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RESEARCH

Functionally divergent melanocortin receptor subtypes and the HPI axis in sea lamprey

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Abstract

The hypothalamus–pituitary–interrenal (HPI) axis is a highly conserved neuroendocrine system in vertebrates, but many details of its physiological role in jawless vertebrates remain unclear. Unlike the proopiomelanocortin (Pomc) gene of jawed vertebrates, lampreys have two divergent melanocortin prohormones, a proopiocortin (Poc) encoding adrenocorticotropic hormone (Acth) and promelanotropin (Pom) encoding two melanocyte-stimulate hormones (Msh-a and Msh-b). This study investigated the HPI axis in sea lamprey (*Petromyzon marinus*) by characterizing the potential involvement of key HPI components through gene expression analyses and functional studies. Phylogenetic analysis revealed that the two lamprey melanocortin receptors, Mcar and Mcbr, occupy basal positions relative to gnathostome melanocortin receptors. We observed that tissue-specific transcriptional patterns of HPI axis genes, including *mcar* and steroidogenic acute regulatory protein (*star*), are both expressed in a 'head kidney' region, suggesting a concomitant role in a corticosteroidogenic HPI axis. Both Mcar and Mcbr receptors can be activated by lamprey Acth, Msh-a and Msh-b peptides. Notably, Mcar was more potently activated by Msh peptides over Acth, and neither receptor required the melanocortin receptor accessory protein (Mrap), which is required for gnathostome Mc2r function. These findings indicate that the strong selectivity for Acth and Mrap1-dependence of Mc2r may be derived traits that evolved after the agnathan–gnathostome divergence. Our results provide evidence for a functional HPI axis in lamprey, albeit with unique features compared to gnathostomes. This study offers insights into the evolutionary origins of the vertebrate HPI axis and highlights the need for further investigation into the regulation and physiological roles of the agnathan HPI axis.

Keywords: HPI; crh; Pomc; Mc2r; evolution

Introduction

The hypothalamus–pituitary–interrenal (HPI) axis in fishes and the homologous hypothalamus–pituitary–adrenal (HPA) axis in tetrapods are essential neuroendocrine systems that regulate the stress response, metabolism and homeostasis in vertebrates (Denver 2009). In the HPI axes of fishes, upon exposure to a stressor, the hypothalamus releases corticotropin-releasing hormone (Crh), which stimulates the secretion of the proopiomelanocortin (Pomc) cleavage product, adrenocorticotropic hormone (Acth), from the pituitary gland. Acth, in turn, stimulates the synthesis and release of corticosteroid hormones, such as cortisol, from the interrenal tissue (Mommsen *et al.* 1999, Bernier *et al.* 2009, Best *et al.* 2024). In fishes, the HPI axis is activated by a wide range of stressors, including physical, chemical and biological factors (Wendelaar Bonga 1997), and plays a crucial role in enabling

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responses to various environmental challenges and maintenance of physiological homeostasis (Barton 2002).

Key to the HPI and HPA axes of vertebrates is the melanocortin 2 receptor (Mc2r), which is expressed in the interrenal/adrenal tissue and receives the pituitary-derived Acth ligand to initiate corticosteroid biosynthesis. The melanocortin receptors are a family of five G protein-coupled receptors (Mc1r, Mc2r, Mc3r, Mc4r and Mc5r) that differentially bind to and are activated by melanocortin peptides, including Acth and the melanocyte-stimulating hormone (Msh) peptides (Cone 2006), which are other cleavage products of Pomc. These receptor subtypes have distinct tissue distribution and functional roles, including regulation of physiological processes such as pigmentation, energy homeostasis and steroidogenesis. Among these subtypes, Mc1r, Mc3r, Mc4r and Mc5r can all be activated by both Acth and α-Msh ligands, but Mc2r can only be activated by Acth. For this reason, Mc2r has been labeled as the 'Acth receptor' (Mountjoy *et al.* 1992).

Recent comparative pharmacological investigations of melanocortin receptors have revealed a complex pattern of functional adaptations that have emerged throughout vertebrate evolution (Dores & Chapa 2021). In bony vertebrates (Osteichthyes), which comprise most of the extant fish species (actinopterygians) and tetrapods (sarcopterygians), Mc2r exhibits highly specific functional characteristics (Shaughnessy *et al.* 2022, 2023*a*). These include an exclusive selectivity for binding adrenocorticotropic hormone and an obligate interaction with the melanocortin receptor accessory protein (referred to here and throughout as 'Mrap1') for proper membrane trafficking and Acth-induced activation. Another ortholog of Mrap exists, Mrap2, but it appears that only Mrap1 is required for Acth activation of Mc2r in osteichthyans (Chan *et al.* 2009, Dores & Chapa 2021).

These functional traits of osteichthyan Mrap1 and Mc2r appear to be derived features in the bony vertebrate lineage. The cartilaginous fishes (Chondrichthyes) are a group of gnathostomes, which radiated approximately 450 million years ago (Inoue *et al.* 2010) that, like Osteichthyes, exhibit an Acth-mediated, stress-responsive HPI axis and a complete family of five melanocortin receptors (Dores *et al.* 2014, Anderson 2015, Bouyoucos *et al.* 2021). However, the Mc2rs of chondrichthyans exhibit markedly different functional properties than those of osteichthyans. Studies in chondrichthyans have revealed that Mc2rs can be activated by either Acth or α-Msh (Reinick *et al.* 2012, Takahashi *et al.* 2016, Hoglin *et al.* 2022, Bouyoucos *et al.* 2023). The chondrichthyan Mc2rs also appears to lack an obligate association with Mrap1. In the elasmobranchs (i.e., sharks and rays), Mc2r activation can be activated when co-expressed with either Mrap1 or Mrap2 (Takahashi *et al.* 2016, Hoglin *et al.* 2022, Bouyoucos *et al.* 2023), and so it is thought that elasmobranch Mc2rs only form associations with Mraps to enhance membrane trafficking. In the lone holocephalan representative that has been studied, the elephant shark (*Callorhinchus milii*), co-expression with either Mrap1 or Mrap2 has no effect on Mc2r function (Reinick *et al.* 2012).

In the jawless vertebrate group Agnatha, represented by hagfishes and lampreys, the presence of an Acth-mediated HPI axis has not been firmly established (Bouyoucos *et al.* 2021). In sea lamprey (*Petromyzon marinus*), as in gnathostomes, genes encoding Crh, Crh-binding proteins and Crh receptors (Crhrs) have been identified and are expressed in the brain (Endsin *et al.* 2017, Cardoso *et al.* 2020, Sobrido-Cameán *et al.* 2023). However, the sea lamprey melanocortins and the melanocortin receptors appear to differ markedly from those of gnathostomes. In contrast to gnathostomes, sea lampreys do not express a singular preproprohormone containing Acth, Msh and opioid cleavage products. Rather, sea lampreys express two smaller preprohormones, proopiocortin (Poc) and proopiomelanotropin (Pom), of which Poc encodes an Acth peptide nearly twice the length of gnathostome Acths and Pom encodes two melanotropins (Msh-a and Msh-b) (Takahashi *et al.* 1995, 2006). In addition, sea lampreys express only two subtypes of melanocortin receptors, Mcar and Mcbr (Haitina *et al.* 2007). Phylogenetic analyses suggest that these receptors may represent ancestral forms of the Mcr family, predating the later diversification of Mcr subtypes in gnathostomes (Haitina *et al.* 2007, Västermark & Schiöth 2011). Finally, sea lampreys appear to express only a single Mrap ortholog (referred to here and throughout as 'Mrap'), with a primary sequence structure more similar to that of the gnathostome Mrap2 (Valsalan *et al.* 2013, Zhu *et al.* 2019, Dores *et al.* 2022). The functional expression of the sea lamprey melanocortins, melanocortin receptors and melanocortin receptor accessory protein and their pharmacological relationships require further investigation to advance our understanding of their functional roles in a sea lamprey HPI axis, if one exists.

The studies presented here are intended to both complement and extend previous studies on the lamprey neuroendocrine system. While earlier work characterized the brain Crh system in sea lamprey (Endsin *et al.* 2017, Sobrido-Cameán *et al.* 2023), our tissue expression analysis sought to reveal the broader distribution of Crh/Crhr and other HPI axis system components across multiple tissues. Similarly, although Mcar function was previously investigated in river lamprey (Haitina *et al.* 2007), the function of Mcbr was not investigated and the role of Mrap was not considered for both Mcar and Mcbr. The function of Mcbr and the role of Mrap were more recently investigated in sea lamprey (Zhu *et al.* 2019); however, only mammalian melanocortin peptides were used. Thus, our investigations sought to provide the first analysis of both receptor subtypes in sea lamprey against sea

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lamprey melanocortin peptides, including their interaction with sea lamprey Mrap. By providing these transcriptional and functional analyses of the sea lamprey HPI axis and melanocortin system, this study aims to contribute to and identify a path forward toward resolving the functional evolution of the HPI axis in vertebrates.

Materials and methods

Sequence discovery and analyses

The genome assembly for *P. marinus* (GCF_010993605.1) was accessed from the National Center for Biotechnology Information (NCBI) GenBank and surveyed for nucleotide sequences corresponding to *crh*, *crhr*, *pom*, *poc*, *mcar*, *mcbr*, *mrap* and *star*. A selection of gnathostome Mc1r, Mc2r, Mc3r, Mc4r, Mc5r, Mrap1 and Mrap2 amino acid sequences obtained from NCBI GenBank were used in multiple sequence alignment and phylogenetic analyses. Multiple sequence alignments were performed using the MUSCLE alignment tool using the MEGA11 software (Tamura *et al.* 2021) and phylogenetic analyses were implemented using the neighbor-joining method (500 bootstrap replicates) in the MEGA11 software.

Live animal care and tissue sampling

Handling and care of sea lamprey followed procedures approved by the Internal Animal Care and Use Committees at the University of Massachusetts (Protocol No. 2016-0009) and the U.S. Geological Survey (Protocol No. C0907). Mid-metamorphic sea lamprey (length: 15.5 ± 0.4 cm; mass: 3.6 ± 1.1 g; n = 3) were collected from the Connecticut River in Turners Falls, Massachusetts, via electrofishing in early autumn. These animals were housed in 1.5 m diameter tanks with flowing, filtered and UV-treated river water at 4 L/min until the completion of metamorphosis. The tanks were maintained at ambient river temperatures with a natural photoperiod. To allow voluntary burrowing, each tank had 10 cm sandy substrate. Sea lampreys were sampled in January, approximately 1 month after the completion of metamorphosis. During sampling, post-metamorphic juvenile sea lamprey were euthanized using a lethal dose of MS-222 (200 mg/L buffered using $NaHCO_3$, pH 7.4), measured for body length and mass and then sampled for tissues. Tissue samples included: brain (all regions except pituitary), pituitary, gill, heart, liver and three regions of the kidney: i) a 'head kidney' region, which consisted of the thin line of darkly pigmented tissue extending anterior from the definitive opisthonephros, where the larval opisthonephros once occupied but since degenerated; ii) an 'anterior kidney' region, which consisted of the most anterior region (approximately 0.5–1 cm) of the definitive opisthonephros, which occupies the posterior two-thirds of the body cavity; and iii) a 'posterior kidney', which consisted of the most posterior (approximately 0.5–1 cm) of the definitive opisthonephros, near the cloaca. As the localization of *crh* has been shown to be expressed in multiple regions of the lamprey brain (Sobrido-Cameán *et al.* 2023), we chose to utilize the whole brain tissue rather than specific regions. Tissues were immediately frozen and stored at −80°C for later RNA extraction.

Gene expression analyses

Protocols for RNA extraction, cDNA synthesis and quantitative PCR closely followed previously described methods (Shaughnessy *et al.* 2023*b*). Total RNA extraction from frozen tissue utilized the TRIzol method (Molecular Research Center Inc., USA), following the manufacturer-provided guidelines. A high-capacity reverse transcription kit (Applied Biosystems Inc.) was used for first-strand cDNA synthesis, adhering to the manufacturer's protocol. Quantitative PCR was conducted using SYBR Select Master Mix and a QuantStudio™ 3 Real-Time PCR System (both from Applied Biosystems, USA). The analyses were conducted in 10 μL reactions, each containing 4 ng cDNA, 150 nM of both forward and reverse primers and 2× SYBR Select Master Mix. The reaction cycle followed the protocol: 2 min at 50°C, 2 min at 95°C for holding and activation, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C and 30 s at 72°C. After the cycling, a melt curve analysis was performed with a thermal ramp from 60 to 95°C 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). Primers for *ef1a* were previously established (Shaughnessy & McCormick 2020), and details of novel primer pairs and PCR products for the *crh-a*, *crh-b*, *crh-c*, *crhr-a*, *crhr-b*, *poc*, *pom*, *mrap*, *mcar*, *mcbr* and *star* PCR assays are presented in Table 1.

Receptor activation analyses

Protocols for cell culture, transfection and reporter gene assay closely followed previously described methods (Shaughnessy *et al.* 2023*b*). Functional properties of sea lamprey Mcar, Mcbr and Mrap were analyzed using a cAMP-responsive luciferase reporter gene assay carried out in Chinese hamster ovary (CHO) cells (ATCC, USA), as previously described (Liang *et al.* 2011, Reinick *et al.* 2012). CHO cells are commonly used for melanocortin receptor functional studies because they do not endogenously express melanocortin receptors or their accessory proteins (Noon *et al.* 2002, Sebag & Hinkle 2007, Reinick *et al.* 2012).

Transfections were performed using commercially produced cDNA constructs of sea lamprey *mcar*, *mcbr* and *mrap* as inserts on a pcDNA3.1+ expression vector (GenScript, USA). Plasmid vectors (2 μg per 1×10^5 cells)

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Table 1 Species-specific (*Petromyzon marinus*) and gene-specific primer details for quantitative PCR analyses.

Gene	Probe	Sequence	T_m (°C)	Product (bp)	Efficiency (%)	C_T (min; max)
ef1a	F	GTGGGTCGTGTTGAGACTGG	57.9	208	96.9	16; 17
	R	GGTCGTTCTTGCTGTCAC	53.7			
crh-a	F	TGTGGAAATGGCACTCCTCC	57.4	256	103.2	27; 32
	R	GATGGTCGCGATTTGTGAGC	56.8			
crh-b	F	TGCCCTTGACATCGGGTTAC	57.4	158	94.1	30; 39
	R	TAGCATTCGACCGAACACCC	57.3			
crh-c	F	TGGCCAACTAATGCCCGATT	57.3	74	99	37; 34
	R	GCAGCTGTCCCCTGTGTTAT	57.4			
crhr-a	F	AGGCGACCATCGTCGCTA	58.8	160	93.1	26; 34
	R	TACACGCTCTTGCTGGTG	55.1			
crhr-b	F	TTCTCACGCAGCGGCTGTG	61.0	165	101.7	24; 36
	R	AGACCACCTGGCCGAC	57.5			
poc	F	CCAACAACAAGTGGTGGCTC	56.8	289	93.3	28; n.d.
	R	ACCCCATTGAAGGCGTAGTC	57.1			
pom	F	GGCTACCGGATGCAACACTT	57.7	216	95	20; n.d.
	R	CGAGTCCTTCTTGGACGGTG	57.9			
mcar	F	AGTGTTCGTCTTCTGCTGGG	57.2	111	101.6	28; 33
	R	AGCAGGTAGAGCGGGAAGTA	57.6			
mcbr	F	CCTGCTGGAGAACATCCTGG	57.8	198	91.8	24; 38
	R	TTGTCCATCTGCTTGAGCGT	57.7			
mrap	F	CGACTATGAGCCCATCTCGTT	59.7	71	98.6	34; 36
	R	CGACCCAGAATCCGATGACG	60.6			
star	F	GTGAATCTCCGCCACTCGAT	57.0	248	94.7	24; 37
	R	AAGTCGAGCTGCATTCGTGA	56.9			

Primers for ef1a were previously published (Shaughnessy & McCormick 2020). Cycle values represent approximate average values for lowest and highest expressing tissue (min and max, respectively). Abbreviations: Tm, melting temperature; n.d., not detected; see text for full gene names.

containing *mcar*, *mcbr* or *mrap* were inserted into CHO cells using a Solution T kit for the Amaxa Nucleofector 2b system (Lonza, USA). Cells were also co-transfected with a cAMP reporter construct, a *luciferase* gene promoted by a cAMP-responsive element (CRE) that was transfected at 2.5 μg per 1×10^5 cells (Chepurny & Holz 2007). Transfected CHO cells were seeded at a density of 3×10^5 cells/cm^2 into opaque 96-well cell culture plates (Cat. No. 3912; Corning Life Sciences, USA). Cells were cultured at 37°C under 5% CO_2 for 48 h in a DMEM/F12 media (Cat. No. 11320-033; Gibco, UK) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

After 48 h in culture post-transfection, the culture media was removed and transfected cells were stimulated in serum-free DMEM/F12 media with sea lamprey melanocortin ligands, Acth, Msh-a or Msh-b (gifts from Dr Stacia Sower, University of New Hampshire). Then, cells were placed back into incubation for an additional 4 h to allow any receptor-mediated cAMP production to occur. After stimulation, media was removed and replaced with a luciferase substrate (BrightGLO; Promega, USA), and the luminescence generated after 5 min was measured by a BioTek Synergy HT microplate reader using the Gen5 software (Agilent Technologies, USA). Luminescence values (measured as relative light units) of an unstimulated (0 M ligand) control for each unique transfection were subtracted from all other luminescence readings as a background correction step.

Calculations and statistical analyses

Dose–response curves for receptor activation were evaluated using nonlinear regression via a three-parameter polynomial model plotting log([ligand]) against luminescence values. The fitted curves yielded values for half-maximal effective concentration (EC_{50}) and maximal response (R_{max}). These values were then compared using the extra-sum-of-squares F test, with a significance level of $\alpha = 0.05$. The PRISM 9 software (GraphPad Inc., USA) was employed for all statistical analyses and figure generation. Results are presented as the mean ± standard error, based on three independent replicates ($n = 3$).

Results

Molecular phylogenetic analyses

We analyzed the phylogenetic relationship of vertebrate Mcrs and Mraps based on their primary amino acid sequences. The primary amino acid sequence identity between sea lamprey Mca and Mcb was 54%. The mean primary sequence identities (%) of Mca to gnathostome Mc1-5r were as follows, respectively: 50, 42, 53, 55 and 56. The mean primary sequence identities (%) of Mcb to gnathostome Mc1-5r were as follows, respectively: 54, 48, 65, 66 and 67. In our phylogenetic analyses of vertebrate Mcrs, there was a

clear grouping of gnathostome Mc1r and Mc2r separately from Mc3r, Mc4r and Mc5r. The sea lamprey Mcar and Mcbr sequences were resolved to basal positions among these groups, respectively (Fig. 1).

In our phylogenetic analyses of the vertebrate Mraps, there were distinct groupings of Mrap1 and Mrap2. The lamprey Mrap sequence resolved to a position basal to the divergence of Mrap1 and Mrap2 (Fig. 2). The primary sequence identities of the lamprey Mrap to gnathostome Mrap1 and Mrap2 were 27 and 30%, respectively.

Gene expression analyses

In a tissue profile, we analyzed mRNA expression of several genes with a potential role in HPI axis signaling. These analyses revealed tissue-specific transcriptional expression of the various component genes of the HPI axis in sea lamprey. We identified three genes encoding a Crh (*crh-a*, *-b* and *-c*) and two genes encoding Crhrs (*crhr-a* and *-b*). The *crh* sequences corresponded to the sequences previously reported by Cardoso *et al.* (2020) as Crh/Ucn_a, _b and _c. The *crhr-a* and *-b* sequences corresponded to the sequences previously reported by Endsin *et al.* (2017) as Crhrα and -β.

In our tissue profile, all three *crh* paralogs were primarily expressed in the brain (Fig. 3A, B, C). Among them, *crh-a* and *-c* were most expressed at similar levels, which were approximately ten-fold higher than *crh-b* (Fig. 3A, B, C; Table 1) Tissue distributions of *crhr-a* and *-b* notably differed. Expression of *crhr-a* was ∼eight-fold higher in

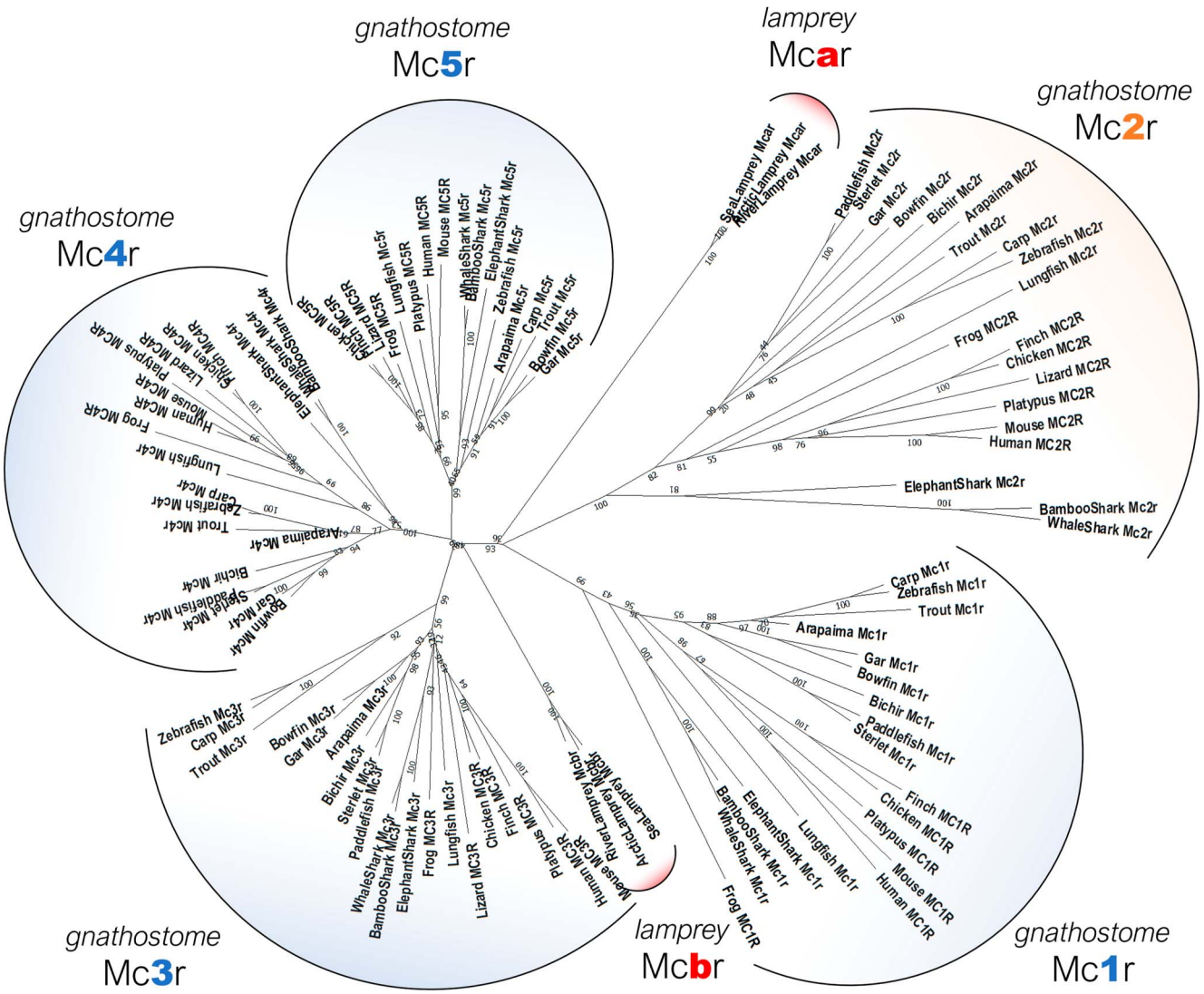

Figure 1

Molecular phylogeny of deduced amino acid sequences of sea lamprey (*Petromyzon marinus*) Mcar and Mcbr among other vertebrate melanocortin receptors (Mc1r, Mc2r, Mc3r, Mc4r and Mc5r). Numbers at nodes indicate bootstrap values (1,000 replicates). See the 'Materials and methods' section for details of analyses and sequence accession numbers. A full color version of this figure available at https://doi.org/10.1530/JME-24-0126.

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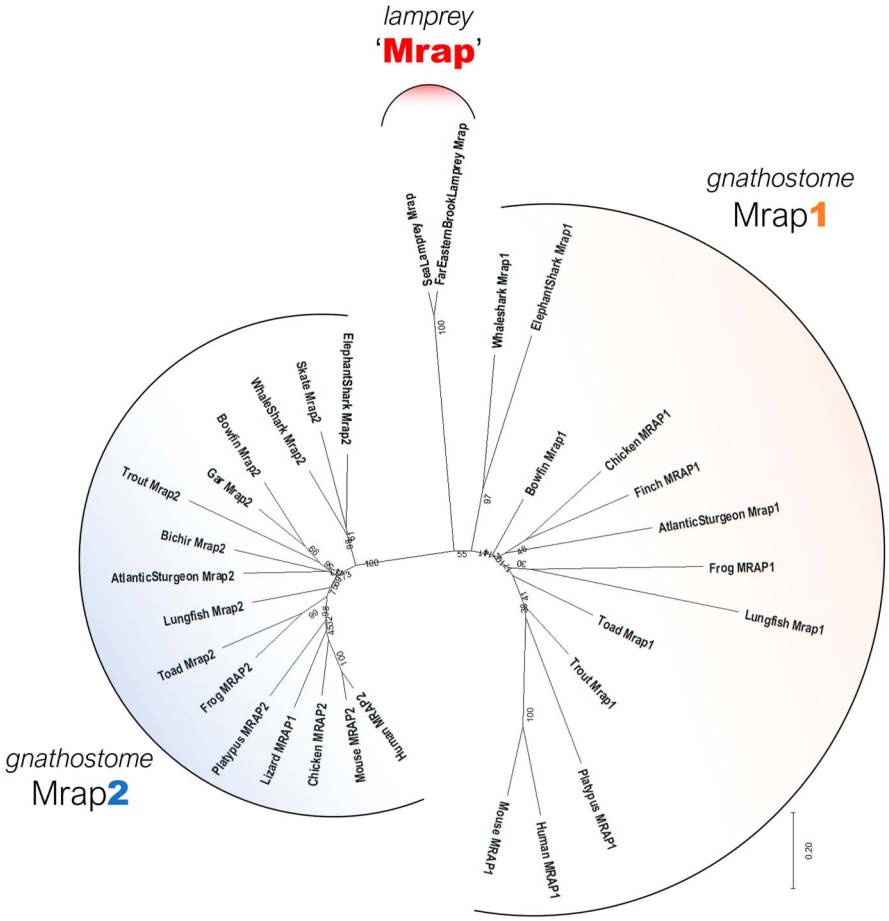

Figure 2

Molecular phylogeny of deduced amino acid sequences of sea lamprey (*Petromyzon marinus*) Mrap among other vertebrate Mraps (Mrap1 and Mrap2). Numbers at nodes indicate bootstrap values (1,000 replicates). See the 'Materials and methods' section for details of analyses and sequence accession numbers. A full color version of this figure available at https://doi.org/10.1530/JME-24-0126.

the brain than in the pituitary or gills, which were ~5–10-fold higher than in all other tissues (Fig. 4A). Expression of *crhr-b* was distributed more broadly among tissues. The most prominent levels of *crhr-b* mRNA were observed in the gills, which were ~two-fold higher than *crhr-b* mRNA levels in the brain and all three kidney regions, which all expressed similar levels of *crhr-b* (Fig. 4B). Sea lamprey Acth- and Msh-encoding genes, *poc* and *pom*, respectively, expressed 2–5 orders of magnitude higher in the pituitary than in any other tissue (Fig. 4C and D; Table 1).

The tissue distribution of the two melanocortin receptor subtypes in lamprey differed considerably. Expression of *mcar* was highest in the head kidney and brain, which had ~3–10-fold higher *mcar* mRNA levels than all other tissues analyzed (Fig. 5A; Table 1). Expression of *mcbr* was ~3–10-fold higher in the brain than all other tissues (Fig. 5B; Table 1). In contrast to the tissue-specific expression of *mcar* and *mcbr*, *mrap* was expressed relatively similarly among all tissues (Fig. 5C). Expression of *star* was primarily restricted to the three kidney regions (Fig. 5D), among which *star* mRNA levels were ~five-fold higher in the head kidney than the other two kidney regions.

The reference gene *ef1a* did not vary significantly across tissues ($P = 0.160$).

Receptor activation experiments

We conducted several functional analyses on the sea lamprey melanocortin receptors. The first was to determine and compare the selectivity of the sea lamprey melanocortin receptors between the known lamprey melanocortins, Acth, Msh-a and Msh-b, and their relative potencies on receptor activation. The second experiment was to determine whether the sea lamprey Mrap affected melanocortin receptor function.

In the first experiment, we observed that all three lamprey melanocortins can activate the sea lamprey Mcar to saturating levels (Fig. 6A). Compared to Acth peptide, Mcar was much more potently activated by Msh-a ($P < 0.001$) and Msh-b ($P < 0.001$) peptides, while no difference in potency was observed between the two Msh peptides ($P < 0.885$) (Table 2). In contrast, all of the melanocortin peptides exhibited a lower potency for activating the sea lamprey Mcbr compared to the Mcar, and no difference between ligands in their potency for activating Mcbr was observed ($P < 0.255$) (Fig. 6B; Table 2).

In our second experiment, we replicated the relative potencies that we observed in our first experiment of Mcar and Mcbr to the three peptides (Fig. 7A, B, C; Table 3). We observed a consistent reduction in

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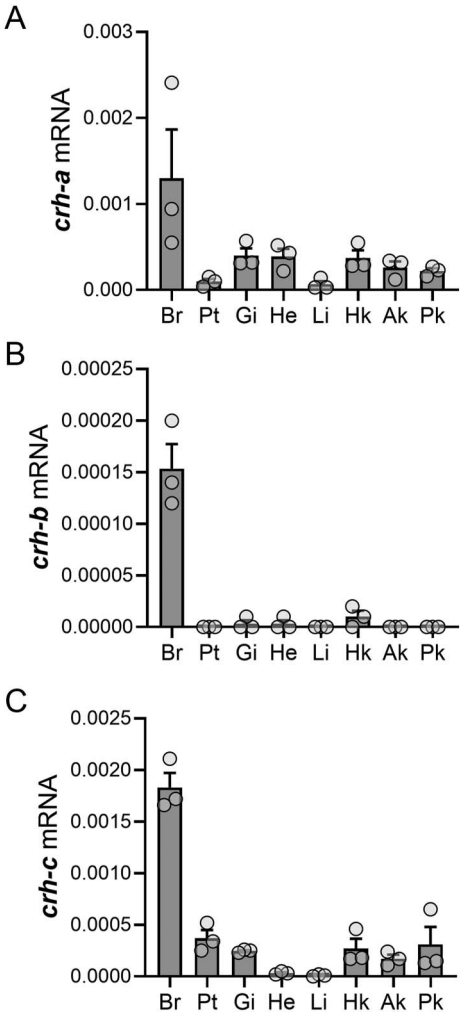

Figure 3

Transcriptional profiles of *crh-a*, *crh-b* and *crh-c* in sea lamprey (*Petromyzon marinus*) are presented for various tissues. Relative mRNA abundance is presented as $2^{-\Delta CT}$ using *ef1a* as a reference gene. Data are presented as the mean ± standard error, with individual points shown; missing data points indicate that no amplification was detected. Abbreviations: *crh*, corticotropin-releasing hormone; *ef1a*, elongation factor 1 alpha; Br, brain; Pt, pituitary; Gi, gill; He, heart; Li, liver; Hk, head kidney; Ak, anterior kidney; Pk, posterior kidney.

R_{max} when Mcar was co-expressed with the sea lamprey Mrap (Fig. 7A; Table 3). The R_{max} of Mcar activation by Acth was reduced to 25% of that of the control lacking Mrap expression (P = 0.002). Likewise, the R_{max} of Mcar activation by Msh-a and Msh-b were reduced to 57% (P < 0.001) and 45% (P < 0.001), respectively. The co-expression of Mrap did not affect the EC_{50} of Mcar to either Acth (P = 0.674) or Msh-a (P = 0.238) but decreased the EC_{50} of Mcar to Msh-b (P = 0.036) (Fig. 7B; Table 3). Again, activation of Mcbr, regardless of Mrap co-expression, did not reach saturating levels in this experiment, and thus EC_{50} and R_{max} could not be estimated (Fig. 7C).

Discussion

Summary of key findings

Our study provides new insights into the aspects of the arrangement and function of the lamprey HPI axis, while highlighting areas that require further investigation. First, we present a comprehensive tissue-specific transcriptional analysis of HPI axis components in the sea lamprey, revealing that both *mcar* and *star* are expressed in a distinctive head kidney region. This finding suggests the presence of a discrete steroidogenic tissue in lamprey, although cellular co-localization studies will be needed to confirm this hypothesis. Second, we provide the first complete pharmacological characterization of both lamprey melanocortin receptors using lamprey-specific peptides. While previous work examined Mcar function in river lamprey (Haitina *et al.* 2007), our study reveals that both Mcar and Mcbr can be activated by lamprey melanocortin peptides, with Mcar showing differential sensitivity to Msh versus Acth peptides. We also demonstrate that the co-expression of Mcar with Mrap reduces receptor activation maxima and reduces the potency of activation by Msh-b activation, nuances in Mcar and Mcbr receptor pharmacology not previously detected. Together, these findings advance our understanding of the molecular components and potential regulatory mechanisms of the lamprey HPI axis.

Expression of HPI axis components

Our gene expression analyses provide compelling evidence for the presence of a functional HPI axis in sea lamprey, albeit with some unique features compared to the classical gnathostome HPI axes. The tissue-specific patterns of transcriptional expression we observed for key HPI axis components largely align with what would be expected based on the gnathostome model, suggesting a considerable degree of conservation in the overall organization of this neuroendocrine system. We acknowledge that analyzing only *crh* genes, rather than including other crh-family genes, may provide an incomplete picture of HPI axis regulation in lamprey. While *crh* is an important signal, other peptides such as urotensin and urocortin (Endsin *et al.* 2017) may also contribute to HPI regulation, as seen with UTS1 in teleosts (Culbert *et al.* 2022). Future studies should examine the relative contributions of these different peptides to the HPI axis in lamprey.

The relatively higher levels of *crh-a*, *-b* and *-c* mRNA in the brain compared to other tissues is consistent with its role as a hypothalamic signal in the HPI axis of gnathostomes (Wendelaar Bonga 1997). Recently, Sobrido-Cameán *et al.* (2023) localized *crh-a*/Crh expression in sea lamprey to the preoptic nucleus/paraventricular nucleus region of the hypothalamus and the reticular formation region of the hindbrain. These Crh-positive neurons appear to

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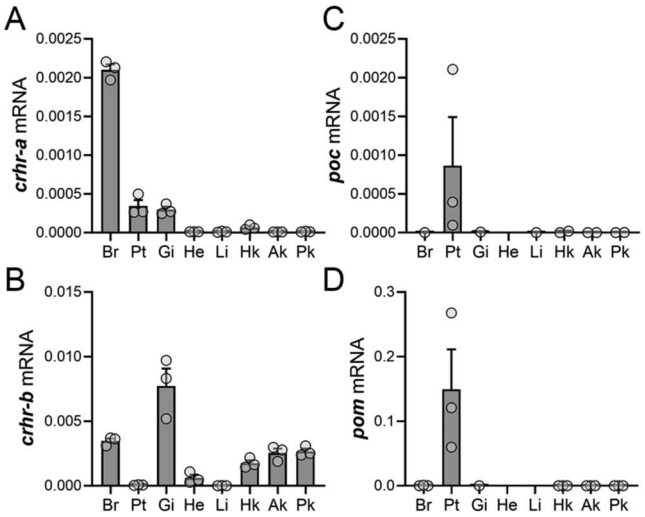

Figure 4

Transcriptional profiles of *crhr-a*, *crhr-b*, *poc* and *pom* in sea lamprey (*Petromyzon marinus*) are presented for various tissues. Relative mRNA abundance is presented as $2^{-\Delta CT}$ using *ef1a* as a reference gene. Data are presented as the mean ± standard error, with individual points shown; missing data points indicate that no amplification was detected. Abbreviations: *crhr*, corticotropin-releasing hormone receptor; *poc*, proopiocortin; *pom*, proopiomelanotropin; *ef1a*, elongation factor 1 alpha; Br, brain; Pt, pituitary; Gi, gill; He, heart; Li, liver; Hk, head kidney; Ak, anterior kidney; Pk, posterior kidney.

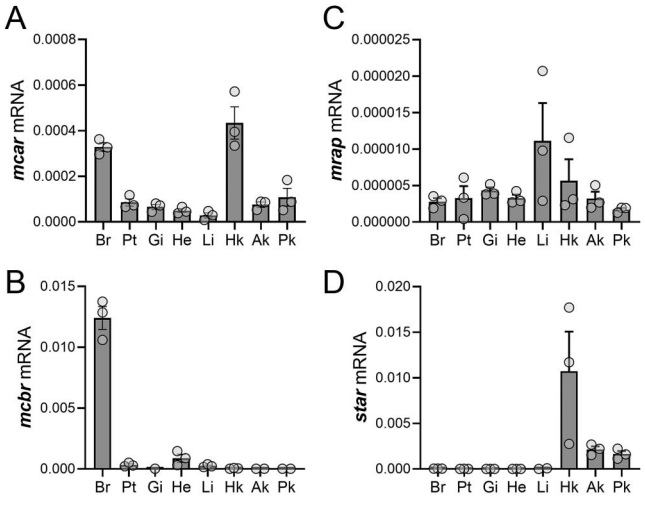

Figure 5

Transcriptional profiles of *mcar*, *mcbr*, *mrap* and *star* in sea lamprey (*Petromyzon marinus*) are presented for various tissues. Relative mRNA abundance is presented as $2^{-\Delta CT}$ using *ef1a* as a reference gene. Data are presented as the mean ± standard error, with individual points shown; missing data points indicate that no amplification was detected. Abbreviations: *mcar*, melanocortin receptor a; *mcbr*, melanocortin receptor b; *mrap*, melanocortin receptor accessory protein; *star*, steroidogenic acute regulatory protein; *ef1a*, elongation factor 1 alpha; Br, brain; Pt, pituitary; Gi, gill; He, heart; Li, liver; Hk, head kidney; Ak, anterior kidney; Pk, posterior kidney.

project from the paraventricular nucleus to the pituitary, indicating a hypothalamus–pituitary Crh signaling pathway is intact in lamprey (Sobrido-Cameán *et al.* 2023). There is some functional evidence to suggest a role for Crh in a corticosteroidogenic HPI axis in lamprey. Intraperitoneal injection of adult lampreys with lamprey or mammalian Crh results in increased plasma 11-deoxycortisol (Close *et al.* 2010, Roberts *et al.* 2014, Rai *et al.* 2015). However, direct evidence that Crh stimulates Acth production in lamprey is still lacking (Roberts *et al.* 2014), representing an important gap in our understanding of the lamprey HPI axis. Which, if any, of the three Crh genes (*crh-a*, *-b*, and *-c*) are involved in stress-responsive HPI signaling will also require further study.

The presumptive receptors to Crh, *crhr-a* and *-b*, have been previously identified (Endsin *et al.* 2017). It has been suggested that these Crh receptor paralogs arose from a lineage-specific gene or genome duplication event in lampreys, rather than being homologous, respectively, to the Crhr1 and -2 of gnathostomes (Endsin *et al.* 2017). Here, we show that *crhr-a* was more highly expressed in the brain than the pituitary, although high levels of mRNA were also detected in the pituitary. On the other hand, *crhr-b* showed very little expression in the pituitary. If an HPI axis exists in sea lamprey, we suspect that the hypothalamic-derived Crh signal likely interacts with the crhr-a receptor ortholog in the pituitary. The regulatory and functional aspects of the Crh–Crhr relationship are ripe for further investigation in agnathans. The nearly exclusive expression of both *poc* and *pom* in the pituitary is consistent with previous work on these lamprey-specific Pomc-like prohormones (Takahashi *et al.* 1995, 2006). Together, the transcriptional patterns of *crh-a*, *-b* and *-c*, *crhr-a*, *poc* and *pom* support the hypothesis that the presence of a hypothalamus–pituitary connection exists and could take part in a lamprey HPI axis, similar to what has been have observed in more derived fishes (Bouyoucos *et al.* 2021).

The kidney of the sea lamprey undergoes substantial changes during metamorphosis. The anterior-positioned opisthonephros of larvae, which contains presumptive interrenal cells (Youson 1973), degenerates and a posterior-positioned opisthonephros appears in post-metamorphic juveniles (Youson 1970). Although the larvae opisthonephros was shown to undergo interrenal cell proliferation in response to mammalian Acth (Youson 1973), a more recent study failed to detect an effect of lamprey Acth on 11-deoxycortisol production using both intraperitoneal injection and ex vivo exposure of kidney tissue (Roberts *et al.* 2014). To our knowledge, no study in lamprey has localized transcriptional expression of *star* or a putative melanocortin receptor to any presumed steroidogenic tissue. Previously, we have shown that *star* is expressed in the opisthonephric kidney of

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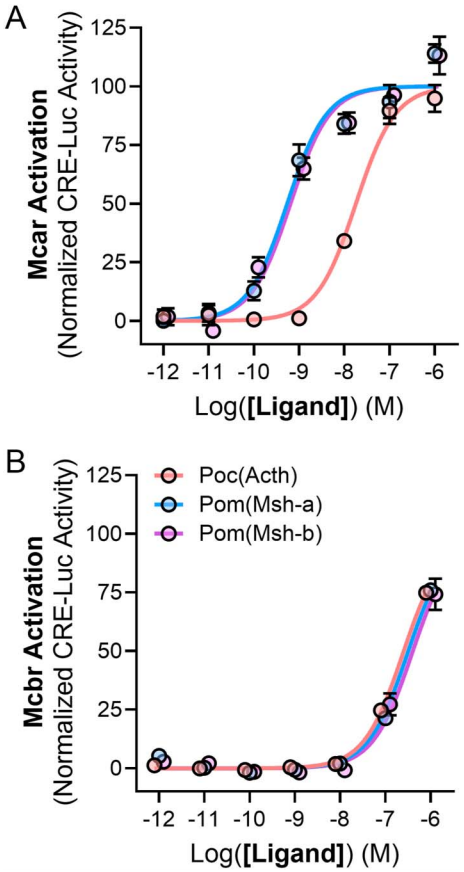

Figure 6

Dose–response of sea lamprey (*Petromyzon marinus*) Mcar (A) and Mcbr (B) expressed in CHO cells stimulated by sea lamprey melanocortin ligands, Acth (encoded by Poc) and Msh-a and Msh-b (encoded by Pom). In both panels, data are presented as normalized cAMP-responsive luciferase activity (R_{max} set to 100 for each curve). Lines represent the fitted dose–response curve (three-parameter polynomial). For clarity, overlapping points/lines have been slightly offset left or right. Data are presented as the mean ± standard error (n = 3). Abbreviations: Mcar, melanocortin receptor a; Mcbr, melanocortin receptor b; Poc, proopiocortin; Pom, proopiomelanotropin; Acth, adrenocorticotropic hormone; Msh, melanocyte stimulating hormone. A full color version of this figure available at https://doi.org/10.1530/JME-24-0126.

juveniles among other tissues (Shaughnessy & McCormick 2021), but this previous study did not seek to finely localize *star* or any melanocortin receptor to any specific region of the juvenile lamprey kidney structure.

In the present experiment in juveniles, we sought to more finely characterize *star* transcriptional expression across the kidney tissue of post-metamorphic sea lamprey. We found that *star* mRNA levels were highest in a 'head kidney'-like region, which is comprised of the highly pigmented tissue extending anterior to the definitive opisthonephros in the juvenile sea lamprey, where the larval opisthonephros once occupied but had since degenerated. Interestingly, this tissue also showed high abundance of *mcar* mRNA. The transcriptional expression of *mcar* and *star* observed in the anterior and posterior opisthonephros may explain how the adult opisthonephros is able to produce some 11-deoxycortisol (Roberts *et al.* 2014), but these opisthonephric regions of the juvenile kidney do not exhibit as high levels of *mcar* and *star* mRNA as were observed the head kidney region. The expression of both *mcar* and *star* in a head kidney region in sea lamprey is reminiscent of the interrenal tissue in gnathostomes and suggests that a head kidney may exist in lamprey and be the primary site of Acth-mediated corticosteroid production. We suspect that future investigations will find that *mcar* and *star* are co-localized to the same interrenal cells and that this head kidney region in juvenile lamprey potently responds to the stimulation by melanocortin peptides (whether Msh-a, Msh-b or Acth) in producing 11-deoxycortisol.

Table 2 Curve fitting analysis of the activation of *P. marinus* Mcar and Mcbr by *P. marinus* melanocortins.

Transfection	Ligand	Log(EC_{50}) (M)
Mcar	Poc(Acth)	−7.74 (−7.91, −7.58)
Mcar	Pom(Msh-a)	−9.26 (−9.54, −8.99)
Mcar	Pom(Msh-b)	−9.29 (−9.6, −9)
Mcbr	Poc(Acth)	−6.53 (−6.65, −6.39)
Mcbr	Pom(Msh-a)	−6.38 (−6.57, −6.12)
Mcbr	Pom(Msh-b)	−6.61 (−6.83, −6.33)

Data are presented as the mean (95% CI; low, high). One-way ANOVA: Mcar, $P < 0.001$; Mcar, $P < 0.001$. Abbreviations: Mcar, melanocortin receptor a; Mcbr, melanocortin receptor b; Poc, proopiocortin; Pom, proopiomelanotropin; Acth, adrenocorticotropic hormone; Msh, melanocyte stimulating hormone.

It is notable that *mcbr* was not transcriptionally expressed in the kidney tissues, with its expression restricted primarily to the brain. A previous study on river lamprey (*Lampetra fluviatilis*) showed expression of *mcar* primarily in the skin and *mcbr* most prominently in the liver and skin (Haitina *et al.* 2007). No kidney tissue was examined in this previous study, but the tissue profile did include brain, although no expression of *mcar* or *mcbr* was detected in the brain. The differences observed between the study by Haitina *et al.* (2007) and the present study in the tissue distribution of *mcar* and *mcbr* are difficult to explain and warrant additional investigation. While sea lamprey and river lamprey are both Northern Hemisphere lampreys of the family Petromyzontidae that diverged approximately 40 million years ago (Brownstein & Near 2023), the methodological differences between the present study and the study by Haitina *et al.* (2007), including differences in life stages studied, methods of tissue collection and preservation and forms of PCR used for detection of mRNA, make direct comparisons challenging.

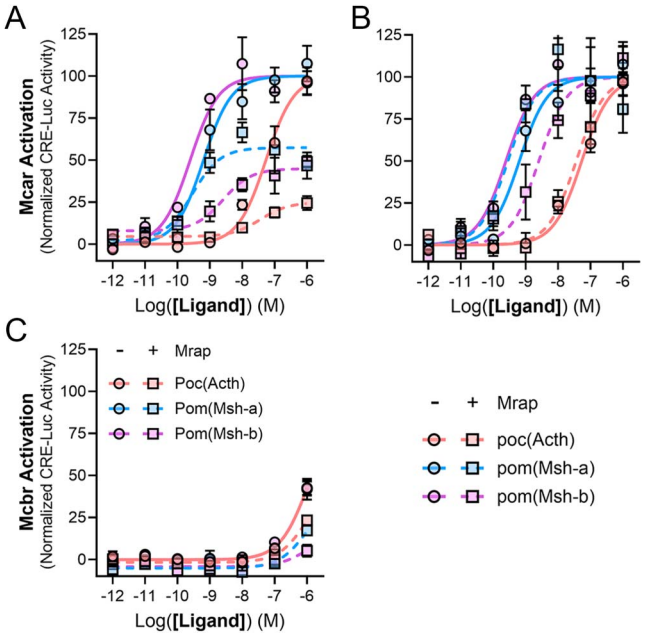

Figure 7

Dose–response of sea lamprey (*Petromyzon marinus*) Mcar (A and B) and Mcbr (C) expressed with or without sea lamprey Mrap in CHO cells stimulated by sea lamprey melanocortin ligands, Acth (encoded by Poc) and Msh-a and Msh-b (encoded by Pom). Panel A and B depict the same assay, but with differing normalizations. In A, R_{max} is set to 100 for the '– Mrap' control but allowed to vary in the '+ Mrap' groups; in B, R_{max} is set to 100 for all groups to more clearly visualize EC_{50} differences. Lines represent the fitted dose–response curve (three-parameter polynomial). Data are presented as the mean ± standard error (n = 3). Abbreviations: Mcar, melanocortin receptor a; Mcbr, melanocortin receptor b; Mrap, melanocortin receptor accessory protein; Poc, proopiocortin; Pom, proopiomelanotropin; Acth, adrenocorticotropic hormone; Msh, melanocyte stimulating hormone. A full color version of this figure available at https://doi.org/10.1530/JME-24-0126.

Phylogenetic and functional characterization of sea lamprey melanocortin receptors

Our molecular phylogenetic analysis of the sea lamprey Mcar and Mcbr describe Mcar as being more similar to Mc1r and Mc2r and Mcbr as being more similar to the gnathostome Mc3r, Mc4r and Mc5r. These relationships have been reported before for Mcar and Mcbr and interpreted as indicating that Mcar and Mcbr were ancestral forms of the Mc1r/Mc2r and Mc3r/Mc4r/Mc5r clades, respectively, which arose after the second whole-genome duplication ('2R') (Haitina *et al.* 2007). The most recent analyses of the agnathan genomes have indicated that indeed, a first whole genome ('1R') occurred in a stem vertebrate and the 2R event occurred after the agnathan–gnathostome divergence (Nakatani *et al.* 2021, Yu *et al.* 2024). However, these analyses and others have also revealed that the agnathan lineage has undergone one or two additional rounds of genome duplication (Yu *et al.* 2024). A deeper syngenetic analysis of the melanocortin receptor family will be needed to more precisely determine the relationship of Mcar and Mcbr to the gnathostome melanocortin receptor counterparts.

Our functional characterization of the sea lamprey Mcar and Mcbr revealed several important features that distinguish them from gnathostome melanocortin receptors, particularly Mc2r. These differences provide valuable insights into the evolution of the melanocortin system and the HPI axis in vertebrates. First, both lamprey Mcar and Mcbr can be activated by Acth, Msh-a and Msh-b. The greater potency of the Msh ligands over Acth in activating the sea lamprey Mcar reflects the findings previously reported for the river lamprey Mcar (Haitina *et al.* 2007). These functional properties of the lamprey Mcar are in stark contrast to the gnathostome Mc2r, which is highly selective for Acth and cannot be activated by Msh peptides (Dores & Chapa 2021). The promiscuity of the lamprey receptors indicates that the ligand selectivity of gnathostome Mc2r may be a derived trait that evolved after the divergence of jawless and jawed vertebrates. That Msh-a and Msh-b exhibited a much greater potency in activating Mcar than Acth is somewhat counterintuitive, given the presumed role of Acth in stimulating corticosteroid production. However, it is important to note that the relative physiological concentrations of these peptides in lampreys are not yet known, and it is possible that Acth could still be the primary endogenous ligand for Mcar in the context of the HPI axis. The present study is the first to report on the relative ability of sea lamprey melanocortin peptides to activate lamprey Mcbr, demonstrating that all

Table 3 Curve fitting analysis of the activation of *P. marinus* Mcar co-expressed with *P. marinus* Mrap by *P. marinus* melanocortins.

Transfection	Ligand	Log(EC_{50}) (M)	R_{max} (%)
Mcar	Poc(Acth)	−7.27 (−7.52, −7.05)	100 (90, 111)
Mcar + Mrap	Poc(Acth)	−7.44 (−7.96, −6.94)	25 (21, 30)
Mcar	Pom(Msh-a)	−9.2 (−9.53, −8.86)	100 (89, 111)
Mcar + Mrap	Pom(Msh-a)	−9.55 (−10.06, −9.05)	57 (49, 66)
Mcar	Pom(Msh-b)	−9.61 (−9.91, −9.3)	100 (91, 109)
Mcar + Mrap	Pom(Msh-b)	−8.59 (−9.28, −7.9)	45 (38, 53)

Data are presented as the mean (95% CI; low, high). Two-way ANOVA results for Log(EC_{50}): Peptide, $P < 0.001$; Mrap, $P = 0.416$; Interaction, $P = 0.015$. Two-way ANOVA results for R_{max}: Peptide, $P = 0.008$; Mrap, $P < 0.001$; Interaction, $P = 0.008$. Abbreviations: Mcar, melanocortin receptor a; Poc, proopiocortin; Pom, proopiomelanotropin; Acth, adrenocorticotropic hormone; Msh, melanocyte stimulating hormone.

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melanocortin ligands had equal potency in activating Mcbr. This is similar to the known properties of the gnathostome Mc3r, Mc4r and Mc5r as 'generic receptors' (Dores *et al.* 2014). Further physiological investigations are needed to determine the activation of a potential HPI axis and regulation of the lamprey melanocortin ligands in response to stress.

Role of Mrap in sea lamprey melanocortin signaling

In bony vertebrates, Mc2r requires Mrap1 for both trafficking to the cell membrane and activation by Acth (Dores & Chapa 2021). The ability of both Mcar and Mcbr to be activated without the co-expression of Mrap is an important functional difference from the gnathostome system. The lamprey Mrap lacks the putative δ-D-Y-δ activation motif (Dores *et al.* 2022), which is located in the N-terminal domain of the Mrap1s of bony vertebrates and is critical in Mc2r activation (as reviewed by Dores & Chapa 2021). Our findings in lamprey, combined with similar observations in cartilaginous fishes (Barney *et al.* 2019, Hoglin *et al.* 2022, Bouyoucos *et al.* 2023), suggest that Mrap dependence is a derived feature of the gnathostome Mc2r that evolved later in vertebrate evolution, possibly coinciding with the specialization of Mc2r as the dedicated 'Acth receptor' (Mountjoy *et al.* 1992).

Interestingly, we did observe transcriptional expression of *mrap* in various lamprey tissues, including the head kidney where *mcar*, *mcbr* and *star* are also expressed. Given the Mrap independence of the lamprey melanocortin receptors, the functional significance of Mrap in lamprey tissues remains unclear. It has been shown that sea lamprey Mrap directly interacts with and decreases the surface expression of sea lamprey Mcar and Mcbr (Zhu *et al.* 2019). This corresponds to our results that co-expression with Mrap reduced the R_{max} of the Mcar when activated by all three melanocortin peptides. In slight contrast to the observation by Zhu *et al.* (2019) that Mrap had no effect on EC_{50} values of Mcar or Mcbr for mammalian α-Msh, we observed that co-expression with Mrap significantly decreased the potency of Msh-a in activating Mcar. This discrepancy could be due to the higher sensitivity of luciferase-mediated transactivation assays used in the present study. Physiologically, the role of Mrap in lampreys is still unclear. Due its role in downregulating Mcar surface expression and reducing the R_{max} and potency to which melanocortins activate Mcar, it appears Mrap may have a inhibitory modulatory role in lamprey melanocortin signaling, similar to the inhibitory effects of Mrap2 on the mammalian Mc5r (Ji *et al.* 2022). Further studies investigating the regulation of the lamprey Mrap and potential interactions between it and other proteins could help elucidate its function in these early vertebrates.

Evolutionary implications of sea lamprey melanocortin system

Our characterization of the sea lamprey melanocortin system provides additional insights into the evolutionary history of this important neuroendocrine signaling pathway. The sea lamprey, as a model jawless vertebrate, offers an opportunity to make comparative evolutionary analyses of the vertebrate melanocortin system and how it has evolved in the vertebrate lineage.

The pattern that has emerged indicates that step-wise shifts in melanocortin receptor specificity and Mrap dependence took place throughout vertebrate evolution. Melanocortin receptor-like proteins that may be the precursor to the vertebrate Mcrs have been identified in the protochordates, as they exhibit mild sequence similarity to vertebrate Mcrs and expected coupling with G proteins but do not exhibit any functional activation by melanocortin peptides (Ji *et al.* 2024). The lamprey melanocortin receptors can be functionally activated by melanocortin peptides, yet still appear to represent the least specialized state among vertebrate Mcrs, with broad ligand specificity and Mrap independence. The cartilaginous fish system represents some intermediate state, with some preference for Acth developing and varying degrees of Mrap dependence (Reinick *et al.* 2012, Hoglin *et al.* 2022, Bouyoucos *et al.* 2023). Finally, in even the earliest bony vertebrates (i.e., chondrostean fishes and lungfishes), we see the highly specialized system with Mc2r being exclusively selective for Acth and strictly dependent on Mrap1 chaperoning for cell surface expression and functional activation (Shaughnessy *et al.* 2022, 2023*a*,*b*).

The evolution of this specialized system in bony vertebrates likely allowed for more precise control of the stress response through the HPA/I axis. By having a dedicated Acth receptor (i.e., Mc2r) that is highly specific in its ligand binding and tissue expression, bony vertebrates can more tightly regulate corticosteroid production in response to stress. This specialization may have provided significant adaptive advantages, allowing for more nuanced and effective stress responses in the face of diverse environmental challenges. However, it is important to note that the more flexible system seen in lampreys and cartilaginous fishes may have its own advantages. The ability to respond to multiple melanocortin peptides through the same receptors could allow for a more diverse range of physiological responses, potentially providing greater adaptability in certain contexts.

The greater potency of Msh peptides compared to Acth in activating Mcar raises an intriguing possibility regarding regulation of the lamprey HPI axis. While previous studies failed to detect increases in plasma 11-deoxycortisol following Acth administration (Roberts *et al.* 2014, Rai *et al.* 2015), injection of pituitary extracts did elevate plasma 11-deoxycortisol (Close *et al.* 2010).

Our findings that Msh peptides more potently activate Mcar than Acth, indicate that Msh peptides, rather than Acth, may serve as the primary endogenous regulators of corticosteroid production in lamprey. This hypothesis would explain why pituitary extracts (that contain both Acth and Msh peptides) effectively stimulated 11-deoxycortisol production (Close *et al.* 2010) while purified Acth alone did not (Roberts *et al.* 2014). Such an arrangement would represent a novel regulatory mechanism compared to gnathostomes, where Acth is the primary regulator of corticosteroid production. However, determining the relative physiological roles of these peptides in the lamprey HPI axis will require careful analysis of their endogenous concentrations, regulation and action.

Limitations and future directions

While our study provides new insights into the sea lamprey HPI axis and melanocortin system, limitations and areas requiring further research should be acknowledged. First, our use in our tissue profile analyses of whole brain rather than more finely regionalized brain tissues (including separating hypophysiotropic from non-hypophysiotropic regions) limits quantitative interpretations that can be made regarding the relative expression of the various *crh* paralogs in hypophysiotropic tissue. Localization and relative expression of each of the Crh paralogs within the hypophysiotropic regions of the brain remains a productive goal for future HPI axis investigations in lampreys. Furthermore, future studies should examine the functional (i.e., binding and activation) relationships between the lamprey Crh and Crhr paralogs. Second, our functional characterization of Mcar and Mcbr was performed in a heterologous cell system *in vitro*. While this approach is widely used and provides valuable information, it may not fully recapitulate the complexity of receptor function *in vivo*. Future studies using lamprey-derived cell lines or *in vivo* approaches could provide additional insights into the physiological roles of these receptors. Third, while we demonstrated that Mcar and Mcbr can be activated by Acth and Msh peptides, we do not yet know the factors controlling their release or their physiological concentrations in lampreys. Understanding the endogenous levels of these peptides would help clarify their relative importance *in vivo* and could provide further insights into the functional evolution of the melanocortin system. Finally, our study focused primarily on mRNA expression of HPI axis components. While this provides valuable information about gene expression, it does not necessarily reflect protein levels or activity. In addition, while our gene expression data suggest the presence of both *star* and *mcar* in the head kidney region, we acknowledge that definitive evidence of cellular co-localization within steroidogenic cells would require microscopic analyses such as *in situ* hybridization or immunohistochemistry. The lamprey head kidney likely contains multiple cell types, as seen in teleost interrenal tissue. Future studies incorporating protein-level analyses and functional assays in lamprey tissues would complement our findings and provide a more definitive account of HPI axis expression and function in these animals.

Conclusions

In the present manuscript, we observed tissue-specific expression of key genes encoding presumed components of an HPI axis in sea lamprey, including expression of both *mcar* and *star* in a 'head kidney' region in juvenile lamprey. We offered additional characterization of the two melanocortin receptor subtypes in sea lamprey, which occupy basal phylogenetic positions relative to the larger family of melanocortin receptors in gnathostomes. Both melanocortin receptors can be activated by sea lamprey Poc-derived Acth and Pom-derived Msh peptides without requiring Mrap, with these peptides exhibiting far greater potencies in activating Mcar than Mcbr. Based on the lower concentrations necessary for activation of Mcar and the expression of *mcar* with *star* in head kidney tissue, it is likely that Mcar is an important component of the lamprey HPI axis. Taken together, our study provides a broad characterization of the HPI axis and melanocortin system in the sea lamprey, offering a plausible arrangement of this axis, and thus providing valuable insight for further investigation into the evolutionary origins of this important neuroendocrine systems in vertebrates.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the work reported.

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References

Anderson WG 2015 Endocrine systems in elasmobranchs. In *Fish Physiology*, vol 34, pp 457–530. Amsterdam, Netherlands: Elsevier. (https://doi.org/10.1016/B978-0-12-801286-4.00008-3)

Barney E, Dores MR, McAvoy D, *et al.* 2019 Elephant shark melanocortin receptors: novel interactions with MRAP1 and implication for the HPI axis. *Gen Comp Endocrinol* **272** 42–51. (https://doi.org/10.1016/j.ygcen.2018.11.009)

Barton BA 2002 Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr Comp Biol* **42** 517–525. (https://doi.org/10.1093/icb/42.3.517)

Bernier NJ, Flik G & Klaren PHM 2009 Regulation and contribution of the corticotropic, melanotropic and thyrotropic axes to the stress response in fishes. In *Fish Physiology*, vol 28, pp 235–311. Amsterdam, Netherlands: Elsevier. (https://doi.org/10.1016/S1546-5098(09)28006-X)

Best C, Culbert BM & Bernier NJ 2024 The hypothalamic-pituitary-interrenal axis and corticosteroids. In *Encyclopedia of Fish Physiology*, 2nd edn. pp 217–232. Cambridge, MA, USA: Elsevier. (https://doi.org/10.1016/B978-0-323-90801-6.00145-2)

Bouyoucos IA, Schoen AN, Wahl R, *et al.* 2021 Ancient fishes and the functional evolution of the corticosteroid stress response in vertebrates. *Comp Biochem Physiol A Mol Integr Physiol* **260** 111024. (https://doi.org/10.1016/j.cbpa.2021.111024)

Bouyoucos IA, Shaughnessy CA, Gary Anderson W, *et al.* 2023 Molecular and pharmacological analysis of the melanocortin-2 receptor and its accessory proteins Mrap1 and Mrap2 in a Squalomorph shark, the Pacific spiny dogfish. *Gen Comp Endocrinol* **342** 114342. (https://doi.org/10.1016/j.ygcen.2023.114342)

Brownstein CD & Near TJ 2023 Phylogenetics and the Cenozoic radiation of lampreys. *Curr Biol* **33** 397–404.e3. (https://doi.org/10.1016/j.cub.2022.12.018)

Cardoso JCR, Bergqvist CA & Larhammar D 2020 Corticotropin-releasing hormone (CRH) gene family duplications in lampreys correlate with two early vertebrate genome doublings. *Front Neurosci* **14** 1–17. (https://doi.org/10.3389/fnins.2020.00672)

Chan LF, Webb TR, Chung TT, *et al.* 2009 MRAP and MRAP2 are bidirectional regulators of the melanocortin receptor family. *Proc Natl Acad Sci U S A* **106** 6146–6151. (https://doi.org/10.1073/pnas.0809918106)

Chepurny OG & Holz GG 2007 A novel cyclic adenosine monophosphate-responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein-coupled receptor drug discovery efforts. *J Biomol Screen* **12** 740–746. (https://doi.org/10.1177/1087057107301856)

Close DA, Yun S-S, McCormick SD, *et al.* 2010 11-Deoxycortisol is a corticosteroid hormone in the lamprey. *Proc Natl Acad Sci U S A* **107** 13942–13947. (https://doi.org/10.1073/pnas.0914026107)

Cone RD 2006 Studies on the physiological functions of the melanocortin system. *Endocr Rev* **27** 736–749. (https://doi.org/10.1210/er.2006-0034)

Culbert BM, Regish AM, Hall DJ, *et al.* 2022 Neuroendocrine regulation of plasma cortisol levels during smoltification and seawater acclimation of atlantic salmon. *Front Endocrinol* **13** 1–22. (https://doi.org/10.3389/fendo.2022.859817)

Denver RJ 2009 Structural and functional evolution of vertebrate neuroendocrine stress systems. *Ann N Y Acad Sci* **1163** 1–16. (https://doi.org/10.1111/j.1749-6632.2009.04433.x)

Dores RM, Londraville RL, Prokop J, *et al.* 2014 Molecular evolution of GPCRs: melanocortin/melanocortin receptors. *J Mol Endocrinol* **52** T29–T42. (https://doi.org/10.1530/JME-14-0050)

Dores RM & Chapa E 2021 Hypothesis and theory: evaluating the Co-evolution of the melanocortin-2 receptor and the accessory protein MRAP1. *Front Endocrinol* **12** 1–10. (https://doi.org/10.3389/fendo.2021.747843)

Dores RM, Mckinley G, Meyers A, *et al.* 2022 Structure/function studies on the activation motif of two non-mammalian Mrap1 orthologs: observations on the phylogeny of Mrap1, including a novel characterization of an Mrap1 from the chondrostean fish, polyodon spathula. *Biomolecules* **12** 1681. (https://doi.org/10.3390/biom12111681)

Endsin MJ, Michalec O, Manzon LA, *et al.* 2017 CRH peptide evolution occurred in three phases: evidence from characterizing sea lamprey CRH system members. *Gen Comp Endocrinol* **240** 162–173. (https://doi.org/10.1016/j.ygcen.2016.10.009)

Haitina T, Klovins J, Takahashi A, *et al.* 2007 Functional characterization of two melanocortin (MC) receptors in lamprey showing orthology to the MC1 and MC4 receptor subtypes. *BMC Evol Biol* **7** 101–114. (https://doi.org/10.1186/1471-2148-7-101)

Hoglin BE, Miner M & Dores RM 2022 Pharmacological properties of whale shark (Rhincodon typus) melanocortin-2 receptor and melancortin-5 receptor: interaction with MRAP1 and MRAP2. *Gen Comp Endocrinol* **315** 113915. (https://doi.org/10.1016/j.ygcen.2021.113915)

Inoue JG, Miya M, Lam K, *et al.* 2010 Evolutionary origin and phylogeny of the modern holocephalans (chondrichthyes: chimaeriformes): a mitogenomic perspective. *Mol Biol Evol* **27** 2576–2586. (https://doi.org/10.1093/molbev/msq147)

Ji L-Q, Hong Y & Tao Y-X 2022 Melanocortin-5 receptor: pharmacology and its regulation of energy metabolism. *Int J Mol Sci* **23** 8727. (https://doi.org/10.3390/ijms23158727)

Ji R-L, Jiang S-S, Kleinau G, *et al.* 2024 Are melanocortin receptors present in extant protochordates? *Biomolecules* **14** 1120. (https://doi.org/10.3390/biom14091120)

Liang L, Sebag JA, Eagelston L, *et al.* 2011 Functional expression of frog and rainbow trout melanocortin 2 receptors using heterologous MRAP1s. *Gen Comp Endocrinol* **174** 5–14. (https://doi.org/10.1016/j.ygcen.2011.07.005)

Mommsen TP, Vijayan MM & Moon TW 1999 Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev Fish Biol Fish* **9** 211–268. (https://doi.org/10.1023/A)

Mountjoy KG, Robbins LS, Mortrud MT, *et al.* 1992 The cloning of a family of genes that encode the melanocortin receptors. *Science* **257** 1248–1251. (https://doi.org/10.1126/science.1325670)

Nakatani Y, Shingate P, Ravi V, *et al.* 2021 Reconstruction of proto-vertebrate, proto-cyclostome and proto-gnathostome genomes provides new insights into early vertebrate evolution. *Nat Commun* **12** 4489. (https://doi.org/10.1038/s41467-021-24573-z)

Noon LA, Franklin JM, King PJ, *et al.* 2002 Failed export of the adrenocorticotrophin receptor from the endoplasmic reticulum in non-adrenal cells: evidence in support of a requirement for a specific adrenal accessory factor. *J Endocrinol* **174** 17–25. (https://doi.org/10.1677/joe.0.1740017)

Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res* **29** e45. (https://doi.org/10.1093/nar/29.9.e45)

Rai S, Szeitz A, Roberts BW, *et al.* 2015 A putative corticosteroid hormone in Pacific lamprey, Entosphenus tridentatus. *Gen Comp Endocrinol* **212** 178–184. (https://doi.org/10.1016/j.ygcen.2014.06.019)

Reinick CL, Liang L, Angleson JK, *et al.* 2012 Identification of an MRAP-independent melanocortin-2 receptor: functional expression of the cartilaginous fish, callorhinchus milii, melanocortin-2 receptor in CHO cells. *Endocrinology* **153** 4757–4765. (https://doi.org/10.1210/en.2012-1482)

Roberts BW, Didier W, Rai S, *et al.* 2014 Regulation of a putative corticosteroid, 17,21-dihydroxypregn-4-ene,3,20-one, in sea lamprey, Petromyzon marinus. *Gen Comp Endocrinol* **196** 17–25. (https://doi.org/10.1016/j.ygcen.2013.11.008)

Sebag JA & Hinkle PM 2007 Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers. *Proc Natl Acad Sci U S A* **104** 20244–20249. (https://doi.org/10.1073/pnas.0708916105)

Shaughnessy CA & McCormick SD 2020 Functional characterization and osmoregulatory role of the Na^+-K^+-$2Cl^-$cotransporter in the gill of sea lamprey (*Petromyzon marinus*), a basal vertebrate. *Am J Physiol Regul Integr Comp Physiol* **318** R17–R29. (https://doi.org/10.1152/ajpregu.00125.2019)

Shaughnessy CA & McCormick SD 2021 11-Deoxycortisol is a stress responsive and gluconeogenic hormone in a jawless vertebrate, the sea lamprey (Petromyzon marinus). *J Exp Biol* **224** jeb241943. (https://doi.org/10.1242/jeb.241943)

Shaughnessy CA, Jensen MF & Dores RM 2022 A basal actinopterygian melanocortin receptor: molecular and functional characterization of an Mc2r ortholog from the Senegal bichir (Polypterus senegalus). *Gen Comp Endocrinol* **328** 114105. (https://doi.org/10.1016/j.ygcen.2022.114105)

Shaughnessy CA, Le K, Myhre VD, *et al.* 2023*a* Functional characterization of melanocortin 2 receptor (Mc2r) from a lobe-finned fish (Protopterus annectens) and insights into the molecular evolution of melanocortin receptors. *Gen Comp Endocrinol* **343** 114356. (https://doi.org/10.1016/j.ygcen.2023.114356)

Shaughnessy CA, Myhre VD, Hall DJ, *et al.* 2023*b* Hypothalamus-pituitary-interrenal (HPI) axis signaling in atlantic sturgeon (acipenser oxyrinchus) and sterlet (acipenser ruthenus). *Gen Comp Endocrinol* **339** 114290. (https://doi.org/10.1016/j.ygcen.2023.114290)

Sobrido-Cameán D, González-Llera L, Anadón R, *et al.* 2023 Organization of the corticotropin-releasing hormone and corticotropin-releasing hormone-binding protein systems in the central nervous system of the sea lamprey Petromyzon marinus. *J Comp Neurol* **531** 58–88. (https://doi.org/10.1002/cne.25412)

Takahashi A, Amemiya Y, Nozaki M, *et al.* 1995 Isolation and characterization of melanotropins from lamprey pituitary glands. *Int J Pept Protein Res* **46** 197–204. (https://doi.org/10.1111/j.1399-3011.1995.tb00589.x)

Takahashi A, Nakata O, Moriyama S, *et al.* 2006 Occurrence of two functionally distinct proopiomelanocortin genes in all modern lampreys. *Gen Comp Endocrinol* **148** 72–78. (https://doi.org/10.1016/j.ygcen.2005.09.003)

Takahashi A, Davis P, Reinick C, *et al.* 2016 Characterization of melanocortin receptors from stingray Dasyatis akajei, a cartilaginous fish. *Gen Comp Endocrinol* **232** 115–124. (https://doi.org/10.1016/j.ygcen.2016.03.030)

Tamura K, Stecher G & Kumar S 2021 MEGA11: molecular evolutionary genetics analysis version 11. *Mol Biol Evol* **38** 3022–3027. (https://doi.org/10.1093/molbev/msab120)

Valsalan R, Krishnan A, Almén MS, *et al.* 2013 Early vertebrate origin of melanocortin 2 receptor accessory proteins (MRAPs). *Gen Comp Endocrinol* **188** 123–132. (https://doi.org/10.1016/j.ygcen.2013.01.004)

Västermark Å & Schiöth HB 2011 The early origin of melanocortin receptors, agouti-related peptide, agouti signalling peptide, and melanocortin receptor-accessory proteins, with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus. *Eur J Pharmacol* **660** 61–69. (https://doi.org/10.1016/j.ejphar.2010.10.106)

Wendelaar Bonga SE 1997 The stress response in fish. *Physiol Rev* **77** 591–625. (https://doi.org/10.1152/physrev.1997.77.3.591)

Youson JH 1970 Observations on the opisthonephric kidney of the sea lamprey of the great lakes, *Petromyzon marinus* L., at various stages during the life cycle. *Can J Zool* **48** 1313–1316. (https://doi.org/10.1139/z70-223)

Youson JH 1973 A comparison of presumptive interrenal tissue in the opisthonephric kidney and dorsal vessel region of the larval sea lamprey, Petromyzon marinus L. *Can J Zool* **51** 796–799. (https://doi.org/10.1139/z73-118)

Yu D, Ren Y, Uesaka M, *et al.* 2024 Hagfish genome elucidates vertebrate whole-genome duplication events and their evolutionary consequences. *Nat Ecol Evol* **8** 519–535. (https://doi.org/10.1038/s41559-023-02299-z)

Zhu M, Xu B, Wang M, *et al.* 2019 Pharmacological modulation of MRAP2 protein on melanocortin receptors in the sea lamprey. *Endocr Connect* **8** 378–388. (https://doi.org/10.1530/ec-19-0019)