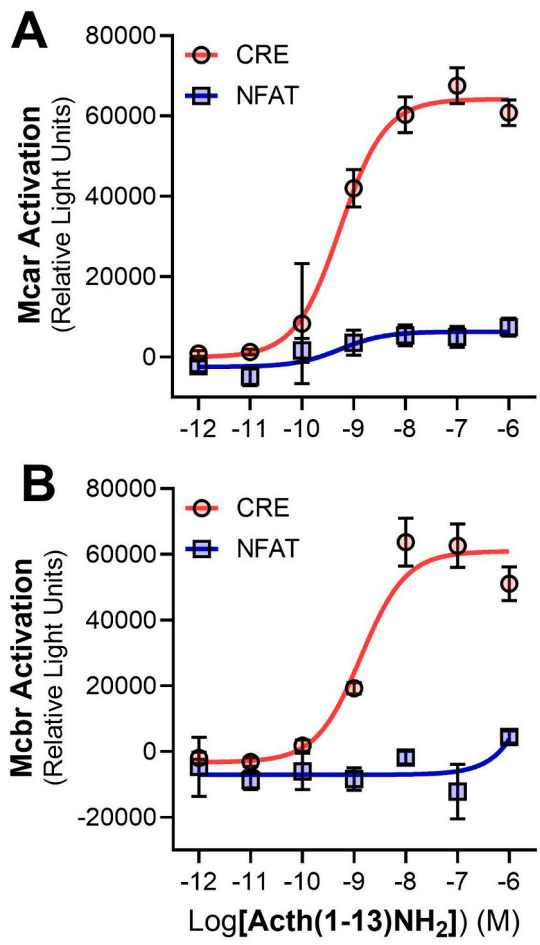

Fig. 8. G protein interactions of hagfish melanocortin receptors. Inshore hagfish (*Eptatretus burgeri*) Mcar (A) or Mcbr (B) were expressed in CHO cells then stimulated by human ACTH(1–13)NH_2 (α-MSH). In both panels, data reflect either a cAMP-responsive luciferase activity (CRE) or Ca^{+}-responsive luciferase activity (NFAT) (presented as relative light units). Lines represent the fitted dose–response curve (unconstrained three-parameter polynomial). Data points are presented as mean ± standard error (n = 3). See Table I for curve fitting analyses.

glucocorticoid synthesis.

The parallel expression of *mcbr* and *mrap* alongside *cyp11a1* and *star* in slime gland is intriguing, but the idea that Mcbr participates in melanocortin regulated steroidogenesis in the slime gland, as we have hypothesized for lamprey Mcar due to its co-expression with *star* in the lamprey interrenal tissue (Shaughnessy et al., 2025), is purely speculative, particularly in the absence of known melanocortins or corticosteroids in hagfish. In both lamprey and hagfishes, it will be necessary to co-localize Mcrs and steroidogenic enzymes to the same cell types to any functional link between melanocortin signaling and corticosteroidogenesis.

In contrast to *mcbr* and *mrap*, *mcar* was predominantly expressed in the brain in hagfish. This is consistent with recent findings for *mcbr* in lamprey (Shaughnessy et al., 2025). We think that it is likely that these agnathan Mcrs have a neuromodulatory role, perhaps in regulating feeding behavior, energy balance, or sensory processing, which appears to be a conserved role for melanocortins among vertebrates (Ji et al., 2022; Tao, 2010). Future studies should seek to more definitively identify the neurophysiological role(s) of Mcrs in agnathans.

4.5. Evolution of the melanocortin receptors in agnathans and vertebrates

The identification of two melanocortin receptors in hagfishes indicates that the Mcar-Mcbr receptor pair is broadly conserved across extant agnathans. Molecular phylogenetic analyses do not definitely place agnathan Mcrs in any particular Mcr clade, and thus they appear distinct from the five paralogous Mcr subtypes found in gnathostomes. Incorporating both the phylogenetic and syntenic evidence clarifies the likely evolutionary relationships between the agnathan melanocortin receptors. Although the phylogenetic analysis shows the two hagfish receptors clustering with lamprey Mcbr rather than with lamprey Mcar, these groupings lack strong bootstrap support. The synteny data provide an overall convincing signal that the hagfish Mcar and Mcbr represent respective orthologs of lamprey Mcar and Mcbr, rather than the products of a lineage-specific duplication.

Multiple WGDs are hypothesized to have occurred in the stem vertebrate lineage, with 1R hypothesized to have occurred prior to the divergence of agnathans and a second round (2R) either just before or just after the divergence of agnathans (Holland and Ocampo Daza, 2018; Marlétaz et al., 2024; Nakatani et al., 2021; Ohno et al., 2009; Yu et al., 2024). It is now widely hypothesized that a single WGD occurred before the agnathan-gnathostome divergence followed by agnathan-specific regional duplications (Smith et al., 2018) or whole-genome duplications or triplications (Marlétaz et al., 2024; Yu et al., 2024). The combined functional, phylogenetic, and syntenic evidence supports a model in which the Mcar/Mcbr receptor pair originated early in vertebrate evolution (as a result of 1R), was retained in both agnathan lineages, and subsequently underwent structural and functional diversification in lampreys.

That agnathans exhibit divergent gene families relative to gnathostomes is well documented, and it is plausible that melanocortin signaling elements duplicated and diverged in parallel with their genomes more broadly. We propose that the ancestral melanocortin receptor was functionally promiscuous, capable of responding to both Acth-like and Msh-like peptides. It is likely that 1R gave rise to the Mcar and Mcbr paralogs in early vertebrates, and this receptor pair has since been retained in both extant agnathan lineages. In both lampreys and hagfish, these two receptors remain broadly responsive to melanocortin peptides, a pattern consistent with ancestral functional promiscuity. In this arrangement, receptor function may be dictated less by ligand specificity and more by localization of expression. For instance, the prominence of *mcar* in the brain and *mcbr* expression in the gonad and slime gland, raises the possibility that melanocortin signaling in hagfish is regulated not by ligand-receptor selectivity but by its cellular context. Depending on where the potential ligand(s), if any, are produced, these receptors could be participating in endocrine, paracrine, or autocrine signaling systems. A non-endocrine arrangement of melanocortin signaling would allow these non-selective receptors in hagfish to participate in distinct physiological processes depending on where they and their ligands are expressed.

In lampreys, however, this system appears to have undergone further specialization. The diversification of melanocortin ligands in lamprey may have exerted selective pressure on receptor function, as evidenced by the increased preference of lamprey Mcar for Msh peptides over Acth. While still responsive to multiple peptides, lamprey Mcar shows a measurable bias in ligand selectivity (Shaughnessy et al., 2025) that is absent in its hagfish counterpart. These observations support a model in which functional divergence of the Mcar paralog occurred within the lamprey lineage, possibly in response to the emergence of functionally distinct melanocortin ligands.

This trajectory from a single, promiscuous receptor to specialized, hormone-dependent subtypes in osteichthyans illustrates how functional constraints and gene duplication events may have shaped the modern vertebrate melanocortin system. The agnathan Mcrs therefore provide critical insight into the early stages of this evolutionary process, occupying a pivotal phylogenetic and functional position between protochordate GPCRs and the specialized melanocortin receptors of the later emerging vertebrates.

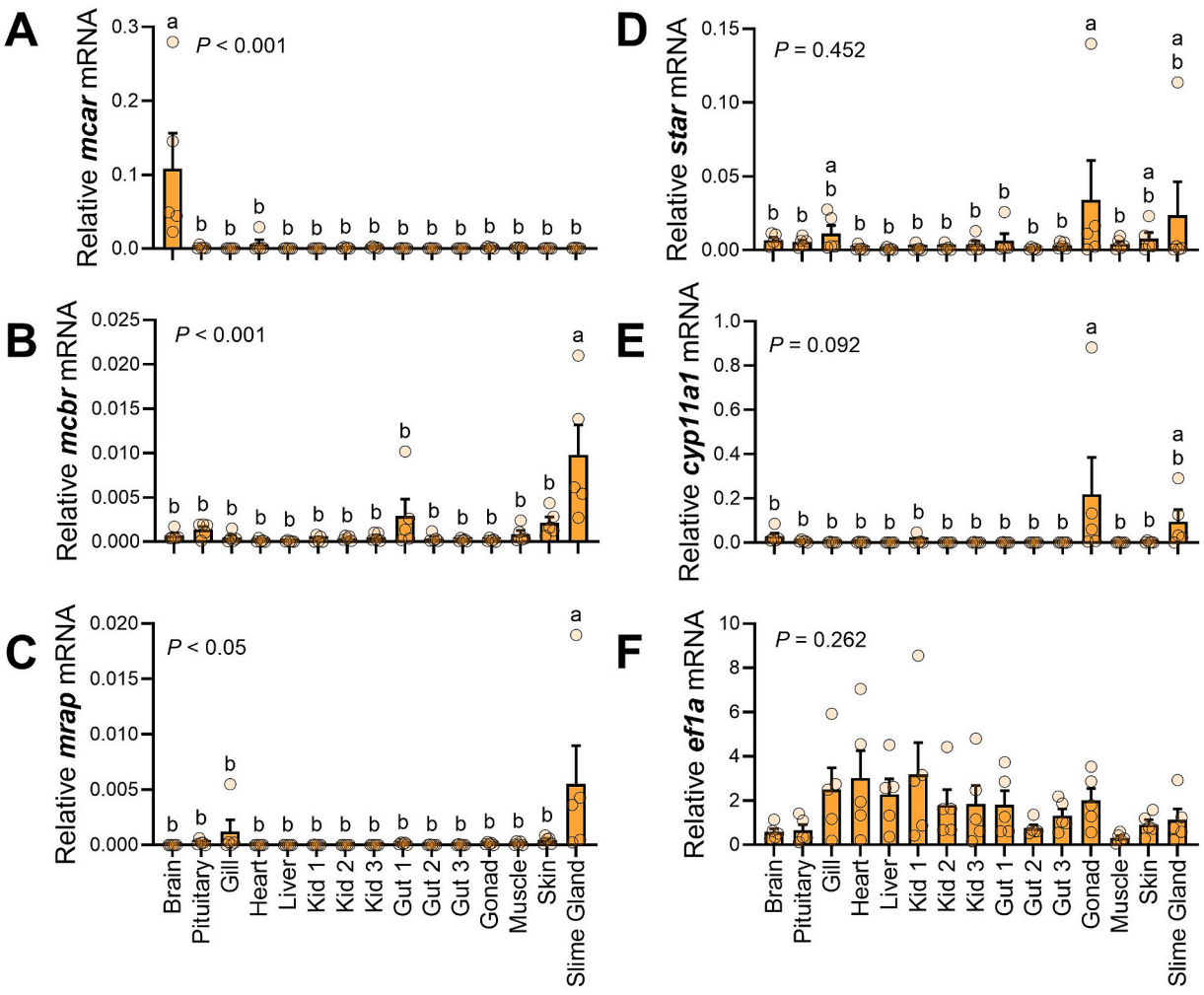

Fig. 9. Tissue profile of hagfish melanocortin receptors and steroidogenic enzymes. Relative abundance of mRNA is presented for *mcar* (A), *mcbr* (B), *mrap* (C), *star* (D), and *cyp11a1* (E) and *ef1a* (F) across various tissues in Pacific hagfish (*Eptatretus stoutii*). Relative mRNA abundance is calculated as $2^{-\Delta CT}$ using *ef1a* as a reference gene. Relative expression data for *ef1a* are presented for reference only; *ef1a* ΔC_T values were calculated as the difference between C_T and the average *ef1a* C_T value (17.3). Data are presented as mean ± standard error, with individual points shown. *P*-values indicate one-way ANOVA results and letters indicate significant pairwise differences, where present. See Supplementary File 2 for additional details.

4.6. Toward resolving the paradox of the missing hormones of the hagfish HPI axis

Despite the present evidence that hagfishes possess functional melanocortin receptors and express key steroidogenic enzymes, a central paradox remains: no functional corticosteroid hormone has been definitively identified in hagfishes (Best et al., 2024; Bridgham et al., 2006; Frost and Goss, 2022). This absence complicates our understanding of how melanocortin signaling might contribute to stress physiology in this lineage. Our detection of *star* and *cyp11a1* expression in both the gonads and slime gland of the Pacific hagfish suggests that at least some tissues might possess the enzymatic capacity for de novo corticosteroid biosynthesis. While gonadal steroidogenesis is well established in agnathans, the expression of *mcbr*, *star*, and *cyp11a1* in the slime gland, is novel and intriguing. Our data lead us to hypothesize that the slime gland may serve as a stress-responsive tissue integrating secretory and steroidogenic functions. Studies seeking to identify a putative corticosteroid in hagfish may consider examining the slime gland as a site of production.

If steroidogenesis is indeed occurring in the slime gland, the nature of the steroid end-product remains unknown. The prevailing wisdom is that cortisol-, corticosterone-, or 11-deoxycortisol-like compounds are not present in hagfish plasma (Best et al., 2024; Bouyoucos et al., 2021; Clifford et al., 2017; Frost and Goss, 2022). This may reflect true absence or simply a limitation of analytical sensitivity or specificity. Alternatively, hagfish may produce a structurally novel corticosteroid, as is the case in the cartilaginous fishes (Anderson, 2012), with a regulatory role in stress physiology. Determining the identity, site of synthesis, and physiological roles of hagfish steroids remains a key objective for future research.

Compounding the enigma of a missing corticosteroid in hagfish is that no *pomc*-like genes have yet been identified in hagfishes. In the present study, we show that hagfish melanocortin receptors respond to both ACTH(1–24) and α-MSH, yet the endogenous source of such peptides is unknown. The presence of functional receptors alongside the apparent absence of ligands is difficult to reconcile. One explanation is that hagfish may utilize an unrecognized ligand that fulfills the role of Acth and/or Msh in activating Mcar and Mcbr. An alternative explanation is that a *pomc*-like gene has simply gone unidentified in hagfish. Given that the hagfish Mcrs have retained functionality to be activated by melanocortin peptides, we posit that there are likely endogenous melanocortin-like ligands for these receptors present in hagfish that have yet been identified.

4.7. Conclusions and future directions

These results demonstrate the importance of integrating additional agnathan models into the broader framework of comparative endocrinology. While studies in lampreys have made significant contributions to understanding endocrine signaling in early vertebrates, studies in hagfishes have historically contributed less to these discussions due to a lack of molecular and functional data. However, hagfishes and lampreys are physiologically distinct in many ways. Thus, phylogenetic or evolutionary inferences based in part on agnathan physiological studies would be strengthened by considering the physiology of both hagfishes and lampreys. By characterizing the expression and function of the melanocortin receptors and their accessory protein in hagfishes, this study adds additional context to understanding the evolution of stress axis regulation.

Together, the present findings in hagfish and previous findings in lamprey studies support the hypothetical model in which the ancestral vertebrate melanocortin system exhibited broadly responsive receptors, limited reliance on accessory proteins, and context-dependent signaling determined by tissue-specific expression rather than strict ligand-receptor specificity. The hagfish system appears to preserve many of these ancestral features and thus may be a useful model in studying the functional evolution of the melanocortin system in stem vertebrates.

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CRediT authorship contribution statement

Ciaran A. Shaughnessy: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Emma E. Kuhn:** Writing – original draft, Investigation, Formal analysis. **Susanne M. Hahs:** Formal analysis, Investigation, Visualization, Writing – review & editing. **Ian A. Bouyoucos:** Investigation, Funding acquisition, Conceptualization. **W. Gary Anderson:** Resources, Project administration, Investigation, Funding acquisition. **Robert M. Dores:** Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

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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.2026.114910.

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