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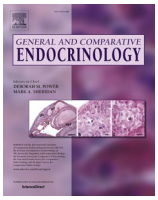

Research paper

Expressions of melanopsins in telencephalon imply their function in synchronizing semilunar spawning rhythm in the mudskipper *Boleophthalmus pectinirostris*

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ABSTRACT

The mudskipper *Boleophthalmus pectinirostris* inhabits intertidal mudflats, exhibiting semilunar reproductive rhythms. To investigate whether melanopsin is possibly involved in the synchronization of the semilunar spawning rhythm in the female mudskipper, we first cloned all four melanopsin subtypes (*opn4m1, opn4m3, opn4x1, opn4x2*) in *B. pectinirostris*. Results from RTq-PCR showed that significantly higher transcription levels of all four melanopsin subtypes were observed in the eyes rather than other tissues. In brain, all four melanopsin subtypes were also detectable in different regions, including the telencephalon, in which the expression of melanopsin has not been reported in other teleosts. The transcription levels of *opn4m3* and *opn4x1* in the telencephalon exhibited a daily fluctuation pattern. When females entered the spawning season, *opn4m1* and *opn4x1* transcript levels increased significantly in the telencephalon. During the spawning season, the transcript levels of *opn4m3* and *opn4x1* in the telencephalon appeared to have a cyclic pattern associated with semilunar periodicity, exhibiting two cycles with a peak around the first or the last lunar quarters. Results from ISH showed that, *opn4x1* mRNA was localized in the medial of dorsal telencephalic area, dorsal nucleus of ventral telencephalic area (Vd), ventral nucleus of ventral telencephalic area (Vv), anterior part of parvocellular preoptic nucleus, magnocellular part of the magnocellular preoptic nucleus (PMmc), habenular and ventral zone of hypothalamus. Intriguingly, *gnrh3* mRNA was also located in Vd, Vv and PMmc. Taken together, our results suggested that melanopsins, e.g. *opn4x1*, expressed in the telencephalon might mediate semilunar spawning activity in the female mudskipper.

1. Introduction

Ambient light plays a particularly important role in almost all animals for mediating their behavior and physiology responses. The detection of light is responsible for image-forming and non-image-forming responses. Non-image-forming vision provides a measure of the ambient luminance for the purposes of synchronizing the animal's biological clock, controlling the pupil size, triggering movement and the seasonal regulation of reproduction (Foster and Hankins, 2002; Perez et al., 2019). In mammals, eyes mediate both image-forming and non-image-forming visual functions (Lucas et al., 1999). In contrast, extraocular photoreception, through photosensitive opsin molecules for non-image-forming, is a particularly common phenomenonin non-mammalian vertebrates, e.g. birds (Foster et al., 1985), reptiles (Underwood, 1973), amphibians (Fraile et al., 1989), and teleost (Frisch, 1911; Nelson and Zucker, 1981; Perez et al., 2019; Shand and Foster, 1999).

A number of photopigments have been localized to extraocular photoreceptors for non-image-forming vision, including neuropsin (Nakane et al., 2010), vertebrate ancient long opsin (Val-Opsin) (Kojima

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et al., 2000), parapinopsin (Blackshaw and Snyder, 1997), pinopsin (Okano et al., 1994), *exo*-rhodopsin (Mano et al., 1999) and melanopsin (Chaurasia et al., 2005; Drivenes et al., 2003; Provencio et al., 1998). Among these photopigments, melanopsin (*opn4*) was a major component of the photoreceptor which was initially discovered in the light-sensitive dermal melanophores of *Xenopus laevis* (Provencio et al., 1998). Since then, related sequences have been identified not only in the human and mouse, but also in the fish, bird, and lizard genome. The phylogeny of the melanopsin gene family can be divided into two evolutionary lineages: *opn4m* (mammalian-like) and *opn4x* (*Xenopus*-like) (Bellingham et al., 2006). Mammalians possess only the *opn4m* ortholog, which is exclusively expressed in a small subset of retinal ganglion cells (RGCs) of the inner retina that are intrinsically photosensitive (ipRGCs), and which are the exclusive conduits for non-visual light inputs to the brain (Hattar et al., 2002). Accumulated evidence from gene ablation studies in mammals shows that melanopsin plays a critical role in the transduction of light information from ipRGCs to regulate a multitude of physiological systems (Lucas et al., 2003; Panda et al., 2002; Ruby et al., 2002). Most non-mammalian vertebrates have both *opn4m* and *opn4x* genes, and both are expressed in many different tissues, including the eye, brain, gill, pineal gland and skin (Bellingham et al., 2002; Chaurasia et al., 2005; Davies et al., 2012; Drivenes et al., 2003; Jenkins et al., 2003; Provencio et al., 1998). Moreover, current studies demonstrate that the expression of melanopsin genes is detected in multiple regions of the brain of non-mammalian vertebrates (Eilertsen et al., 2014; Matos-Cruz et al., 2011; Sandbakken et al., 2012). Study in masu salmon (*Oncorhynchus masou masou*) demonstrates that *opn4*, which it expressed in the saccus vasculosus (SV), may play a role as a deep-brain photoreceptor to sense photoperiodic information and further regulate seasonal reproduction (Nakane et al., 2013). Therefore, it would be interesting to examine potential function of *opn4* in mediating light period related spawning rhythm in other teleosts.

Circalunar and circasemilunar reproductive rhythms are widespread among marine animals including corals, annelid worms, midges, and fishes, and rely on endogenous oscillators and environmental cues (Harrison et al., 1984; Zantke et al., 2013). The mudskipper *Boleophthalmus pectinirostris*, a burrow-dwelling species, is widely distributed in marine intertidal regions and exhibits amphibious behavior and numerous physiological and morphological specializations adapted for amphibious life (Clayton, 1993; Hong et al., 2007; Li et al., 2016). The spawning season of this species is between May and September each year, with spawning peaks in May and July (Xie and Zhang, 1990). In our previous study, we found that *B. pectinirostris* possesses a semilunar spawning rhythm (i.e. spawns either around the first or the last lunar quarters during the spawning season), accompanied by semilunar periodicity variations of steroid hormones in the blood plasma (Wang et al., 2008). Moreover, the transcript levels of melatonin receptor subtypes in the diencephalon and ovary displayed two cycles in a lunar month, and peaked around the first and last lunar quarters, which suggests that melatonin signals are involved in the regulation of ovary development (Hong et al., 2014). It is well-known that melatonin is regulated by light. Therefore, it is possible that the semi-lunar spawning activity of *B. pectinirostris* is related to a cyclic change of ambient light. Studies in Glodlined spinefoot, *Siganus guttatus*, which spawns synchronously around the first quarter moon during the reproductive season, indicated that repetitive moonlight illumination is important for the lunar-phase-synchronized spawning rhythm, and *Cryptochrome*3, a deep brain photoreceptor, could be an internal signal for recognizing external lunar phase (Takeuchi et al., 2018).

Therefore, our study aimed to investigate whether, in addition to melatonin signals, melanopsin is involved in the synchronization of the semilunar spawning rhythm in the female *B. pectinirostris*. We first cloned all four subtypes of the melanopsin gene in the mudskipper and examined their distribution in the different regions of the brain and peripheral tissues. The diurnal variations of these melanopsin genes in eyes and brain were analyzed. Moreover, the monthly and semilunar variations, localization of melanopsin subtypes in brain were also examined. Besides, in order to investigate a potential correlation between melanopsin and reproduction system, we also examine the localization of gonadotrophin-releasing hormone 3 (GnRH3) neurons, which has also been implicated in the control of reproductive behavior in teleost (Okubo and Nagahama, 2008).

2. Materials and methods

2.1. Experimental fish and sampling protocol

Specimens of adult female mudskipper *B. pectinirostris* were captured from mudflat of Xiapu, Fujian, China (26°53′N, 120°03′E), using trap net or burrowing. A trap net is 8 m long and consists of several chambers (length 25 cm × width × 20 cm × height 12 cm) with an opening on the side. The trap net is placed on the mudflat at low tide. When the fish go out of the burrow at low tide for food, they randomly enter the chambers and are caught. Fish were collected from chambers at the next low tide. Scale rings were used to determine the ages of sampled fish. 2-year-old females (lengths 107–143 mm and weights 17.0–36.1 g) were selected from the catch and used in subsequent experiments. Gonadosomatic index (GSI) was calculated as GSI (%) = [gonad weight (g) / total body weight (g)] × 100% (GSI: 0.71–9.79%). All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Xiamen University.

To examine the tissue distribution of the melanopsin mRNAs, gill, eyes, brain, heart, spleen, intestine, liver, kidney, muscle, skin, and ovary were collected on April 29, 2015. As previously described, the whole brain was divided into five portions: telencephalon, diencephalon, optic tectum (mesencephalon), cerebellum, and medulla oblongata (Hong et al., 2014). We severed the optic nerve and removed the optic tectum which was weakly connected to other regions. Then, we cut at the boundary between the telencephalon, diencephalon, cerebellum, and medulla oblongata (Kawabata et al., 2012). Each time, at least six female mudskippers were randomly sampled. Female mudskippers were anesthetized by immersion in seawater containing 0.1% (w/v) MS-222 (Tricaine mesylate, Sigma-Aldrich, Carlsbad, CA, USA) until opercula movements stopped and then sacrificed. All the samples collected were immediately dipped into liquid nitrogen and stored at −80 °C. Total RNA was extracted from the tissue samples using the RNAzol method (MRC, USA). The same amount of total RNA (1 μg) was treated with DNase I (Thermo Scientific, USA) to remove genomic DNA. Total RNA (1 μg) was used for the synthesis of the first strand cDNAs synthesis primed with oligo(dT) using the ReverAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions. Real-time PCR was performed as described in section 2.4.

To examine the daily variation of melanopsin subtypes mRNA levels in the telencephalon, diencephalon, and eyes, live female mudskippers captured were transferred to an outdoor muddy pond under natural temperature and photoperiod. Samples were collected at 3 h intervals in a 24 h period from 13:30. Anesthetized and sacrificed method was conducted as above and the eyes, telencephalon, and diencephalon of the same fish were collected sequentially. During the dark periods, samples were collected under dim red light.

To examine the semilunar cycles variation of melanopsin subtypes, brain samples were collected at 3 days intervals from May 19 to June 18, 2015. To examine the monthly variation of melanopsin subtypes, fishes were collected at one month intervals from March 1 to July 1, 2016. After captured from mudflat, female mudskippers were immediately transported to laboratory within the same day. Before sampling brain tissue, mudskippers were held in a plastic tank (length 60 cm × width × 30 cm × height 15 cm) filled with 3 cm thick silt and 2 cm of artificial brackish water (salinity of 15‰) at 26–28 °C. Fish brain samples were collected from 19:00 to 21:00 on the captured day, and sampling method was conducted as above.

2.2. Molecular cloning

Eyes collected from female *B. pectinirostris* were used for cloning the melanopsin subtype genes. Total RNA was extracted using RNAzol reagent (MRC, Cincinnati, OH, USA) and treated with DNase I (Thermo Scientific, Madison, WI, USA) to eliminate genomic DNA. Reverse transcribed into first strand cDNA using a SMARTer®RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) following the manufacturer's instructions. A similar strategy was used for obtaining the full cDNA sequences of all four melanopsin subtypes. For example, a partial *opn4m1* cDNA sequence was first cloned using primers which were designed based on the sequences from the genome data of *B. pectinirostris* (You et al., 2014), using the software Primer3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The PCR amplification was carried out in 20 μL volume using recombinant Taq™ DNA polymerase (Takara, Shuzo, Otsu, Japan) under the following cycling conditions: 94 °C for 5 min (1 cycle); 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min (30 cycles), followed by a final extension step at 72 °C for 10 min. The PCR products with expected size were extracted from agarose gel following electrophoresis, sub-cloned into vector pMD-19 T (Takara), and then transformed into *Escherichia coli* DH5α (Promega, Madison, WI, USA). Several positive clones were selected, and sequenced by Invitrogen Ltd (Guangzhou, China). Based on the partial cDNA sequence obtained above, gene-specific primers were designed for further extension by 5′-and 3′-RACE products. The PCR amplification for 5′-or 3′-RACE was performed using a universal primer in the kit and a gene-specific primer and carried out in a 50 μL volume using the Advantage®2 PCR Kit (Clontech). The PCR reaction was performed under the following cycling conditions: 5 cycles of 94 °C for 30 s, 72 °C 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The RACE products were subcloned and sequenced as previously described. The primers for cloning are listed in Table 1.

2.3. Sequence and phylogenetic analyses

After obtaining the cDNAs for the mudskipper's four melanopsin subtypes, the deduced amino acid sequences were obtained using the ExPASy Translate Tool (http://www.expasy.ch/tools/dna.html). A homology search was performed using the BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The alignment of known melanopsin subtypes was performed using the Megalign program of the Lasergene software package (DNASTAR, Madison, WI, USA) and the Clustal W method. Phylogenetic analysis was carried out by constructing a neighbor-joining tree with a bootstrap value of 1000 trials for each position and rooted by the *B. pectinirostris* Rhodopsin (GenBank accession number: XM_020922320). The four melanopsins identified in *B. pectinirostris* were designated following the nomenclature introduced by Bellingham (Bellingham et al., 2006). We applied TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/) to predict transmenbrane units of the four melanopsins subtypes (Krogh et al., 2001).

Table 1
PCR primers used for cloning four melanopsin subtypes.

Gene name	Primer name	Primer sequence (5′→3′)
opn4m1	Forward	GTGGGCAACTTTCTGGTCAT
	Reverse	CGTCCAATGGGAATAAAGGA
	3′-GSP	ATCCTATGACACTGACTTCTCTATG
	5′-GSP	CATGCTGGTGGTGAAGAAGATGGGTGT
opn4m3	Forward	TCCCAGACCACGCCCACTACAT
	Reverse	GGGCCCACGAGATCACGAACAG
	3′-GSP	CATCATCTTCTGCTACTTCTGCATC
	5′-GSP	TTTCGCCGAGTCCCTGGTTCCGTGG
opn4x1	Forward	ACTGGGAATGCCTTGGTTATGTA
	Reverse	ATCAACAGACATTCGGGAAAGTG
	3′-GSP	CTCCATGTCTACATCTGACACACAG
	5′-GSP	GAGAATCAGTGCTAAGCAGACAGTC
opn4x2	Forward	GGAGCTCTCTTTGGCATCAC
	Reverse	AGGGACAAAAACCTCGTCCT
	3′-GSP	AGTCCAATCATAGATCCAACCACAG
	5′-GSP	GGCGAGGGGGATGAAGAAGACGAAG

2.4. Real-time quantitative PCR (RTq-PCR)

Specific primers for detecting target genes were designed and examined for their specificity and amplification efficiency on serial dilutions of respective target gene plasmid DNA (10^3 –10^8 copies/μL) (Table 2). All RTq-PCRs was conducted on an ABI 7500 fast real-time PCR System with a 20 μL reaction system using the protocol of SYBR ® Select Master Mix (Applied Biosystems, Foster City, CA, USA). In detail, the reaction contained 10 μL SYBR ® Select Master Mix (2X), 400 nM Forward primers, 400 nM reverse primers, 100 ng cDNA template, add RNase-free water to a total volume 20 μL. The thermal cycling conditions were: 1 cycle of 95 °C for 2 min, and 40 cycles of 95 °C for the 30 s, 60 °C for 30 s and 72 °C for 30 s. Primer amplification efficiency ranged 81.594 to 104.378 with $R^2 > 0.994$. The primers for elongation factor 1α (*ef1α*) were designed based on the *B. pectinirostris* genome sequences. (GenBank accession numbers: XM_020921512, XM_020921310). The efficiency and specificity of primers used for RTq-PCR were shown in Table 2. The relative abundance of *melanopsins* mRNA transcripts was evaluated using the formula: $R = 2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The arithmetic mean of the copy number of the reference gene (*ef1α*) was used to normalized the expression values. The reference gene (ef1a) was stable in multiple brain areas during diurnal, lunar, and monthly cycles, as well as in diverse tissues (Fig. S3).

2.5. Cellular location of opn4x1 and gnrh3 in brain analysis by in situ hybridization (ISH)

Probes for both sense and antisense digoxigenin-labeled RNA strands were transcribed *in vitro* from a linearized pGEM-T-easy plasmid (Promega) containing partial sequence of *opn4x1* and *gnrh3* (GenBank accession numbers: XM_020921310.1) cDNA using the RNA labeling kit (Roche, Mannheim, Germany). Sense and antisense RNA probes were transcribed using SP6 and T7 RNA polymerase (Roche), respectively. The *opn4x1* and *gnrh3* gene-specific primers for ISH are listed in Table 4. The freshly dissected brain was fixed in 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4) overnight at 4℃, and then dehydrated, embedded in paraffin and serially sectioned at 10 μm thickness. We prepared sections of the whole brain, which were mounted on glass slides. After deparaffinized, slides were hydrated and treated with Proteinase K (10 ug/mL) (Roche), then incubated in hybridization buffer with DIG-labeled probe overnight at 55 ℃. The slides were washed with 2X SSC buffer and then incubation anti-digoxigenin antibody fab fragments conjugated to alkaline phosphatase overnight at 4 ℃. The hybridization signals were detected using NBT/BCIP (Roche). Photomicrographs were taken in optic microscope (Leica DM 2500) with a digital camera (Leica DFC 7000T, Leica Application Suite X, software version 3.7.2)

2.6. Statistical analysis

All data are presented as the means ± the standard error of the mean (SEM). The values were analyzed via one-way ANOVA followed by Tukey's post hoc test to assess statistically significant differences among the individual groups. Student's *t*-test was also conducted to determine any significant differences between the two groups. The statistical analyses were performed using the GraphPad Prism8 software package (San Diego, CA, USA). $P < 0.01$ and $P < 0.05$ were considered to be statistically extremely significant and significant, respectively.

Table 2
Primers used for the gene expression analyses.

Gene name	Primer name	Primer sequence (5′→3′)	Length (bp)	GenBank No.
ef1α	Forward	TGATCACTGGAACCTCTCAG	283	XM_020932525.1
	Reverse	ATCCAGAGATGGGCACAAAG		MF796241
opn4m1	Forward	CACCCACCCGAAGTACAGAATAG	200	
	Reverse	AGACGGAGGAGAGGCGGGACTT		MF796240
opn4m3	Forward	GCATCTTCAGGGCCATACGACACA	159	
	Reverse	GGGCCCACGAGATCACGAACAG		MF796239
opn4x1	Forward	ATCCGCCATACTAGTAGAGACATA	146	
	Reverse	GGCGACCAGGAAAGCACA		MF796242
opn4x2	Forward	ATCGTGGTTTATGTCCTCTCC TGATGGCGTAGATGAAGGGGTTGT	154	
	Reverse			

Table 3
Sequence information of four melanopsin genes in *B. pectinirostris*.

Name	Predicted ORF	Predicted aa length	cDNA length	GenBank No.
opn4m1	1491 bp	496 aa	1540 bp	MF796241
opn4m3	1641 bp	546aa	1870 bp	MF796240
opn4x1	1260 bp	419 aa	1413 bp	MF796239
opn4x2	1551 bp	516 aa	1758 bp	MF796242

ORF: open reading frame; aa: amino acid.

Table 4
Primers used for *in situ* hybridization.

Gene name	Primer name	Primer sequence (5′→3′)	Probe length	GenBank No.
opn4x1	Forward	CCCACTACATTGTCGCCTTT	871bp	MF796239
gnrh3	Reverse	GTGTCCCTGTATTTCGAATG		
	Forward	ATGGAAGCTAGCAACAGTAG	336bp	XM_020921310.1
	Reverse	GGCAGCGTCTTCACTTATCA		

3. Results

3.1. Cloning and phylogenetic analyses of melanopsin cDNAs

Full-length cDNAs of the four melanopsins of *B. pectinirostris* were cloned from the eyes. The length of cDNA, predicted open reading frame, the amino acid sequence, and GenBank accession number of the four melanopsin subtype genes are listed in Table 3. An alignment (Fig. 1) of the deduced amino acid sequences of the four melanopsin subtypes in *B. pectinirostris* together with previously published melanopsin amino acid sequences of Atlantic halibut (*Hippoglossus hippoglossus*) showed that *opn4m1*, *opn4m3*, *opn4x1* and *opn4x2* encode a seven putative transmembrane α-helical domain (TM 1–7) structure. The alignment further showed that residues considered as critical for photoreceptor function were also conserved. Tyrosine in TM3 and glutamic acid between TM4 and TM5 are characteristic of two potential Schiff base counterions of the melanopsin family (Provencio et al., 1998; Terakita et al., 2000). The conserved lysine in TM7, which can bind to 11-*cis*-retinal chromophore via a Schiff base linkage (Menon et al., 2001), together with the asparagine residue in the TM2 and tripeptide DRY region at the interface of TM3 and the second intracellular loop play important roles in the G-protein activation process (Bockaert and Pin, 1999).

The phylogenetic tree was constructed using the neighbor-joining method on the basis of deduced amino acid sequences of vertebrate melanopsin genes. The phylogenetic tree classified all melanopsin into two clades named as *opn4m* and *opn4x* (Fig. 2). The *B. pectinirostris* melanopsins were positioned in these two clades and arose from duplication of melanopsins, which is also present in other teleosts.

3.2. Tissue distribution of melanopsin subtypes in female B. pectinirostris

Tissue distribution using RTq-PCR analysis revealed that all four melanopsin subtypes were expressed dominantly in the eyes and brain while barely detectable in the skin, muscle, spleen, kidney, gill, heart and ovary (Fig. 3). Importantly, the expression of four melanopsin genes in different regions of the brain was variable. Besides, three melanopsin subtypes (*opn4m1*, *opn4x1*, *opn4x2*) were predominantly expressed in the telencephalon, and the diencephalon was the major expression region for *opn4m1*, *opn4m3* and *opn4x1*.

3.3. Diurnal expression of the melanopsin subtypes in eyes, telencephalon, and diencephalon

Since the melanopsin subtypes were expressed mainly in the eyes, telencephalon, and diencephalon, we further examined the diurnal expression of those genes in these tissues. In the eyes, the expression levels of *opn4x2* mRNA were significantly higher ($p < 0.05$) in the early afternoon (13:30), decreased significantly and were maintained low in the late half of the light phase and the dark phase, and then followed by a significant increase ($p < 0.05$) in the middle of the morning (10:30). The expression levels of *opn4m1*, *opn4m3* and *opn4x1* mRNAs displayed the same pattern that decreased in the dark phase followed by an increase in the light phase, but without significant differences (Fig. 4a–d).

In the telencephalon, the expression of *opn4m3* mRNA reached the highest level at 13:30, significantly decreased ($p < 0.05$) at dusk (19:30) and then maintained at the lower expression in the dark phase and first half of the light phase. In contrast, the expression of *opn4x1* mRNA reached the highest level at dusk (19:30), and showed the lowest level in the morning (04:30–10:30). No significant diurnal fluctuations of *opn4m1* and *opn4x2* mRNA were observed in the telencephalon (Fig. 4e-h).

However, in the diencephalon, the expression levels of *opn4m3* and *opn4x1* mRNA were similar: significantly higher in the afternoon and at the first half of the dark phase, and significantly lower in the morning (04:30–10:30). Diurnal fluctuations of *opn4m1* and *opn4x2* mRNAs were also observed in the diencephalon. The levels of *opn4m1* reached a peak at dusk (19:30), while a peak of *opn4x2* mRNA was observed soon after midnight (01:30) (Fig. 4i-l).

3.4. Lunar expression of the melanopsin subtypes mRNA in eyes, telencephalon, and diencephalon

Within one lunar month during the peak spawning period (from May to June), in the eyes, the expression levels of *opn4m1* mRNA exhibited one peak at first quarter (May 28) (Fig. 5a). The expression levels of *opn4m3* mRNA started to increase to higher levels around the first lunar quarter and continuously increased significantly to the highest levels between the last lunar quarter and the new noon (from June 9 to 15) (Fig. 5b). The *opn4x1* mRNA expression varied slightly throughout the study period but exhibited an obvious peak on June 9 (the last lunar quarter) (Fig. 5c). No significant lunar variations of *opn4x2* mRNA were

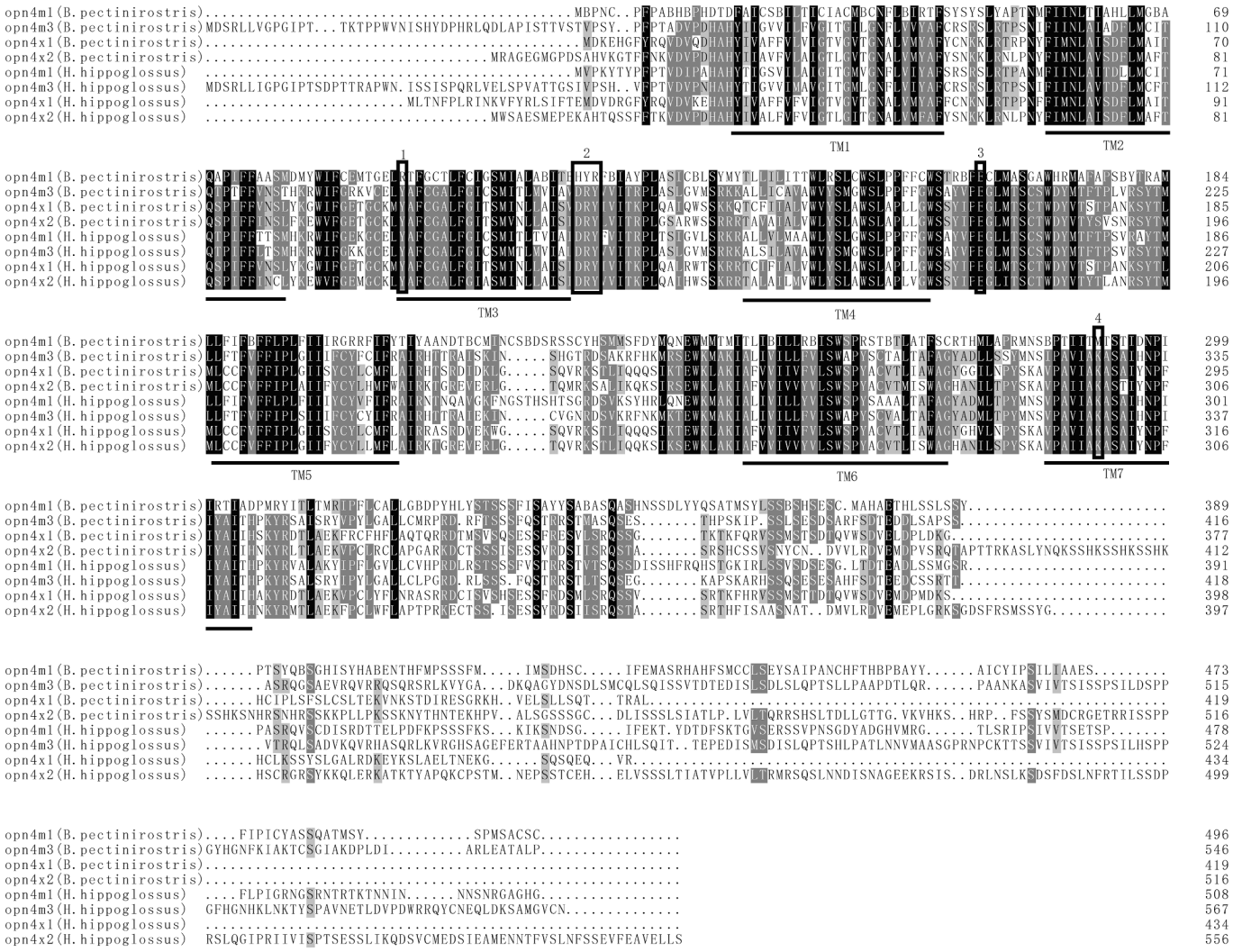

Fig. 1. Alignment of the deduced amino acid sequence of *B. pectinirostris* melanopsins against previously published melanopsin sequences of Atlantic halibut (*H. hippoglossus*). TM1-7 are the seven transmembrane regions; 1 and 3 are the potential Schiff base counterions tyrosine (Y) and glutamic acid (E); 2 is the DRY tripeptide, and 4 is the retinal attachment site (K).

observed in the eyes (Fig. 5d).

In the telencephalon, the expression levels of *opn4m1*, *opn4m3* and *opn4x1* mRNAs displayed the two similarly regular cycle variations within one lunar month, but only *opn4m3* and *opn4x1* mRNA exhibited significant differences (Fig. 5e-h). Each of these three genes exhibited two peaks in the two regular cycles; the first peak was observed on May 28 (the day between the first lunar quarter and full moon), and the second one on June 15 (the day between the last lunar quarter and new moon). The *opn4x2* mRNA levels in the telencephalon also showed a distinct cyclic pattern. The first peak appeared on May 28, the second on June 6, and the third on June 15. Thus, lunar fluctuated expression patterns of *opn4x1* and *opn4m3* showed semilunar rhythm during the peak spawning period.

In the diencephalon, the expression patterns of the four melanopsin subtypes exhibited obvious variations and did not show clear semilunar rhythmicity (Fig. 5i-l). Relatively low levels of four melanopsin subtypes mRNA initially appeared between the new moon and the first lunar quarter (from May 19 to 25), then increased on May 28, but only *opn4x2* exhibited significant changes. The level of *opn4m1* was highest on June 6, and relatively high levels of *opn4m3* were observed in the period from June 3 to 9. The second peak of the *opn4x1* gene occurred on June 3, and then it decreased significantly ($p < 0.05$). Thereafter, it increased again, reaching the third peak ($p < 0.05$) on June 9 (around the last quarter). Compared with the expression pattern of *opn4x1*, the last two peaks of *opn4x2* mRNA occurred later.

3.5. Monthly expression changes of melanopsin subtypes mRNA in the telencephalon and diencephalon

The GSI values of female fish were significantly higher in April than in March and continued to increase significantly in May, reaching the highest levels in June, and slightly decreased in July (Fig. S1). To identify whether melanopsin subtypes genes are involved in the synchronization with the peak spawning season, we examine melanopsin genes monthly fluctuation expression patterns. The *opn4m1* transcription levels failed to show any significant difference in the diencephalon, while in the telencephalon the *opn4m1* gene transcripts were significantly up-regulated in May (Fig. 6a, e). The expression of *opn4m3* and *opn4x2* did not significantly change in the telencephalon and diencephalon from March to July (Fig. 6b, d, f, h). The expression of *onp4x1* significantly increased towards a peak around the spawning season in the telencephalon and diencephalon (Fig. 6c, g). However, the patterns of the *opn4x1* gene varied in the telencephalon and diencephalon. The expression of *opn4x1* was low before the spawning season (March) and significantly up-regulated from April to June in the telencephalon (Fig. 6c). In the diencephalon, the *opn4x1* transcript levels increased

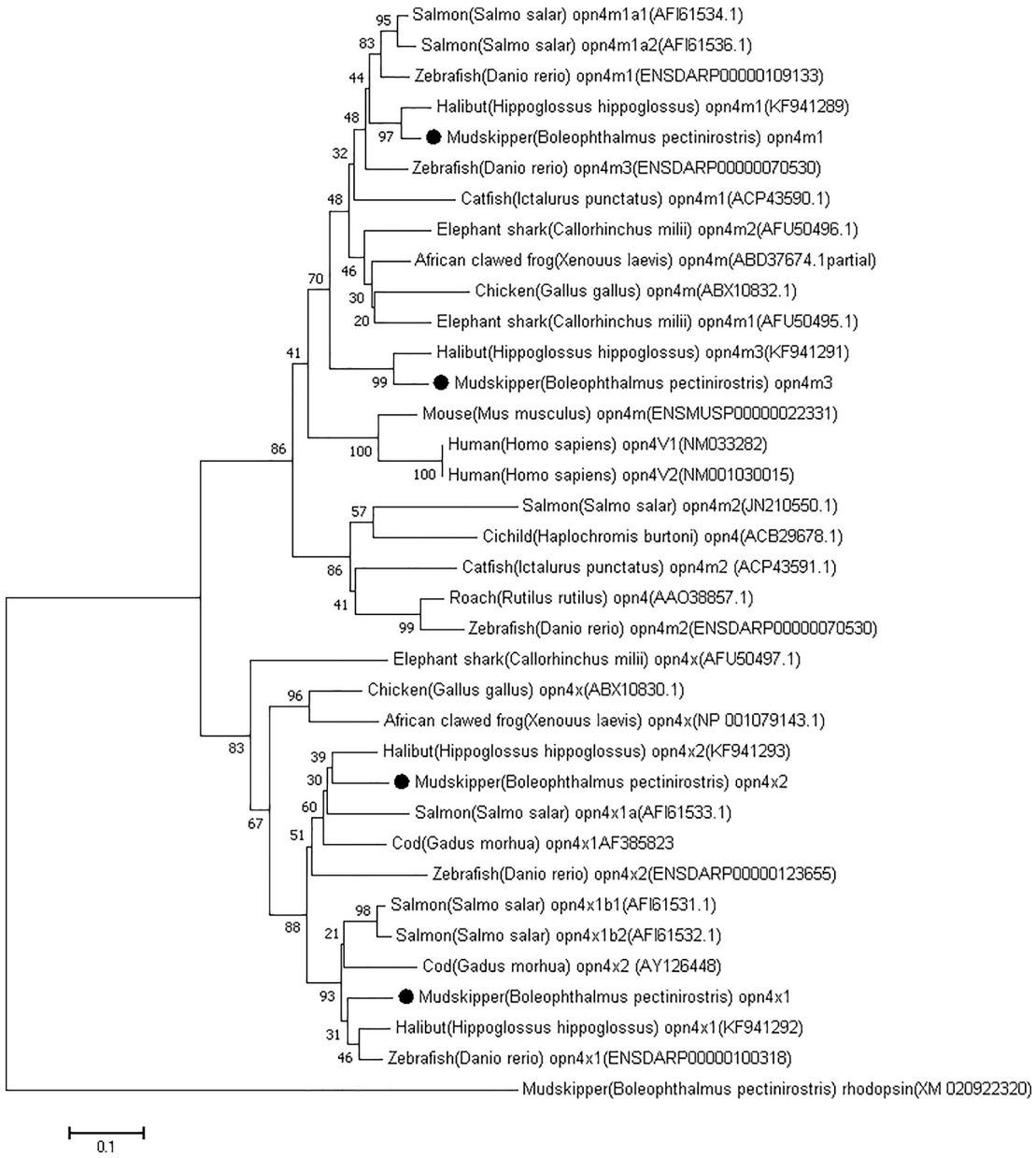

Fig. 2. Phylogenetic analysis of melanopsin subtypes in *B. pectinirostris.* The amino acid sequences of melanopsins of *B. pectinirostris* and other species (GenBank/Ensembl accession numbers are included) were aligned using ClustalW and analyzed using the neighbor-joining method. Node values represent an analysis of 1000 bootstrap trials. *B. pectinirostris* rhodopsin was used as an outgroup for the tree. GenBank/Ensembl accession numbers for sequences are depicted above.

significantly in April, then decreased slightly in the peak spawning months (May and June), and were raised again in July (Fig. 6g).

3.6. Cellular location of opn4x1 and gnrh3 in the telencephalon and diencephalon

Only melanopsin subtype *opn4x1* gene mRNA levels increased varied synchronously with the semilunar spawning cycle and its expression patterns display two peaks in a lunar period in the telencephalon. Therefore, we examined the cellular location of *opn4x1* in brain. The positive signals for *opn4x1* mRNA were located in the dorsal nucleus of ventral telencephalic area (Vd), ventral nucleus of ventral telencephalic area (Vv) (Fig. 7a–d) and medial of dorsal telencephalic area (Dm) (Fig. 7e-h). In the diencephalon, anterior part of the parvocellular preoptic nucleus (PPa) of preoptic area (POA) (Fig. 7i-l), magnocellular part of the magnocellular preoptic nucleus (PMmc) of POA (Fig. 7m-p), habenular nucleus (Hb) (Fig. 7q-t), and ventral zone of periventricular hypothalamus (Hv) (Fig. 7u-x) showed positive *opn4x1* mRNA signals.

GnRHs are neuropeptides mainly responsible for reproduction in both vertebrates and non-vertebrates. Most teleost fishes have three GnRH subtypes (GnRH1, GnRH2, and GnRH3). Among them, GnRH3 neurons, which are found only in teleosts, are located in the terminal nerve ganglion near the olfactory bulb and project primarily to the telencephalon (Okubo and Nagahama, 2008). GnRH3 performs different functions in different brain regions and plays a key role in gonadal development, maturation (Feng et al., 2020). By determining the co-expression regions of *opn4x1* and *gnrh3*, it is inferred that opn4 functions in different brain regions and at different time points. Therefore, we further examined the distribution of GnRH3 neurons in the brain tissue, especially in the telencephalon. In the telencephalon, the positive signals for *gnrh3* mRNA were located in glomerular layer (Gl) of olfactory bulb (Fig. 8a-d), also located in Vd and Vv (Fig. 8e-h). In the diencephalon, *gnrh3* mRNA were located PMmc (Fig. 8i-l), specifically in gigantocellular part of the magnocellular preoptic nucleus (PMgc) of

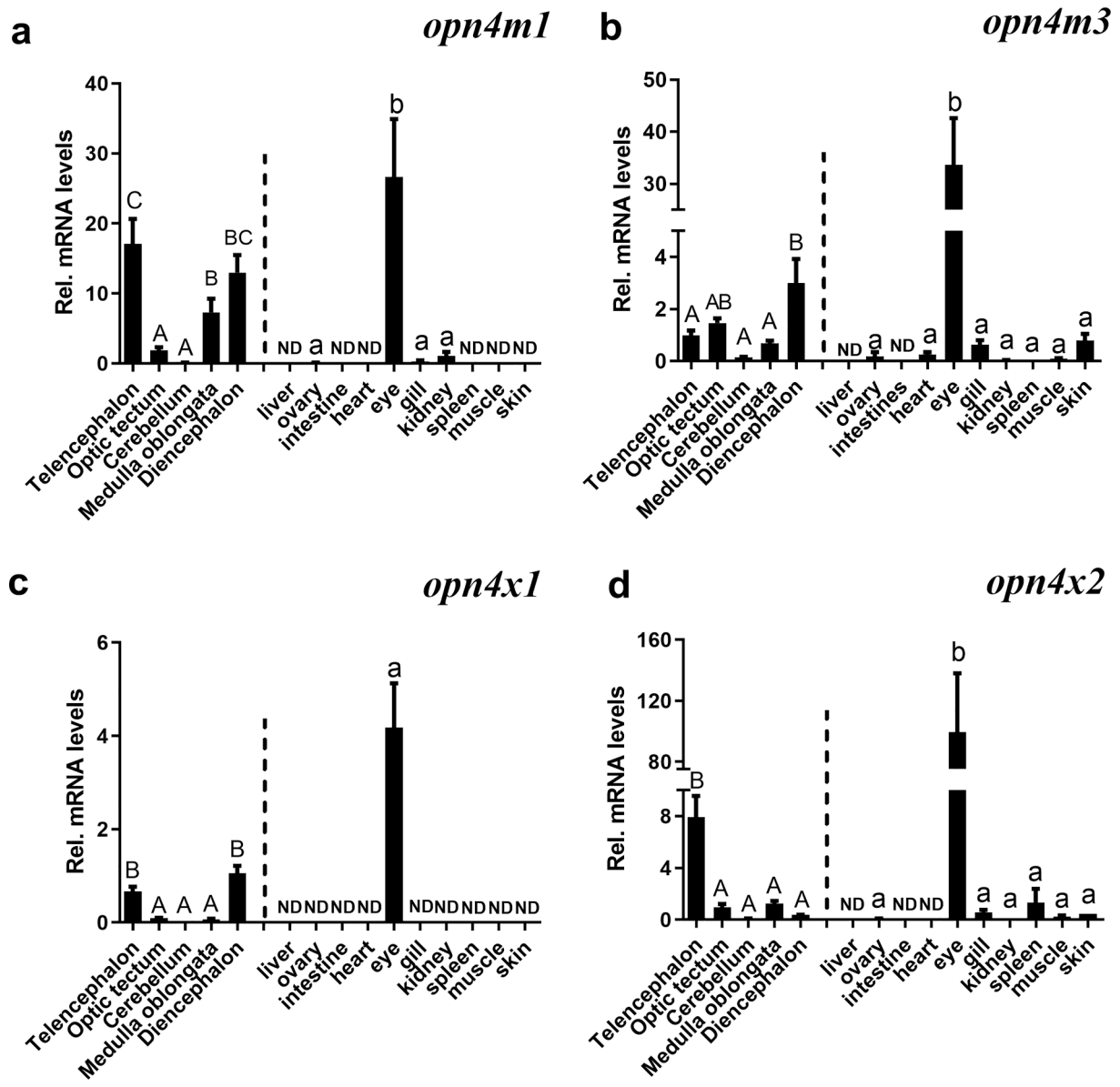

Fig. 3. Tissue specific expression of melanopsin subtypes in female *B. pectinirostris*. The levels of the respective mRNAs in the brain (on the left side of the dashed line) and peripheral tissues (on the right side of the dashed line) were determined using RTq-PCR and normalized to the reference gene (*ef1α*). Data are expressed as the mean ± SEM (n = 6). Bars marked with different upper case letters are significantly different among tested brain regions ($p < 0.05$). Bars marked with different lower case letters are significantly different among tested peripheral tissues ($p < 0.05$). ND, not detectable.

POA and parvocellular preotic nucleus posterior part (PPp) of POA (Fig. 8m-p). No signal was detected using the sense probe for *opn4x1* and *gnrh3* in brain sections (Fig. S2).

4. Discussion

In this study, we cloned, from *B. pectinirostris*, four melanopsin subtype genes which belong to the two distinct groups of melanopsin, i.e. *opn4m* and *opn4x*. In agreement with previous publications on other teleosts (Davies et al., 2011; Drivenes et al., 2003; Eilertsen et al., 2014; Sandbakken et al., 2012), there were two *B. pectinirostris* melanopsins in each group, due to a whole-genome duplication event early in the evolution of ray-finned fish (Eilertsen et al., 2014). The deduced amino acid sequences of these genes showed that all four melanopsin subtypes were encoding elements essential for opsin function, suggesting that they were functional photopigments. All melanopsin subtype genes were expressed mainly in *B. pectinirostris* eyes and brains, and *opn4m3* and *opn4x2* transcripts were found in a wide range of tissues with lower but detectable levels. The wide expression of melanopsins in peripheral tissues suggested that they might be involved in the regulation of multiple light-dependent physiologies in *B. pectinirostris*.

Similar to other vertebrates, the highest expression levels of all melanopsin subtype genes were observed in *B. pectinirostris* eyes. Moreover, all four melanopsin mRNAs showed a similar daily fluctuation pattern, i.e. decrease in the dark phase followed by an increase in the light phase, where only *opn4x2* exhibited a statistically significant difference. Interestingly, it was worth noting that the *opn4m3* mRNAs displayed the different trends in the telencephalon and diencephalon and that *opn4m3* in the telencephalon exhibited a daily fluctuation pattern as same as all four melanopsin genes in the eyes. A few studies showed that the expression of melanopsin genes is regulated by light. Thus, during the photophase, melatonin suppresses *opn4m* and *opn4x* mRNAs *in vitro* from embryonic *X. laevis* melanophores (Moraes et al., 2014); in white leghorn chickens (*Gallus domesticus*), melanopsin mRNA levels are highest at night in both the pineal and retinal photoreceptors, and light exposure at night suppresses the increase of melanopsin mRNA

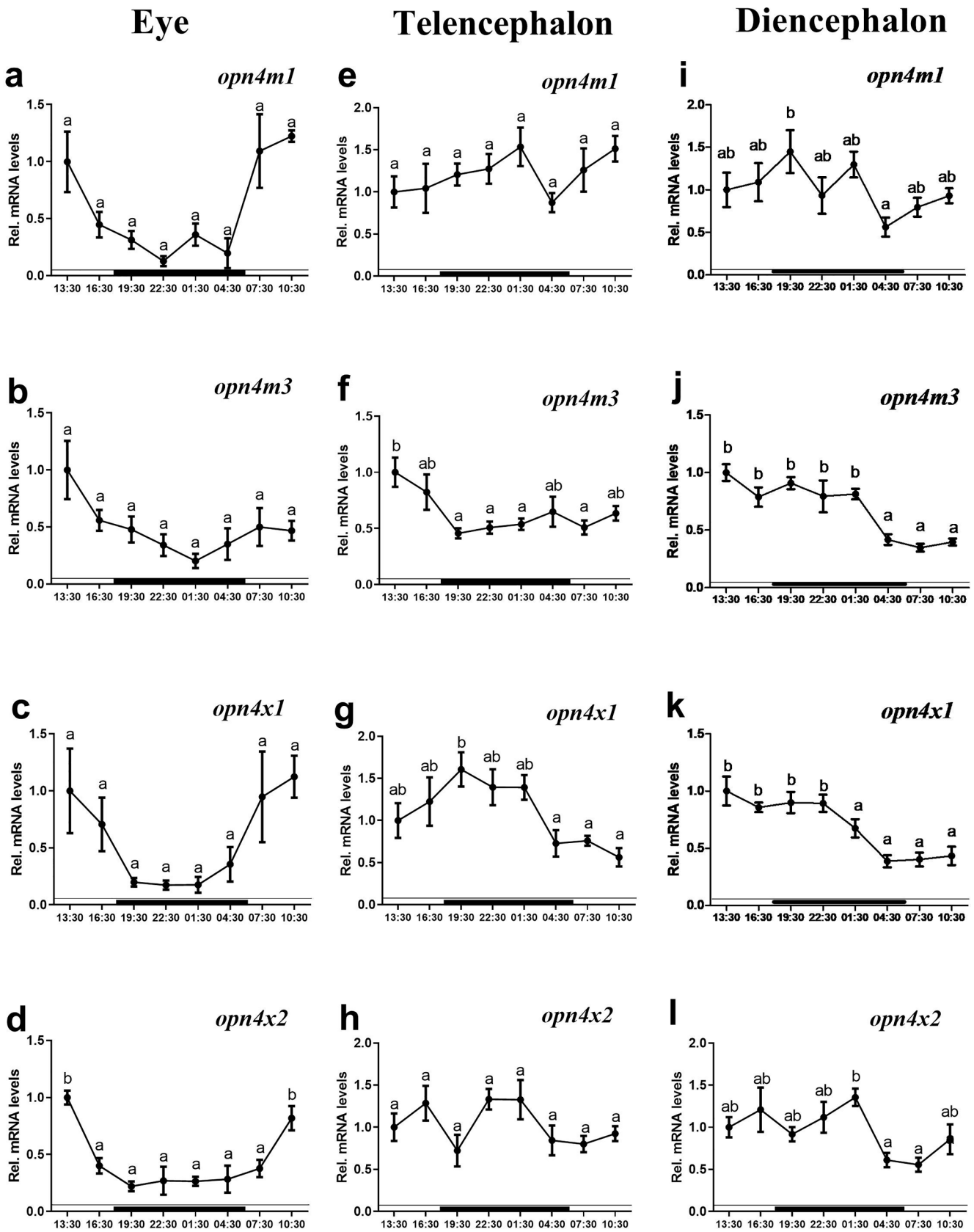

Fig. 4. Diurnal expression of the four melanopsin subtypes mRNA in the eyes (a to d), telencephalon (e to h) and diencephalon (i to l) of female *B. pectinirostris*. Solid and open bars along the X-axis represent the dark phase and the light phase. The expression level was normalized using the reference gene *ef1α*. Data are expressed as the mean ± SEM (n = 6) relative to the respective transcript levels of the time point at 13:30. Values marked with different letters are significantly different from each other ($p < 0.05$).

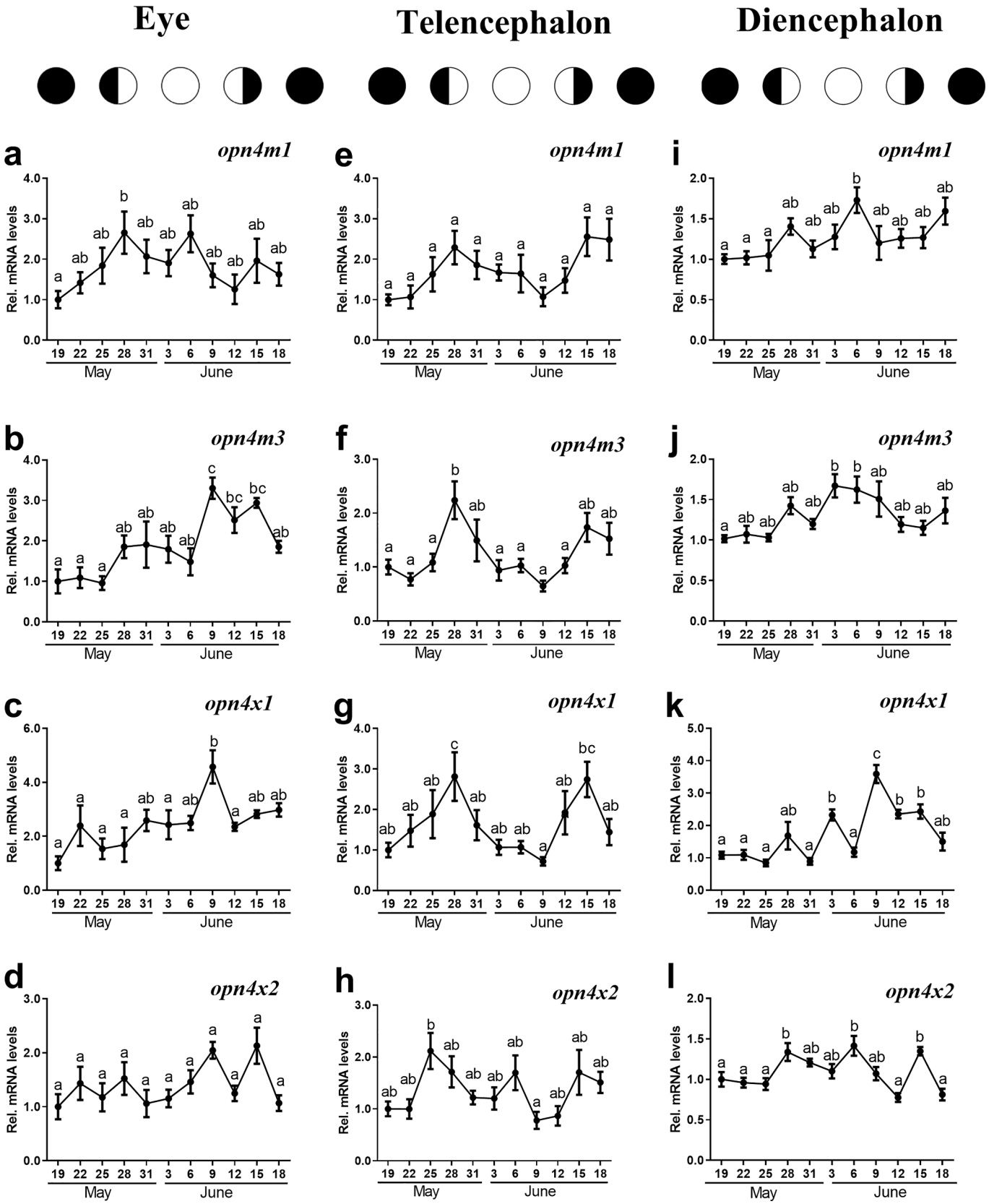

Fig. 5. Semilunar expression of the four melanopsin subtypes in the eyes (a to d), telencephalon (e to h), and diencephalon (i to l) of female *B. pectinirostris*. Lunar phases are indicated with the following symbols: ● new moon; ◐ first lunar quarter; ○ full moon; and ◑ last lunar quarter. The expression level was normalized using the reference gene *ef1α*. Data are expressed as the mean ± SEM (n = 6) relative to the respective transcript levels of the date on May 19. Values marked with different letters are significantly different from each other ($p < 0.05$).

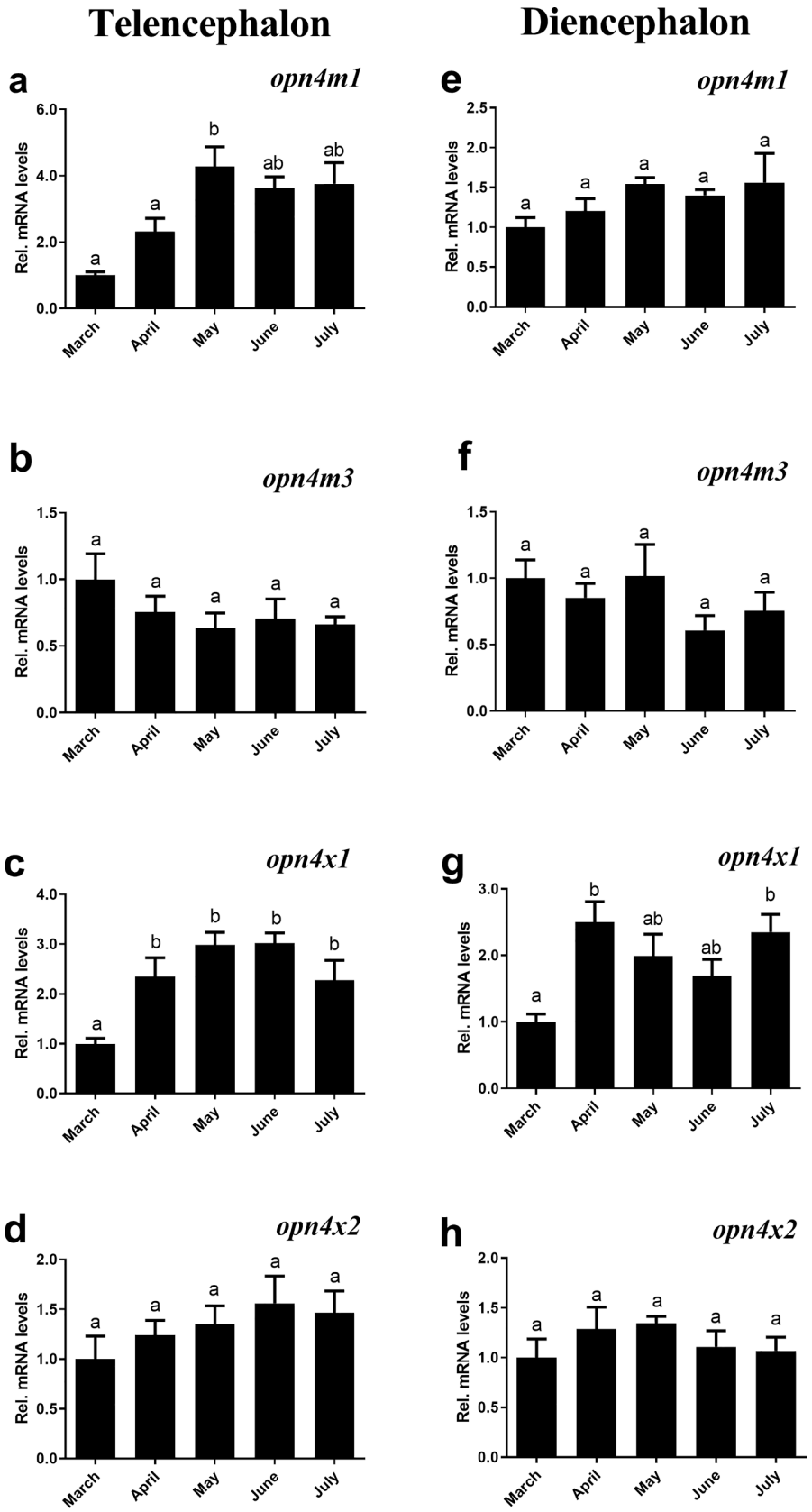

Fig. 6. The relative abundance of the four melanopsin genes in the telencephalon (a to d) and diencephalon (e to h) of female *B. pectinirostris* from March to July. The expression level was normalized using the reference gene *ef1α*. Data are expressed as the mean ± SEM (n = 6) relative to the respective transcript levels of March. Bars marked with different letters are significantly different from each other ($p < 0.05$).

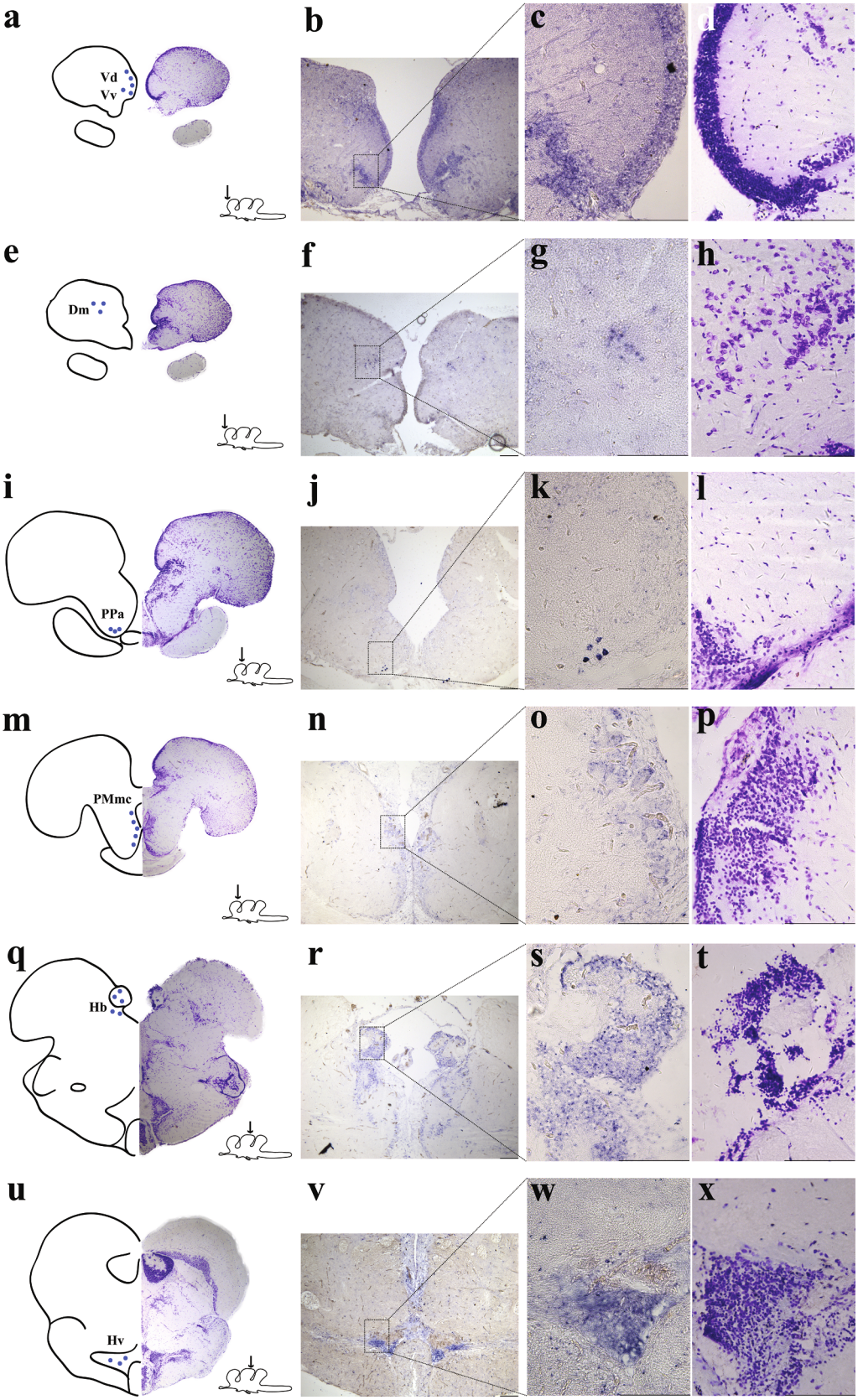

Fig. 7. Cellular location of *opn4x1* in female *B. pectinirostris* telencephalon and diencephalon by *in situ* hybridization. a, e, i, m, q, u: Nissl-stained transverse sections (right) at the equivalent level of *opn4x1* positive cells illustrated by purple dots are shown to the left patterns. *opn4x1* positive cell populations with a lower magnification (b, f, j, n, r, v). *opn4x1* positive cell populations with a higher magnification (c, g, k, o, s, w). Nissl-stained cell populations of interest with a higher magnification at the same level and the same high magnification (d, h, l, p, t, x). a-d: Expression in dorsal nucleus of ventral telencephalic area (Vd) and ventral nucleus of ventral telencephalic area (Vv). e-h: Expression in medial of dorsal telencephalic area (Dm). i-l: Expression in the parvocellular preotic nucleus anterior part (PPa). m-p magnocellular part of the magnocellular preoptic nucleus (PMmc). q-t Expression in habenular nucleus (Hb). u-x: Expression in the ventral zone of the hypothalamus (Hv). Scale bar = 100 μm.

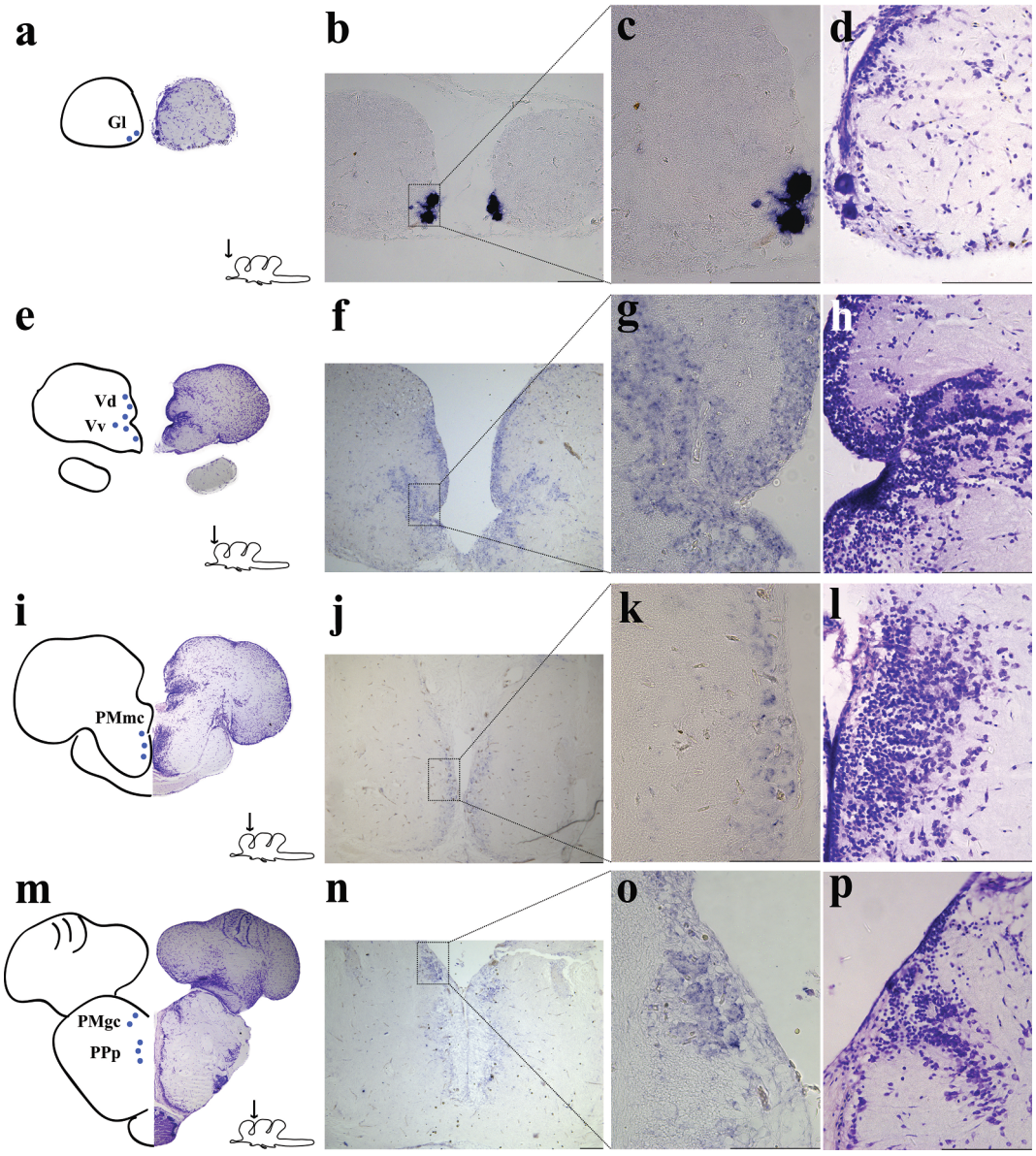

Fig. 8. Cellular location of *gnrh3* in female *B. pectinirostris* telencephalon and diencephalon by *in situ* hybridization. a, e, i, m: Nissl-stained transverse sections (right) at the equivalent level of *gnrh3* positive cells illustrated by purple dots are shown to the left patterns. *gnrh3* positive cell populations with a lower magnification (b, f, j, n). *gnrh3* positive cell populations with a higher magnification (c, g, k, o). Nissl-stained cell populations of interest with a higher magnification at the same level and the same high magnification (d, h, l, p). a-d: Expression in glomerular layer of olfactory bulb (Gl). e-h: Expression in Vd and Vv. i-l: Expression in PMmc. m-p Expression in gigantocellular part of the magnocellular preoptic nucleus (PMgc) and the parvocellular preotic nucleus posterior part (PPp). Scale bar = 100 μm.

in the pineal gland, but not in the retina (Chaurasia et al., 2005); and in the African cichlid fish (*Astatotilapia burtoni*), where light is essential for daily up-regulation of retinal *opn4* mRNA, *opn4* mRNA levels in the retina fluctuate significantly, but do not fluctuate significantly in the brain (Grone et al., 2007). Further study is necessary to investigate the expression in the eyes of melanopsin genes regulated by light.

All four melanopsin subtypes were clearly detected in different regions of the *B. pectinirostris* brain and were expressed mainly in the telencephalon and diencephalon. In order to better understand the potential function of melanopsin in the female neuroendocrine reproductive system, we further examined the monthly expression patterns of the four subtypes in the telencephalon and diencephalon before and during peak spawning periods. The results showed that, in both telencephalon and diencephalon, the levels of *opn4x1* increased during the peak spawning periods. In addition, the *opn4m1* mRNA was significantly up-regulated during May in the telencephalon, but not in the diencephalon. Moreover, ISH results showed *opn4x1* transcripts were expressed in ventral zone of hypothalamus. In birds, the hypothalamus is considered to be a major photoreceptive brain region, since it contains multiple deep brain opsins, i.e. VA-opsin, melanopsin, and Opsin5 (Halford et al., 2009; Kang et al., 2010; Nakane and Yoshimura, 2010), which are proposed to play a role in the control of seasonal reproduction. One recent study in masu salmon demonstrates that the SV, which expresses several opsins including *opn4*, is involved in the regulation of seasonal gonad development (Nakane et al., 2013). The lineages of *opn4m* and *opn4x* are accompanied by gene-specific duplications. In mammals, the *opn4x* gene has been lost over the course of evolution (Bellingham et al., 2006; Pires et al., 2007), which might suggest that *opn4x1* may have some conserved functions in non-mammalian species. Therefore, the *opn4x1* expressed in the diencephalon might be involved in the regulation of ovary development in female *B. pectinirostris*.

The major focus of this study was to investigate whether melanopsin

is involved in the synchronization of the semilunar spawning periodicity in the female *B. pectinirostris*. One interesting result in the present study is that melanopsin genes are expressed in the telencephalon of *B. pectinirostris*, which has not been reported in other teleostes (Davies et al., 2012; Drivenes et al., 2003; Eilertsen et al., 2014; Eilertsen et al., 2018; Fernandes et al., 2012; Moore et al., 2014; Nakane et al., 2013; Sandbakken et al., 2012), but has been reported in the ruin lizard (*Podarcis sicula*) (Frigato et al., 2006). More interestingly, the transcript levels of *opn4m3* and *opn4x1* melanopsin genes in the *B. pectinirostris* telencephalon displayed semilunar fluctuation pattern closely related with semilunar spawning rhythm. Until 1930, the telencephalon of teleost fish was considered as an olfactory brain part. However, since 1930, many experiments clearly indicated the importance of the telencephalon for schooling, learning, and reproductive and aggressive behavior. Study in hime salmon (*Oncorhynchus nerka*) suggested that sexual behavior is integrated in specific parts of the telencephalon and POA (Satou et al., 1984). The localization of *opn4x1* in brain suggested that light could be detected by *opn4x1* positive neurons in both telencephalon and diencephalon. However, photons of longer wavelengths penetrate approximately 1000 times more effectively into deep brain area than photons of shorter wavelengths (Hartwig and Van Veen, 1979). Study in mouse showed that melanopsin forms a functional short-wavelength photopigment which was activated most efficiently by light in the mid-blue range (420–440 nm) (Newman et al., 2003). Therefore, it is reasonable for subpallial structure of telencephalon to be a major brain area to detect ambient light penetrated through the overlying tissue (e.g. the pineal window) without dramatic decrease of light transmission and spectral absorption by brain substance.

Our ISH results showed that *opn4x1* transcripts expressed in the Dm, Vd, and Vv of telencephalon in *B. pectinirostris*. Previous study in zebrafish demonstrated that, the telencephalic Dm and Vd in teleost are proposed homologs of mammalian striatum (Rink and Wullimann, 2002; Wullimann and Mueller, 2004), and is involved in light avoidance behavior and specifically, the Dm plays a critical role in mediating behavioral choices (Lau et al., 2011). Selective tracing experiments in the weakly electric fish *Eigenmannia virescens* showed that Vv, the teleost homologue to the basal forebrain nuclei of other vertebrates, has strong interconnections with the medial hypothalamus and POA (Wong, 1997). The POA has been implicated in sexual behavior across vertebrates, and the expression of *ptgfr* mRNA in this region is consistent with a role in spawning behavior (Juntti et al., 2016). Interestingly, we also observed *opn4x1* transcripts express in POA, which may suggest that *opn4x1* plays a role in female *B. pectinirostris* spawning behavior. In vertebrates, The GnRH has also been implicated in reproductive behavior (Okubo and Nagahama, 2008). The paralogous GnRH genes (GnRH1, GnRH2 and GnRH3) have undergone subfunctionalization during the evolution of vertebrates (Fernald and White, 1999; Kah et al., 2007). GnRH3 has been identified only in the teleost lineage, and several evidences showed GnRH3 would have functioned as neuromodulators, affecting reproductive behavior (Abraham et al., 2008; Zohar et al., 2010). The results in the present study showed that *gnrh3* mRNA was co-distributed in the same brain region with *opn4x1* mRNA in the Vd, and Vv of telencephalon. Moreover, in POA, both *gnrh3* and *opn4x1* mRNAs were located in PMmc. These results suggested *opn4x1* positive neurons in Vd, Vv and PMmc may sense light cues and further regulate the activity of GnRH3 neuron. Taken together, it is possible that *opn4x1* in brain may has a role in the neural control of reproductive behavior in the mudskipper.

In conclusion, this study reported the cloning of four melanopsin subtypes, i.e., *opn4m1, opn4m3, opn4x1* and *opn4x2* in *B. pectinirostris*. For the first time, we reported the expression of melanopsin genes in the telencephalon of teleost species. In the telencephalon, *opn4x1* mRNA levels increased during the peak spawning periods, and displayed a semilunar fluctuation pattern. Moreover, expression areas of *opn4x1* and *gnrh3* mRNAs were highly overlapped in the telencephalon and POA. Our results suggested that *opn4x1* appears to be one of the deep brain photoreceptive molecules that synchronizes semilunar spawning rhythm in the mudskipper *B. pectinirostris*. This unique neurophysiological mechanism seems to be an evolutionary adaptation for *B. pectinirostris* that exhibits amphibian and burrow-dwelling habits in intertidal mudflats.

Uncited references

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CRediT authorship contribution statement

He Ma: Investigation, Data curation, Writing – original draft, Writing – review & editing. **Ming Shu Yang:** Investigation, Data curation, Writing – original draft, Writing – original draft. **Yu Ting Zhang:** Investigation. **Heng Tong Qiu:** Investigation. **Xin Xin You:** Investigation. **Shi Xi Chen:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Wan Shu Hong:** Conceptualization, Supervision, Writing – review & editing.

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.2021.113926.

References

Abraham, E., Palevitch, O., Ijiri, S., Du, S.J., Gothilf, Y., Zohar, Y., 2008. Early development of forebrain gonadotrophin-releasing hormone (GnRH) neurones and the role of GnRH as an autocrine migration factor. J. Neuroendocrinol. 20 (3), 394–405. https://doi.org/10.1111/j.1365-2826.2008.01654.x.

Bellingham, J., Chaurasia, S.S., Melyan, Z., Liu, C.M., Cameron, M.A., Tarttelin, E.E., Iuvone, P.M., Hankins, M.W., Tosini, G., Lucas, R.J., 2006. Evolution of melanopsin photoreceptors: Discovery and characterization of a new melanopsin in nonmammalian vertebrates. PLoS. Biol. 4(10), 1874–1874. 10.1371/journal.pbio.0040320.

Bellingham, J., Whitmore, D., Philp, A.R., Wells, D.J., Foster, R.G., 2002. Zebrafish melanopsin: isolation, tissue localisation and phylogenetic position. Mol. Brain Res. 107 (2), 128–136. https://doi.org/10.1016/S0169-328x(02)00454-0.

Blackshaw, S., Snyder, S.H., 1997. Parapinopsin, a novel catfish opsin localized to the parapineal organ, defines a new gene family. J. Neurosci. 17 (21), 8083–8092. https://doi.org/10.1523/jneurosci.17-21-08083.1997.

Bockaert, J., Pin, J.P., 1999. Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO J. 18 (7), 1723–1729. https://doi.org/10.1093/emboj/18.7.1723.

Chaurasia, S.S., Rollag, M.D., Jiang, G., Hayes, W.P., Haque, R., Natesan, A., Zatz, M., Tosini, G., Liu, C., Korf, H.W., Iuvone, P.M., Provencio, I., 2005. Molecular cloning, localization and circadian expression of chicken melanopsin (Opn4): differential regulation of expression in pineal and retinal cell types. J. Neurochem. 92 (1), 158–170. https://doi.org/10.1111/jnc.2005.92.issue-110.1111/j.1471-4159.2004.02874.x.

Clayton, D., 1993. Mudskippers. Oceanography Marine Biology: an annual review.

Davies, W.I.L., Tay, B.-H., Zheng, L., Danks, J.A., Brenner, S., Foster, R.G., Collin, S.P., Hankins, M.W., Venkatesh, B., Hunt, D.M., Burgess, H.A., 2012. Evolution and functional characterisation of melanopsins in a deep-sea chimaera (Elephant Shark, Callorhinchus milii). PLoS ONE 7 (12), e51276. https://doi.org/10.1371/journal.pone.0051276.

Davies, W.I.L., Zheng, L., Hughes, S., Tamai, T.K., Turton, M., Halford, S., Foster, R.G., Whitmore, D., Hankins, M.W., 2011. Functional diversity of melanopsins and their global expression in the teleost retina. Cell. Mol. Life Sci. 68 (24), 4115–4132. https://doi.org/10.1007/s00018-011-0785-4.

Drivenes, Ø., Søviknes, A.M., Ebbesson, L.O.E., Fjose, A., Seo, H.-C., Helvik, J.V., 2003. Isolation and characterization of two teleost melanopsin genes and their differential expression within the inner retina and brain. J. Comp. Neurol. 456 (1), 84–93. https://doi.org/10.1002/cne.10523.

Eilertsen, M., Drivenes, Ø., Edvardsen, R.B., Bradley, C.A., Ebbesson, L.O.E., Helvik, J.V., 2014. Exorhodopsin and melanopsin systems in the pineal complex and brain at early developmental stages of Atlantic halibut (Hippoglossus hippoglossus). J. Comp. Neurol. 522 (18), 4003–4022. https://doi.org/10.1002/cne.23652.

Eilertsen, M., Valen, R., Drivenes, Ø., Ebbesson, L.O.E., Helvik, J.V., 2018. Transient photoreception in the hindbrain is permissive to the life history transition of hatching in Atlantic halibut. Dev. Biol. 444 (2), 129–138. https://doi.org/10.1016/j.ydbio.2018.10.006.

Feng, K., Cui, X., Song, Y., Tao, B., Chen, J., Wang, J., Liu, S., Sun, Y., Zhu, Z., Trudeau, V.L., Hu, W., 2020. Gnrh3 regulates PGC proliferation and sex differentiation in developing zebrafish. Endocrinology 161 (1). https://doi.org/10.1210/endocr/bqz024.

Fernald, R.D., White, R.B., 1999. Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Front. Neuroendocrinol. 20 (3), 224–240. https://doi.org/10.1006/frne.1999.0181.

Fernandes, A.M., Fero, K., Arrenberg, A.B., Bergeron, S.A., Driever, W., Burgess, H.A., 2012. Deep brain photoreceptors control light-seeking behavior in zebrafish larvae. Curr. Biol. 22 (21), 2042–2047. https://doi.org/10.1016/j.cub.2012.08.016.

Foster, R.G., Follett, B.K., Lythgoe, J.N., 1985. Rhodopsin-like sensitivity of extra-retinal photoreceptors mediating the photoperiodic response in quail. Nature 313 (5997), 50–52. https://doi.org/10.1038/313050a0.

Foster, R.G., Hankins, M.W., 2002. Non-rod, non-cone photoreception in the vertebrates. Prog. Retin. Eye Res. 21 (6), 507–527. https://doi.org/10.1016/S1350-9462(02)00036-8.

Fraile, B., Paniagua, R., Rodriguez, M.C., Saez, F.J., 1989. Effect of photoperiod on testicular function in blinded and non-blinded marbled newts, Triturus marmoratus (caudata, salamandridae). J. Morphol. 200 (2), 151–162. https://doi.org/10.1002/(ISSN)1097-468710.1002/jmor.v200:210.1002/jmor.1052000205.

Frigato, E., Vallone, D., Bertolucci, C., Foulkes, N.S., 2006. Isolation and characterization of melanopsin and pinopsin expression within photoreceptive sites of reptiles. Naturwissenschaften 93 (8), 379–385. https://doi.org/10.1007/s00114-006-0119-9.

Frisch, K.V., 1911. Beiträge zur Physiologie der Pigmentzellen in der Fischhaut. Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere 138 (7), 319–387. https://doi.org/10.1007/BF01680752.

Grone, B.P., Sheng, Z., Chen, C.-C., Fernald, R.D., 2007. Localization and diurnal expression of melanopsin, vertebrate ancient opsin, and pituitary adenylate cyclase-activating peptide mRNA in a teleost retina. J. Biol. Rhythms 22 (6), 558–561. https://doi.org/10.1177/0748730407308285.

Halford, S., Pires, S.S., Turton, M., Zheng, L., Gonzalez-Menendez, I., Davies, W.L., Peirson, S.N., Garcia-Fernandez, J.M., Hankins, M.W., Foster, R.G., 2009. VA opsin-based photoreceptors in the hypothalamus of birds. Curr. Biol. 19 (16), 1396–1402. https://doi.org/10.1016/j.cub.2009.06.066.

Harrison, P.L., Babcock, R.C., Bull, G.D., Oliver, J.K., Wallace, C.C., Willis, B.L., 1984. Mass spawning in tropical reef corals. Science 223 (4641), 1186–1189. https://doi.org/10.1126/science.223.4641.1186.

Hartwig, H.G., Van Veen, T., 1979. Spectral characteristics of visible radiation penetrating into the brain and stimulating extraretinal photoreceptors. J. Comp. Physiol. 130 (3), 277–282. https://doi.org/10.1007/BF00.

Hattar, S., Liao, H.W., Takao, M., Berson, D.M., Yau, K.W., 2002. Melanopsin-containing retinal. ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295 (5557), 1065–1070. https://doi.org/10.1126/science.1069609.

Hong, L.Y., Hong, W.S., Zhu, W.B., Shi, Q., You, X.X., Chen, S.X., 2014. Cloning and expression of melatonin receptors in the mudskipper Boleophthalmus pectinirostris: their role in synchronizing its semilunar spawning rhythm. Gen. Comp. Endocrinol. 195, 138–150. https://doi.org/10.1016/j.ygcen.2013.11.004.

Hong, W.S., Chen, S.X., Zhang, Q.Y., Qiong, W., 2007. Reproductive ecology of the mudskipper Bolephthalmus pectinirostris. Acta Oceanol. Sin 26 (4), 72–81. https://doi.org/10.1109/UT.2007.370822.

Jenkins, A., Munoz, M., Tarttelin, E.E., Bellingham, J., Foster, R.G., Hankins, M.W., 2003. VA opsin, melanopsin, and an inherent light response within retinal interneurons. Curr. Biol. 13 (15), 1269–1278. https://doi.org/10.1016/S0960-9822(03)00509-8.

Juntti, S.A., Hilliard, A.T., Kent, K.R., Kumar, A., Nguyen, A., Jimenez, M.A., Loveland, J. L., Mourrain, P., Fernald, R.D., 2016. A neural basis for control of cichlid female reproductive behavior by prostaglandin F-2 alpha. Curr. Biol. 26 (7), 943–949. https://doi.org/10.1016/j.cub.2016.01.067.

Kah, O., Lethimonier, C., Somoza, G., Guilgur, L.G., Vaillant, C., Lareyre, J.J., 2007. GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective. Gen. Comp. Endocrinol. 153 (1–3), 346–364. https://doi.org/10.1016/j.ygcen.2007.01.030.

Kang, S.W., Leclerc, B., Kosonsiriluk, S., Mauro, L.J., Iwasawa, A., El Halawani, M.E., 2010. Melanopsin expression in dopamine-melatonin neurons of the premammillary nucleus of the hypothalamus and seasonal reproduction in birds. Neuroscience 170 (1), 200–213. https://doi.org/10.1016/j.neuroscience.2010.06.082.

Kawabata, Y., Hiraki, T., Takeuchi, A., Okubo, K., 2012. Sex differences in the expression of vasotocin/isotocin, gonadotropin-releasing hormone, and tyrosine and tryptophan hydroxylase family genes in the Medaka Brain. Neuroscience 218, 65–77. https://doi.org/10.1016/j.neuroscience.2012.05.021.

Kojima, D., Mano, H., Fukada, Y., 2000. Vertebrate ancient-long opsin: a green-sensitive photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. J. Neurosci. 20 (8), 2845–2851. https://doi.org/10.1523/JNEUROSCI.20-08-02845.2000.

Krogh, A., Larsson, B., von Heijne, G., Sonnhammer, E.L.L., 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305 (3), 567–580. https://doi.org/10.1006/jmbi.2000.4315.

Lau, B.Y.B., Mathur, P., Gould, G.G., Guo, S., 2011. Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proc. Natl. Acad. Sci. U. S. A. 108 (6), 2581–2586. https://doi.org/10.1073/pnas.1018275108.

Li, Z., Hong, W.S., Qiu, H.T., Yu, T.Z., Yang, M.S., You, X.X., Chen, S.X., 2016. Cloning and expression of two hepcidin genes in the mudskipper (Boleophthalmus pectinirostris) provides insights into their roles in male reproductive immunity. Fish Shellfish Immunol. 56, 239–247. https://doi.org/10.1016/j.fsi.2016.07.025.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25 (4), 402–408. https://doi.org/10.1006/meth.2001.1262.

Lucas, R.J., Freedman, M.S., Munoz, M., Garcia-Fernandez, J.M., Foster, R.G., 1999. Regulation of the mammalian pineal by non-rod, non-cone, ocular photoreceptors. Science 284 (5413), 505–507. https://doi.org/10.1126/science.284.5413.505.

Lucas, R.J., Hattar, S., Takao, M., Berson, D.M., Foster, R.G., Yau, K.W., 2003. Diminished pupillary light reflex at high irradiances in melanopsin-knockout mice. Science 299 (5604), 245–247. https://doi.org/10.1126/science.1077293.

Mano, H., Kojima, D., Fukada, Y., 1999. Exo-rhodopsin: a novel rhodopsin expressed in the zebrafish pineal gland. Mol. Brain Res. 73 (1–2), 110–118. https://doi.org/10.1016/S0169-328x(99)00242-9.

Matos-Cruz, V., Blasic, J., Nickle, B., Robinson, P.R., Hattar, S., Halpern, M.E., Nitabach, M.N., 2011. Unexpected diversity and photoperiod dependence of the Zebrafish Melanopsin system. PLoS ONE 6 (9), e25111. https://doi.org/10.1371/journal.pone.0025111.

Menon, S.T., Han, M., Sakmar, T.P., 2001. Rhodopsin: structural basis of molecular physiology. Physiol. Rev. 81 (4), 1659–1688. https://doi.org/10.1152/physrev.2001.81.4.1659.

Moore, H.A., Whitmore, D., Ebihara, S., 2014. Circadian rhythmicity and light sensitivity of the zebrafish brain. PLoS ONE 9 (1), e86176. https://doi.org/10.1371/journal.pone.0086176.

Moraes, M.N.d.C.M., Santos, L.R.D., Mezzalira, N., Poletini, M.O., Castrucci, A.M.d.L., 2014. Regulation of melanopsins and Per1 by α -MSH and melatonin in photosensitive Xenopus laevis melanophores. Biomed Res. Int. 2014, 1–10. https://doi.org/10.1155/2014/654710.

Nakane, Y., Ikegami, K., Iigo, M., Ono, H., Takeda, K., Takahashi, D., Uesaka, M., Kimijima, M., Hashimoto, R., Arai, N., Suga, T., Kosuge, K., Abe, T., Maeda, R., Senga, T., Amiya, N., Azuma, T., Amano, M., Abe, H., Yamamoto, N., Yoshimura, T., 2013. The saccus vasculosus of fish is a sensor of seasonal changes in day length. Nat. Commun. 4 (1) https://doi.org/10.1038/ncomms3108.

Nakane, Y., Ikegami, K., Ono, H., Yamamoto, N., Yoshida, S., Hirunagi, K., Ebihara, S., Kubo, Y., Yoshimura, T., 2010. A mammalian neural tissue opsin (Opsin 5) is a deep brain photoreceptor in birds. Proc. Natl. Acad. Sci. U. S. A. 107 (34), 15264–15268. https://doi.org/10.1073/pnas.1006393107.

Nakane, Y., Yoshimura, T., 2010. Deep brain photoreceptors and a seasonal signal transduction cascade in birds. Cell Tissue Res. 342 (3), 341–344. https://doi.org/10.1007/s00441-010-1073-6.

Nelson, R.J., Zucker, I., 1981. Absence of extraocular photoreception in diurnal and nocturnal rodents exposed to direct sunlight. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 69 (1), 145–148. https://doi.org/10.1016/0300-9629(81)90651-4.

Newman, L.A., Walker, M.T., Brown, R.L., Cronin, T.W., Robinson, P.R., 2003. Melanopsin forms a functional short-wavelength photopigment. Biochemistry 42 (44), 12734–12738. https://doi.org/10.1021/bi035418z.

Okano, T., Yoshizawa, T., Fukada, Y., 1994. Pinopsin is a chicken pineal photoreceptor molecule. Nature 372 (6501), 94–97. https://doi.org/10.1038/372094a0.

Okubo, K., Nagahama, Y., 2008. Structural and functional evolution of gonadotropin-releasing hormone in vertebrates. Acta Physiol. 193 (1), 3–15. https://doi.org/10.1111/j.1748-1716.2008.01832.x.

Panda, S., Sato, T.K., Castrucci, A.M., Rollag, M.D., DeGrip, W.J., Hogenesch, J.B., Provencio, I., Kay, S.A., 2002. Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. Science 298 (5601), 2213–2216. https://doi.org/10.1126/science.1076848.

Perez, J.H., Tolla, E., Dunn, I.C., Meddle, S.L., Stevenson, T.J., 2019. A comparative perspective on extra-retinal photoreception. Trends Endocrinol. Metab. 30 (1), 39–53. https://doi.org/10.1016/j.tem.2018.10.005.

Pires, S.S., Shand, J., Bellingham, J., Arrese, C., Turton, M., Peirson, S., Foster, R.G., Halford, S., 2007. Isolation and characterization of melanopsin (Opn4) from the Australian marsupial Sminthopsis crassicaudata (fat-tailed dunnart). Proc R Soc Biol Sci Ser B 274 (1627), 2791–2799. https://doi.org/10.1098/rspb.2007.0976.

Provencio, I., Jiang, G.S., De Grip, W.J., Hayes, W.P., Rollag, M.D., 1998. Melanopsin: an opsin in melanophores, brain, and eye. Proc. Natl. Acad. Sci. U. S. A. 95 (1), 340–345. https://doi.org/10.1073/pnas.95.1.340.

Rink, E., Wullimann, M.F., 2002. Connections of the ventral telencephalon and tyrosine hydroxylase distribution in the zebrafish brain (Danio rerio) lead to identification of an ascending dopaminergic system in a teleost. Brain Res. Bull. 57 (3–4), 385–387. https://doi.org/10.1016/s0361-9230(01)00696-7.

Ruby, N.F., Brennan, T.J., Xie, X.M., Cao, V., Franken, P., Heller, H.C., O'Hara, B.F., 2002. Role of melanopsin in circadian responses to light. Science 298 (5601), 2211–2213. https://doi.org/10.1126/science.1076701.

Sandbakken, M., Ebbesson, L., Stefansson, S., Helvik, J.V., 2012. Isolation and characterization of melanopsin photoreceptors of atlantic salmon (Salmo salar). J. Comp. Neurol. 520 (16), 3727–3744. https://doi.org/10.1002/cne.v520.1610.1002/cne.23125.

Satou, M., Oka, Y., Kusunoki, M., Matsushima, T., Kato, M., Fujita, I., Ueda, K., 1984. Telencephalic and preoptic areas integrate sexual-behavior in hime salmon (Landlocked Red Salmon, Oncorhynchus-Nerka) – results of electrical brain-stimulation experiments. Physiol. Behav. 33 (3), 441–447. https://doi.org/10.1016/0031-9384(84)90167-7.

Shand, J., Foster, R.G., 1999. The extraretinal photoreceptors of non-mammalian vertebrates. In: Archer, S.N., Djamgoz, M.B.A., Loew, E.R., Partridge, J.C., Vallerga, S. (Eds.), Adaptive Mechanisms in the Ecology of Vision. Springer, Netherlands, Dordrecht, pp. 197–222.

Takeuchi, Y., Kabutomori, R., Yamauchi, C., Miyagi, H., Takemura, A., Okano, K., Okano, T., 2018. Moonlight controls lunar-phase-dependency and regular oscillation of clock gene expressions in a lunar-synchronized spawner fish. Goldlined spinefoot. Sci. Rep. 8 (1), 6208. https://doi.org/10.1038/s41598-018-24538-1.

Terakita, A., Yamashita, T., Shichida, Y., 2000. Highly conserved glutamic acid in the extracellular IV-V loop in rhodopsins acts as the counterion in retinochrome, a member of the rhodopsin family. Proc. Natl. Acad. Sci. U. S. A. 97 (26), 14263–14267. https://doi.org/10.1073/pnas.260349597.

Underwood, H., 1973. Retinal and extraretinal photoreceptors mediate entrainment of the circadian locomotor rhythm in lizards. J. Comp. Physiol. 83 (2), 187–222. https://doi.org/10.1007/BF00696895.

Wang, Q., Hong, W.S., Chen, S., Zhang, Q.Y., 2008. Variation with semilunar periodicity of plasma steroid hormone production in the mudskipper Boleophthalmus pectinirostris. Gen. Comp. Endocrinol. 155 (3), 821–826. https://doi.org/10.1016/j.ygcen.2007.10.008.

Wong, C.J.H., 1997. Connections of the basal forebrain of the weakly electric fish, Eigenmannia virescens. J. Comp. Neurol. 389 (1), 49–64. https://doi.org/10.1002/(SICI)1096-9861(19971208)389:1<49::AID-CNE4>3.0.CO;2-E.

Wullimann, M.F., Mueller, T., 2004. Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior (vol 475, pg 143, 2004). J. Comp. Neurol. 478 (4), 427–428. https://doi.org/10.1002/cne.20291.

Xie, X.Y., Zhang, Q.Y., 1990. Studies on the gonadal development in female mudskipper, Boleophthalmus pectinirostris. J. Oceanogr. Taiwan Strait 3, 004.

You, X., Bian, C., Zan, Q., Xu, X., Liu, X., Chen, J., Wang, J., Qiu, Y., Li, W., Zhang, X., Sun, Y., Chen, S., Hong, W., Li, Y., Cheng, S., Fan, G., Shi, C., Liang, J., Tom Tang, Y., Yang, C., Ruan, Z., Bai, J., Peng, C., Mu, Q., Lu, J., Fan, M., Yang, S., Huang, Z., Jiang, X., Fang, X., Zhang, G., Zhang, Y., Polgar, G., Yu, H., Li, J., Liu, Z., Zhang, G., Ravi, V., Coon, S.L., Wang, J., Yang, H., Venkatesh, B., Wang, J., Shi, Q., 2014. Mudskipper genomes provide insights into the terrestrial adaptation of amphibious fishes. Nat. Commun. 5, 5594-5594. 10.1038/ncomms6594.

Zantke, J., Ishikawa-Fujiwara, T., Arboleda, E., Lohs, C., Schipany, K., Hallay, N., Straw, A.D., Todo, T., Tessmar-Raible, K., 2013. Circadian and circalunar clock interactions in a marine annelid. Cell Rep. 5 (1), 99–113. https://doi.org/10.1016/j.celrep.2013.08.031.

Zohar, Y., Munoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165 (3), 438–455. https://doi.org/10.1016/j.ygcen.2009.04.017.