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SHORT NOTE

Melanin-concentrating hormone is not involved in luminescence emission in the velvet belly lanternshark, *Etmopterus spinax*

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Abstract
Luminous deep-sea etmopterid sharks use hormonal control to regulate bioluminescence. Melatonin and prolactin trigger light emission and, conversely, α-melanocyte stimulating hormone actively reduces ongoing luminescence. Interestingly, these hormones are also known as regulators of skin pigment motion in teleost fish and epipelagic elasmobranchs. On the other hand, the melanin-concentrating hormone (MCH) is another regulator of the skin pigment motion in fish melanophores. Here, we studied the putative effect of MCH on the light emission control of the velvet belly lanternshark, *Etmopterus spinax* (Etmopteridae). In parallel, the presence of the MCH receptor in our model is investigated through database searches. Our results show that MCH is not involved in the bioluminescence triggering in the velvet belly lanternshark. Moreover, no MCH receptor transcript was found in a specific transcriptome of the luminous ventral skin of *E. spinax*.

Introduction

Etmopterid sharks are known to possess the ability to emit an intrinsic luminescence thanks to thousands of light organs, the photophores, spread mainly within the ventral epidermis (Claes and Mallefet 2009a, b; Renwart et al. 2014; Duchatelet et al. 2019a). Ultrastructurally depicted by Renwart et al. (2014), photophores are composed of emitting cells, called photocytes, embedded in a cup-shaped pigmented sheath, upholstered by a guanine crystal layer reflecting the light toward the outside. A specific shutter-like area, called the iris-like structure (ILS), is located between photocytes and lens cells topping the organ (Claes and Mallefet 2009a; Renwart et al. 2014). ILS cells include melanophores which control the amount of light emitted through pigment motion regulation (Claes and Mallefet 2010; Renwart et al. 2015). Dispersion of melanosome corresponds to the photophore closure, while pigment aggregation results in a photophore open state allowing light to pass toward the outside (Claes and Mallefet 2010; Renwart et al. 2015). Counterillumination, aposematism, and intraspecific communication are the main assumed bioluminescent function for this shark family (Claes and Mallefet 2008, 2009a; Claes et al. 2010a, 2013; Duchatelet et al. 2019b). Etmopterids regulate their light emission through a hormonal control. While melatonin (MT) and prolactin (PRL) trigger light emission, α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) decrease luminescence (Claes and Mallefet 2009a; Duchatelet et al. 2019c). In addition, pharmacological studies highlighted the involvement of neuromodulators such as nitric oxide (NO) and γ-aminobutyric acid (GABA) in the etmopterid light emission control (Claes et al. 2010b, 2011; Claes and Mallefet 2015).

Interestingly, the previously mentioned actors are regulators of the pigment motion in teleost, amphibian, and to a lesser extent, mammals (Nery and de Lauro Castrucci 1997; Fujii 2000; Sköld et al. 2002; Takahashi and Kawauchi 2006; Aspengren et al. 2008; Slominski et al. 2008; Cal et al. 2017). Other actors also regulate pigment aggregation or dispersion in teleost chromatophores such as the melanin-concentrating hormone (MCH), catecholamines or light cues through opsin-based photoreception (Fujii 2000, for review). MCH has been shown to be involved in melanophore pigment aggregation in all investigated teleost species

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to date (Nagai et al. 1986; Oshima et al. 1986; Baker 1993; Kawauchi 2006; Mizusawa et al. 2011, 2013) while it is also acting, in mammals, on different taxa-specific functions such as appetite and homeostasis regulation (Rossi et al. 1997; Griffond and Baker 2002; Saito and Nagasaki 2008). In elasmobranchs, although the presence of this hormone has been confirmed in the shark brain (Vallarino et al. 1989; Mizusawa et al. 2012), no effect on skin pigment motion has been observed in two Neoselachii species [i.e. (1) the batoid freshwater stingray *Potamotrygon reticulatus* (Visconti and Castrucci 1993; Visconti et al. 1999) and (2) the Galeomorphii two banded houndshark *Triakis scyllium* (Mizusawa et al. 2012)]. Although no effect of MCH on skin pigment motion has been described in few swallow water sharks previously investigated, no study clearly states the potential involvement of this hormone on skin melanophores of deep-sea sharks. Here, the putative effect of MCH on the light emission and the skin pigment motion of the lanternshark species *Etmopterus spinax* was tested and searches for MCH receptors were performed within the recently available *E. spinax* skin transcriptome. Results demonstrated the inefficiency of MCH to trigger light emission. No pigment movements in the ILS melanophore cells were induced by MCH applications. Furthermore, MCH receptor mRNAs were not found within the ventral skin reference transcriptome of this shark.

Materials and methods

Animal collection

Sixteen adult velvet belly lanternsharks, *E. spinax* were sampled during a field session in January 2017 by longlines in the Raunefjord, Norway (60°15′54″N; 05°07′46″E). Collected specimens were maintained in dark conditions in tanks filled with running cold sea water at the Bergen University Marine Station (Espegrend, Norway). All sharks were euthanised by a blow to the head followed by a full incision of the spinal cord, following the local rules for experimental vertebrate care and the European rules for animal handling. Sharks were sexed, measured and weighed before experimentation took place.

Pharmacological tests

Skin tissues were dissected from the ventral luminous area of each shark. Round shape skin patches were sampled from the skin using a 6 mm diameter metal cap driller (Claes and Mallefet 2009c; Duchatelet et al. 2019c). These ventral skin patches were rinsed with shark saline [292 mmol l^{-1} NaCl, 3.2 mmol l^{-1} KCl, 5 mmol l^{-1} CaCl2, 0.6 MgSO4, 1.6 mmol l^{-1} Na_2SO_4, 300 mmol l^{-1} urea, 150 mmol l^{-1} trimethylamine N-oxide, 10 mmol l^{-1} glucose, 6 mmol l^{-1} $NaHCO_3$; total osmolarity: 1.080 mosmol; pH 7.7 (Bernal et al. 2005)] in a 96-well plate. Afterward, ventral skin patches were soaked in either MT 10^{-6} mol l^{-1}, PRL 10^{-6} mol l^{-1}, MCH 10^{-8} to 10^{-6} mol l^{-1} or shark saline (as control). Drugs were obtained at Sigma, St. Louis, Missouri, USA. Application of either MT or PRL served as control of the light emission induction since these hormones are known to trigger shark bioluminescence (Claes and Mallefet 2009c, 2010; Duchatelet et al. 2019c). Luminescence of ventral skin patches subjected to the various treatments was measured using a microplate luminometer (Berthold MPL12/Orion; Pforzheim, Germany) calibrated using a standard 470 nm light source (Beta light; Saunders Technology, Hayes, UK). Light outputs were recorded using Simplicity software (Berthold, Pforzheim, Germany) during 60 min with a measurement every minute. Luminescence responses were characterised according to the total amount of light emitted during this experimentation [Ltot, in Gigaquanta per hour (Gq h^{-1}); Claes and Mallefet 2009c; Duchatelet et al. 2019c]. This light parameter was standardised according to the skin patch surface area [(in cm^2); Duchatelet et al. 2019c].

At the end of each pharmacological experiments, skin-associated photophore states were pictured thanks to a Lumix DMC-FZ300 camera (Panasonic Corporation, Osaka, Japan) mounted on a stereomicroscope.

Transcriptomic analyses

Parallel to pharmacological assays, in silico searches for MCH receptors were undergone. Using available *E. spinax* transcriptomic data (SRA/NCBI accession number: SRX4379543; Delroisse et al. 2018), homology-based local tBLASTn searches (Altschul et al. 1990) for MCH receptor sequences were performed using vertebrate reference sequences as queries. Top hit candidates (i.e. *e* values $< 10^{-5}$) were used as request in reciprocal BLASTx searches (i.e. first top hit) against online NCBI databases (https://www.ncbi.nlm.nih.gov/) to emphasise sequence potentially homologous to MCH receptors. All BLAST analyses were performed using default parameters. In silico translations were performed for the putative MCH receptor sequences retrieved from the shark transcriptome using the ExPaSY online tool (http://web.expasy.org/translate; Gasteiger et al. 2005).

MCH receptor phylogenetic inference

Reference metazoan MCH receptors, as well as closely related somatostatin receptors (i.e. SSTR), were collected in NCBI databases. A multiple alignment was performed with the MAFFT algorithm in Geneious Prime 2019.1.1. (https://www.geneious.com). A strict trimming was then performed

using TrimAL algorithm (Capella-Gutiérrez et al. 2009) implemented in Metapiga 3.1. (Helaers and Milinkovitch 2010). Maximum likelihood phylogenetic analysis was performed using PhyML 3.0. using the JTT + G + I + F model of amino acid substitution [(Akaike Information Criterion (Lefort et al. 2017)] and optimised with SPR tree searching (Guindon et al. 2005). Bootstrap analysis (1000 replicates) was performed. The urotensin-2 receptors (UTR2) of the whale shark, the elephant shark and the cattle were selected as a global outgroup based on Sailer et al. 2001.

Statistical analyses

All analyses [ANOVA, post-hoc Tukey tests] were performed with the software JMP pro v.14 (SAS Institute Inc., Cary, NC, USA, 1989–2007). The Gaussian distribution condition was obtained for all analyses after logarithm transformation allowing the use of parametric tests. ANOVA was used to show a significant difference between treatments while post-hoc Tukey tests allowed the differentiation of clusters.

Results

The effect of MCH on the ventral skin patches of *E. spinax* ($n = 16$) was tested. Typical long-lasting weak luminescence is obtained with MT 10^{-6} mol l^{-1} applications, while applications of PRL 10^{-6} mol l^{-1} trigger fast and strong luminescent responses (Fig. 1a, b, Table 1). Conversely, MCH applications (dose–response at 10^{-8} to 10^{-5} mol l^{-1}) do not induce a significant light production and stay similar to the control level (shark saline; p value > 0.05; Fig. 1b, Table 1). PRL and MT Ltot present a value statistically different from all other experiments (p value < 0.05; Fig. 1b).

End-experiment ILS melanophore states show fully open photophores with a bluish reflection (i.e. resulting from the reflection of the stereomicroscope light system on

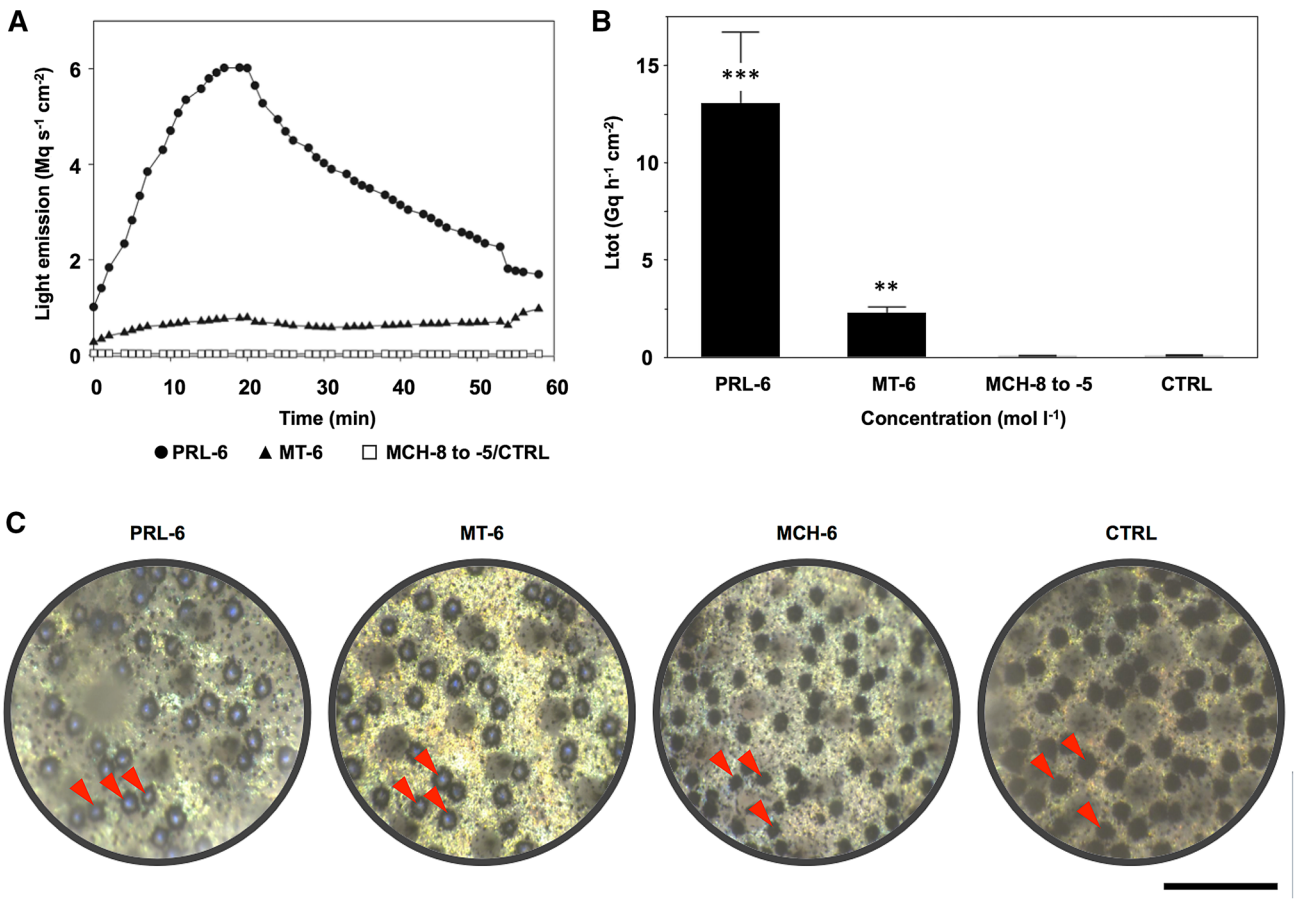


Fig. 1 Effects of MT, PRL and MCH applications on E. spinax light emission. **a** Mean time course evolution of the light emissions (Mq s^{-1} cm^{-2}) from ventral skin patches of velvet belly lanternshark after drug applications ($n = 16$). **b** Total amount of light produced (Ltot; Gq h^{-1} cm^{-2}) by ventral skin patches under hormonal treatments. Ltot is integrated on 60 min. **c** Representative ventral skin patch pictures after the end of each experimental procedures presenting photophores (red arrowhead). CTRL, shark saline, $n = 16$, error bars correspond to SEM. ** and *** correspond to statistically different groups. Scale bar, 1 mm

Table 1 Total amount of light emitted after melatonin (MT), prolactin (PRL) or melanin-concentrating hormone (MCH) applications integrated on 60 min

Drug	Concentration (mol l^{-1})	Ltot (Gq h^{-1} cm^{-2})
MT	10^{-6}	2.32 ± 0.29
PRL	10^{-6}	13.07 ± 3.65
MCH	10^{-8}	0.08 ± 0.02
	10^{-7}	0.06 ± 0.01
	10^{-6}	0.08 ± 0.02
	10^{-5}	0.06 ± 0.01
Shark saline (control)	–	0.10 ± 0.02

$n = 16$, data presented as mean ± SEM

the guanine layer) in both MT and PRL experiments. Conversely, ventral skin photophores do not present black pigment location modifications after MCH treatment, similar to control with shark saline (Fig. 1c).

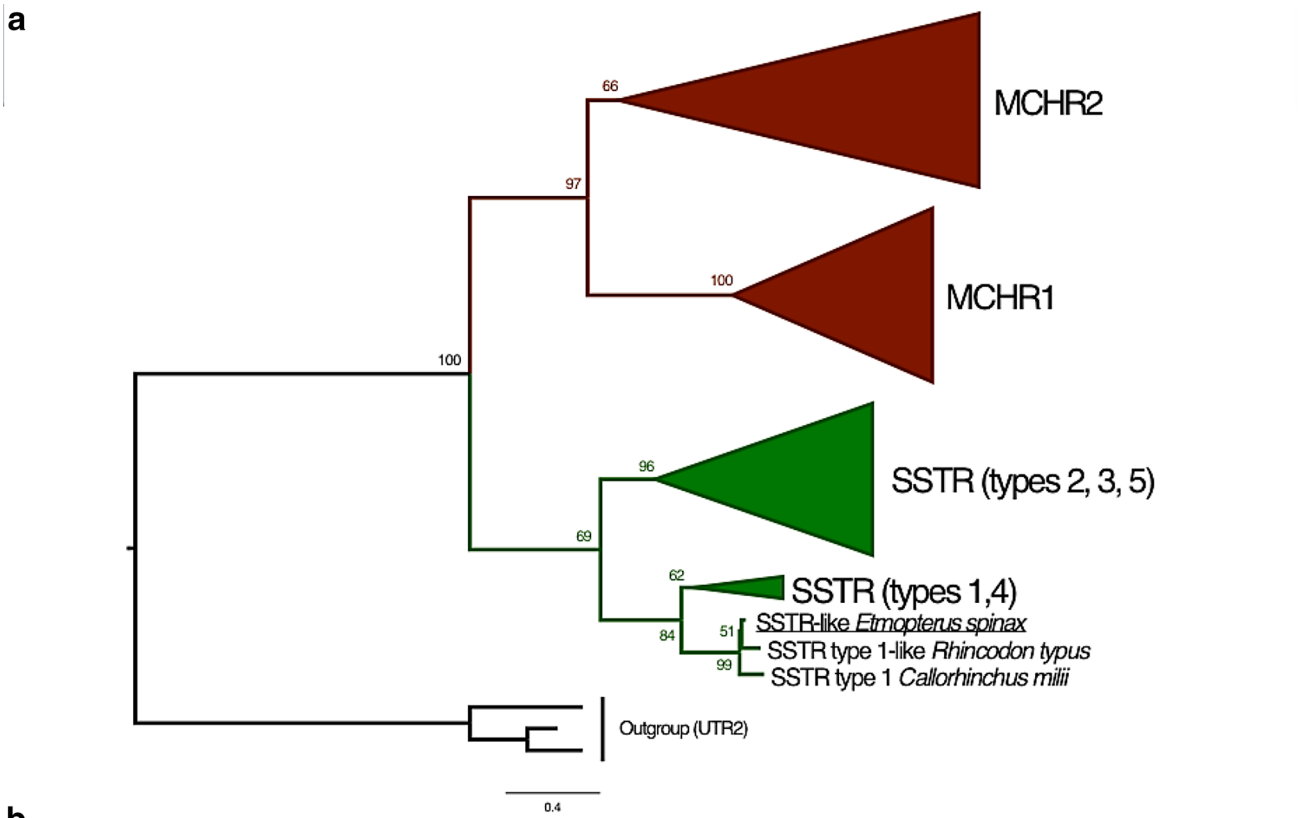
Top hit mRNA search for MCH receptor within the available *E. spinax* skin transcriptome (Delroisse et al. 2018) lead to one putative mRNA sequence. After in silico translation and reciprocal BLASTx analysis, this sequence is shown to rather be associated with somatostatin receptor than MCH receptor. Phylogenetic inference reveals the clustering of the retrieved sequence within the closely related somatostatin receptor (Es-somatostatin receptor) rather than MCH receptor, confirming the absence of an MCH receptor within the skin of *E. spinax* (Fig. 2a). Reference metazoan MCH receptors, as well as closely related somatostatin receptors, are listed in Fig. 2b.

Discussion

A common core mechanism, based on hormone activity, regulates both bioluminescence and pigment motion in lanternsharks (Claes and Mallefet 2009c, 2010). Hormones regulating luminescence in sharks possess two distinct targets within the photophore: (1) the photocytes, for the light emission itself, and (2) the ILS melanophores, for the regulation of the shutter function (Claes and Mallefet 2009c; Claes et al. 2012; Renwart et al. 2015). MT, PRL, α-MSH and ACTH were shown to act at both levels in the velvet belly lanternshark (Claes and Mallefet 2009c; Duchatelet et al. 2019c). Therefore, we hypothesised the involvement of MCH on light emission and pigment motion in *E. spinax*. Here, to complete the understanding of the bioluminescence control, we pharmacologically demonstrated the inefficiency of MCH to modify (1) *E. spinax* skin color and (2) light emission. While MT and PRL trigger light emission and open the ILS (i.e. through ILS melanophore pigment aggregations), MCH was then unable to modify pigment position within the ILS. Moreover, via transcriptome analyses, the absence of MCH receptor mRNA was highlighted in *E. spinax*. To date, MCH and associated receptors are involved in physiological pigment aggregation in Neopterygii fish melanophores (Vallarino et al. 1989; Qu et al. 1996; Rossi et al. 1997; Takahashi et al. 2004; Matsuda et al. 2006; Baker and Bird 2002). Only two other elasmobranch species have been tested based on pharmacological applications of MCH (i.e. at 10^{-8} to 10^{-6} mol l^{-1}) and pigment cell responses revealed the inefficiency of this hormone to regulate color change and aggregate melanosomes within skin melanophores (Visconti and Castrucci 1993; Mizusawa et al. 2012). Mizusawa et al. (2012), also showed that MCH and its receptor, located in the brain of the scalloped hammerhead shark (*Sphyrna lewini*), affect feeding behaviour, but not skin pigment migration. The absence of MCH receptor in the velvet belly lanternshark skin transcript is in agreement with previous works on other Neoselachii species revealing the absence of skin-related MCH receptors hence MCH inefficiency on skin color modulation. Present results confirm the absence of MCH activity on skin pigment motion in sharks, particularly here, for a deep-sea shark species. Additionally, MCH was demonstrated to be inefficient on the *E. spinax* light emission triggering. MCH hormone is ancestrally associated with the central nervous system in gnathostomes while the peripherical function (i.e. skin pigment potion regulator) appears to be a Neopterigii-specific apomorphy (Sherbrooke et al. 1988). The name "melanin-concentrating hormone", given after its discovery and function characterization within the melanophores of the chum salmon (Kawauchi et al. 1983), now appears slightly confusing when considering the whole gnathostome clade.

Other actors also involved in pigment aggregation or dispersion are suggested to be part of the mechanical control of light emission through pigment location modulation, such as the extraocular opsin-based photoreception (e.g. Es-Opn3; Delroisse et al. 2018; Duchatelet et al. 2019d). Efforts are still needed to fully understand the whole mechanistic framework behind light emission and ILS cell shutter actions in the velvet belly lanternshark and luminous elasmobranchs, in general.

a



b

RECEPTORS	SPECIES	ACCESSION NUMBERS (NCBI)
MCHR1	*Homo sapiens*	NP_005288.3
	Xenopus tropicalis	NP_001297042.1
	Danio rerio	XP_692413.3
	Carassius auratus	BAH70338.1
	Oreochromis niloticus	XP_019213013.1
	Verasper moseri	BAF49517.1
	Paralichthys olivaceus	AKO69803.1
	Callorhinchus milii	XP_007907777.1
	Sphyrna lewini	BAM63325.1
MCHR2	*Homo sapiens*	NP_115892.2
	Xenopus tropicalis	NP_001297035.1
	Danio rerio	XP_001921716.2
	Carassius auratus	BAH70339.1
	Oreochromis niloticus	XP_003445904.1
	Verasper moseri	BAF49518.1
	Paralichthys olivaceus	AKO69806.1
	Callorhinchus milii	XP_007891227.1
	Sphyrna lewini	BAM63326.1

RECEPTORS	SPECIES	ACCESSION NUMBERS (NCBI)
SSTR1	*Oncorhynchus mykiss*	NP_001118115.1
	Callorhinchus milii	XP_007891558.1
	Rhincodon typus	XP_020380104.1
SSTR2	*Rattus norvegicus*	AAA42165.1
	Haplochromis burtoni	NP_001274338.1
	Callorhinchus milii	XP_007887096.1
	Rhincodon typus	XP_020374914.1
SSTR3	*Homo sapiens*	NP_001042.1
SSTR4	*Xenopus tropicalis*	XP_017949635.1
SSTR5	*Anabarilius grahami*	ROL53034.1
	Callorhinchus milii	XP_007901730.1
	Rhincodon typus	XP_020378793.1
UTR2	*Bos taurus*	NP_001035574.1
	Callorhinchus milii	XP_007887083.1
	Rhincodon typus	XP_020370820.1

Fig. 2 MCH receptor phylogenetic inference. **a** Maximum likelihood tree based on an amino acid sequence alignment of MCH receptors (MCHR) and somatostatin receptors (SSTR). Tree is calculated by PhyML using JTT + G + I + F model of evolution. SPR setting was used for tree optimization. Numbers at the nodes indicate bootstrap percentages based on 1000 replicates. The scale bar represents the percentage of amino acid substitutions per site. The Whale shark, elephant shark and cattle urotensin-2 receptor (UTR2) sequences were used to root the tree. **b** MCH and somatostatin receptor sequences from various organisms used as references for the maximum likelihood phylogenetic analysis

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Author contributions LD performed, analysed and interpreted the pharmacological tests, performed transcriptome data analysis and was a major contributor in writing the manuscript. JD performed transcriptome and phylogenetic analyses, contributed to and revised the manuscript. JM supervised the work, contributed to and revised the manuscript. All authors read and approved the final manuscript.

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Data accessibility The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Animal Ethics Committee of the Catholic University of Louvain in Louvain-la-Neuve, in agreement with the European directive 2010/63/UE).

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