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Cloning and expression of melatonin receptors in the mudskipper *Boleophthalmus pectinirostris*: Their role in synchronizing its semilunar spawning rhythm

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ABSTRACT

The mudskipper *Boleophthalmus pectinirostris*, a burrow-dwelling fish inhabiting intertidal mudflats, spawns only once during the spawning season around either the first or last lunar quarters. To understand the molecular mechanisms regulating this semilunar spawning rhythm, we cloned all melatonin receptor subtypes (*mtnr1a1.4*, *mtnr1a1.7*, *mtnr1b*, and *mtnr1c*). Expression of three melatonin receptor subtypes (except *mtnr1c*) was found in the ovaries. In contrast, the expression of all receptor subtypes was found in the diencephalon and the pituitary. In the fully-grown follicles, only *mtnr1a1.7* mRNA was detected in both the isolated follicle layers and denuded oocytes. Interestingly, the transcript levels of both *mtnr1a1.4* in the diencephalon and *mtnr1a1.7* in the ovary displayed two cycles within one lunar month, and peaked around the first and last lunar quarters. We used 17α,20β-dihydroxy-4-pregnen-3-one (DHP), a maturation-inducing hormone, as a biomarker to examine the involvement of melatonin receptors in the control of the spawning cycle. Melatonin significantly increased the plasma DHP level 1 h post intraperitoneal injection. Melatonin also directly stimulated ovarian fragments *in vitro* to produce a significantly higher amount of DHP. Taken together, these results provided the first evidence that melatonin receptors were involved in the synchronization of the semilunar spawning rhythm in the female mudskipper by acting through the HPG axis and/or directly on ovarian tissues to stimulate the production of DHP.

1. Introduction

Environmental changes caused by lunar cycle are important for reproductive success, hatching and dispersal of larvae, survival, and growth of progeny (Takemura et al., 2004). Therefore, many coral reef fishes and intertidal fishes synchronize their spawning according to the lunar cycle (Takemura et al., 2010; Taylor, 1984).

Circadian oscillators and their messengers are essential for synchronizing reproductive events in order to correspond to changes in the environment factors. In brief, environmental factor(s) provide external cues to the circadian clocks, which in turn produce rhythmic messengers that synchronize the activities of target cells and tissues. Melatonin (N-acetyl-5-methoxytryptamine) is a well-recognized messenger of the vertebrate circadian clock, which plays an important role in regulating reproductive events in seasonally breeding vertebrates including the teleosts (Bromage et al., 2001; Matthews et al., 1993; Reiter, 1991; Reiter et al., 2009). In teleosts, melatonin is produced mainly in the pineal gland; though the retinas also produce a meaningful amount of melatonin (Kezuka et al., 1989).

The effects of melatonin are mediated through its high- and low-affinity receptors (Patino et al., 2012; Vanecek, 1998; von Gall et al., 2002). The high-affinity melatonin receptors belong to a superfamily of G-protein coupled receptors (GPCRs) coupled to several intracellular pathways (Iigo et al., 1994; Reppert et al., 1996; Shiu and Pang, 1998). In contrast, the low-affinity melatonin receptor identified in mammals corresponds to quinone reductase-2, a cytosolic enzyme that might be involved in detoxification processes. Three different melatonin receptor subtypes have been identified in vertebrates. The Mtnr1a (also known as Mel_{1a}) and Mtnr1b (also known as Mel_{1b}) subtypes have been identified in all the vertebrates examined (Ebisawa et al., 1994; Gaildrat et al., 2002), while the presence of the Mtnr1c (also known as Mel_{1c}) subtype has been found only in non-mammalian species (Ebisawa et al., 1994; Park et al., 2007b). In addition, two subtypes of

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Mtnr1a, i.e. Mtnr1a1.4 (also known as Mel_{1a}1.4) and Mtnr1a1.7 (also known as Mel_{1a}1.7), have been identified in zebrafish, *Danio rerio* (Reppert et al., 1995), rainbow trout, *Oncorhynchus mykiss* (Mazurais et al., 1999), grass puffer, *Takifugu niphobles* (Ikegami et al., 2009b), and goldfish, *Carassius auratus* (Ikegami et al., 2009a).

The mudskipper *Boleophthalmus pectinirostris*, a burrow-dwelling fish inhabiting intertidal mudflats, is an exceptional model among fishes for their amphibious behavior and numerous physiological and morphological specializations adapted for amphibious life (Chen et al., 2006, 2007; Hong et al., 2007, 2013). In previous field investigations, we found that the ovaries of wild *B. pectinirostris* developed to the tertiary yolk stage prior to the spawning season. The fish spawn once, either at the first or the last lunar quarters during the spawning season (Hong et al., 2007). The peak levels of plasma steroid hormones, including estrogens, androgens, and progestins, also correspond well with the first and last lunar quarters. Our results suggest that plasma steroid hormones are regulated according to the lunar cycle (Wang et al., 2008), as reported in other teleosts (Rahman et al., 2000a,b). However, the endocrine signalings initiated by the lunar cycle that control this semilunar reproductive cycle are still largely unknown.

In the present study, we first cloned all four subtypes of melatonin receptor cDNAs, and examined their distribution in the different regions of the brain and peripheral tissues. The diurnal variations of these receptors in the brain, retina, pituitary and ovary were subsequently determined using real-time quantitative PCR (qPCR). In addition, the effects of melatonin on the production of 17α,20β-dihydroxy-4-pregnen-3-one (DHP), a maturation-inducing hormone (MIH) in teleosts (Nagahama, 1997), were examined both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Experimental fish

Adult female mudskipper *B. pectinirostris* were captured from Funing Bay, Fujian, China (26°53′N; 120°03′E) using trap nets during low tide between May and June, 2012. Body length and body weight were measured (110–166 mm and 16.6–31.0 g). Gonadosomatic index (GSI) was calculated as GSI (%) = [gonad weight (g)/total body weight (g)] × 100%. Females with tertiary yolk stage oocytes (GSI: 6.42–8.09%; oocyte diameter: 310–500 μm) (Wang et al., 2008) at the same age (2 years old) were selected for the present study. All experiment protocols were reviewed and approved by the Institute Animal Care and Use Committee of Xiamen University.

2.2. Cloning of melatonin receptor subtypes

Brain tissue collected from mature mudskippers was used for cloning the *mtnr1a1.4* and *mtnr1a1.7*, whereas retina tissue was used for cloning the *mtnr1b* and *mtnr1c* cDNAs. Total RNA was extracted using the RNAzol reagent (Molecular Research Center Inc. (MRC), Cincinnati, OH, USA) and reverse transcribed into first strand cDNA using a 5′-Full RACE Kit (TaKaRa, Japan) and 3′-Full RACE Core Set (TaKaRa) following the manufacturer's instructions. A similar strategy was used for obtaining the full cDNA sequences of all four melatonin receptor subtypes. For example, a partial *mtnr1a1.7* cDNA sequence was first cloned from brain cDNAs using degenerated primers (Table 1), designed based on the highly conserved domain of known *mtnr1a1.7* cDNA sequences in vertebrates. The PCR amplification was carried out in a 25 μl volume using recombinant *Taq*™ DNA polymerase (TaKaRa). The PCR reaction was performed under the following cycling conditions: 1 cycle of denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s, followed by a 10 min extension at 72 °C. The PCR products of expected size were extracted from the agarose gel following electrophoresis, sub-cloned into vector pTZ57R/T (InsTAclone™ PCR Cloning Kit, Fermentas, Canada), and then transformed into *Escherichia coli* DH5α (Promega, Madison, WI, USA). Several positive clones were selected and sequenced (Invitrogen Ltd, Guangzhou, China). The first PCR amplification for 5′ or 3′ RACE was performed using a universal primer in the kit and a gene specific primer, which was designed based on the partial cDNA sequence obtained above. These initial 5′ or 3′ RACE products were then used for nested PCR amplifications using the respective gene-specific nested primers, in combination with a nested universal primer. All RACE reactions were carried out following the manufacturer's instructions. RACE products were subcloned and sequenced as described above.

Table 1
PCR primers used for cloning melatonin receptor subtypes.

Genes name	Primer name	Primer sequence(5′→3′)
mtnr1a 1.4	Forward	TGBCCGACCTGGTGGTGGCG
	Reverse	CCATGAAGTAGCTGGCTGTGA
	3′GSP	CTACATCTGCCACAGCCTGA
	3′GSNP	TCCAATGCCAATACCATGTG
mtnr1a 1.7	Forward	AGCGTCATCGGCTCCATC
	Reverse	GCTGGCCACRAANARCCACTC
	3′GSP	CGTGAGGAACTTTGTCACCA
	3′GSNP	TGTTGTGTTCGTGCTCTTCG
	5′GSP	ACTGCAAGGAGCCTACGAAA
	5′GSNP	ATTGGGCACGATGGCTACTA
mtnr1b	Forward	ATYTTCAACATCACNGGSATYG
	Reverse	RCTGACCACRAAVAGCCACTC
	3′GSP	GCCCAGTGACATGAGGAACT
	3′GSNP	CCTGACCATGTTTGTGGTGT
	5′GSP	GGAGCCCACAAAGAAATTGG
	5′GSNP	ACTATGGCGGCTATGGTGAG
mtnr1c	Forward	CTACCTGGGYCTCACCTG
	Reverse	GTAGCTBGTGACAAABAGCCACTC
	3′GSP	TGCGAATATGGGTGCTAGTG
	3′GSNP	CACAGGGTGAAACCAGAACA
	5′GSP	GGGCACAACTTTACTCGGATT
	5′GSNP	AGGCCTATGAGGTTGAGTGG

2.3. Sequence analysis

After obtaining the cDNAs for the mudskipper's four melatonin receptor 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 melatonin receptor subtypes was performed using the Megalign program of the Lasergene software package (DNASTAR Inc., Madison, WI, USA) and the Clustal W method. Phylogenetic analyses were carried out using the aligned amino acid sequences and the neighbor-joining algorithm (Saitou and Nei, 1987) with a boot-strap value of 1000 trials for each position and rooted by the mudskipper thyrotropin receptor (GenBank accession number KC686694).

2.4. Tissue distribution of the melatonin receptor mRNAs

To examine the tissue distribution of the melatonin receptor mRNAs, adult fish were anesthetized in 0.01% MS222 (Sigma–Aldrich) buffered solution with an equal amount of sodium bicarbonate and decapitated humanely around midnight (00:00). The brains were trimmed to collect the pituitary, telencephalon, diencephalon, optic tectum, cerebellum and medulla oblongata separately (Fig. 1). The retina was collected from the eyeballs. The gill, heart, stomach, spleen, intestine, muscle, skin and ovary were collected and immediately dipped into liquid nitrogen and stored

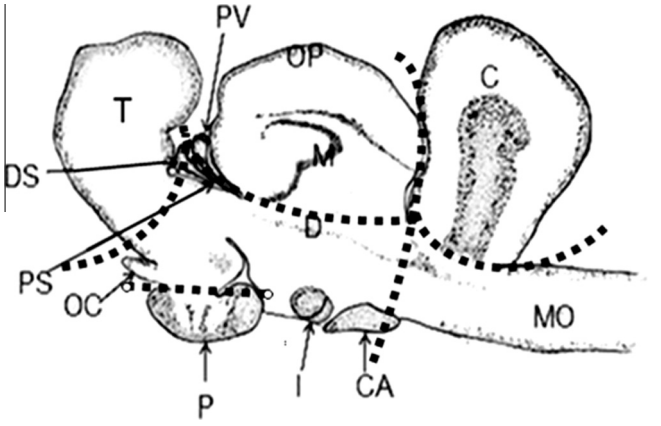

Fig. 1. Schematic illustration of a sagittal section of the mudskipper brain. T: Telencephalon; OP: Optic tectum; M: Mesencephalon; D: Diencephalon; C: Cerebellum; MO: Medulla oblongata; P: Pituitary; PS: Pineal stalk; PV: Pineal ending vesicle; DS: Dorsal sac; OC: Optic chiasma; I: Infundibulum; CA: Corpus mamillare.

at −80 °C. Total RNA was extracted from the tissue samples using the RNAzol method (MRC). The same amount of total RNA (1.5 μg) was used for synthesis of the first strand cDNAs using the M-MLV RTase cDNA Synthesis Kit (TaKaRa). Real-time qPCR was performed as described in Section 2.7.

2.5. Expression of melatonin receptor mRNAs during the diurnal cycle

Freshly captured adult females were transferred into an outdoor muddy pool under natural temperature and photoperiod. To examine the diurnal variation of melatonin receptor subtype mRNA levels in the brain and retina, samples were collected at 3 h intervals during a 24 h period following the procedures described in Section 2.5. Total RNA extraction and cDNA synthesis for the brain and retina samples were conducted as described in Section 2.4. Real-time qPCR was performed as described in Section 2.7.

2.6. Expression of melatonin receptor mRNAs during the lunar cycle

Samples were collected from freshly captured fish that were anesthetized and humanely decapitated at 20:00–21:00 h. The brain and ovary were removed and soaked immediately in RNAlater (Ambion, TX, USA). After the samples were brought back to the laboratory, the brains were further trimmed to collect the diencephalon and pituitary. Total RNA extraction and cDNA synthesis for the brain and retina samples were conducted as described in Section 2.4. Real-time qPCR was performed as indicated in Section 2.7.

2.7. Real-time qPCR

Specific primers for detecting target genes (Table 2) were designed and examined for their specificity and amplification efficiency on serial dilutions of respective target gene plasmid DNA (2×10^2–2×10^7 copies/2 μl). All qPCR was performed in 20 μl reactions employing SYBR Premix Ex Taq (TaKaRa), and C_t values were determined in a 7500 fast Real-Time PCR System (Applied Biosystems, USA) using default settings. The relative mRNA levels of the target genes were determined using the comparative C_t method (Schmittgen and Livak, 2008) with the *β-actin* gene as the internal control. The cDNA sequence for mudskipper's *β-actin* was kindly provided by BGI (Shenzhen, China) (GenBank accession number: KC622028).

Table 2
PCR primers used for the gene expression analyses.

Genes name	Primer name	Primer sequence(5′→3′)
β-actin	Forward	CAGGCTGTGCTGTCCTTGTA
	Reverse	GGAAGAGTAGCCACGCTCTG
mtnr1a1.4	Forward	CTCGTTTGGGTGCTAACCAT
	Reverse	CCGTAGATAGCAATGCGTGA
mtnr1a1.7	Forward	GCAACCTCCTCGTCATCTTC
	Reverse	TGAGGAAGCCACTGATCTGA
mtnr1b	Forward	CTTGGGTCATTGGCATCTTG
	Reverse	CCAGCCGTCATGAAAGAGAG
mtnr1c	Forward	CACTTCTCCCGGAGTGTCC
	Reverse	GCAGTGAGCACCAGTGGATA
vasa	Forward	AGAGGACAGGCAGACCTTGA
	Reverse	CGTTCGGTTCCTGTCACTTT
cyp19a	Forward	CTGAGATTTCACCCCGTTGT
	Reverse	GTCACCAGGACCGACTTCAT
gdf9	Forward	ATCAAAGCTCTTTCCCAGCA
	Reverse	CAGCAGGGTGGATTTTAGGA

2.8. Cellular localization of melatonin receptors in the ovarian follicles

Ovaries with tertiary yolk stage oocytes were collected from female mudskippers following deep anesthetization and humane decapitation, and placed in a 100 mm culture dish that contained 60% Leibovitz L-15 (GIBCO, Invitrogen) medium. The fully grown follicles (~500 μm in diameter) were manually isolated from the ovary, and the follicle layer was then carefully peeled off with fine forceps without damaging the oocyte. The isolated follicle layers and denuded but intact oocytes were pooled separately and subjected to RNA extraction and cDNA synthesis as described in Section 2.4. To localize the expression of the melatonin receptor mRNAs in different compartments of the follicles, PCR amplification of the cDNAs was performed using primer sets designed for qPCR (Table 2). To ensure a clean separation of follicle layers from oocytes, we used ovarian aromatase A (*cyp19a*), *growth differentiation factor 9* (gdf9) (Liu and Ge, 2007) and *vasa homolog* (*vasa*) (Gustafson and Wessel, 2010) as markers for the follicle layer and oocytes, respectively. The cDNA sequences of mudskipper *cyp19a*, *gdf9*, and *vasa* were kindly provided by BGI which is running a genome project for the mudskipper *B. pectinirostris* (GenBank accession number: *cyp19a*, KC633183; *vasa*, KC633182; *gdf9*, KC633181). PCR amplification was performed using a set of specific forward (F) and reverse (R) primers, designed from the cDNA sequences (Table 2) under the following conditions: 1 cycle of denaturation at 94 °C for 5 min, 32 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s, followed by a 10 min extension at 72 °C. The PCR products were analyzed using gel electrophoresis, and fragments of the expected size were extracted, sub-cloned into vector pTZ57R/T and then transformed into *E. coli* DH5α. The plasmid DNA of several positive clones was selected and sequenced as indicated in Section 2.2 to confirm their identities.

2.9. Effects of melatonin on plasma DHP levels in vivo

Melatonin (Sigma–Aldrich) was first dissolved in a small amount of ethanol and then diluted in 0.7% saline to the desired concentrations. For the time course experiment, female fish were randomly divided into the treated and the control group. Around 12:00, the fish were intraperitoneally injected with 100 μl of saline containing melatonin at a dose of 50 ng/g body weight, or saline only as the control. Plasma samples were collected at 1, 2, and 4 h following injection to determine the effects of melatonin on DHP production *in vivo*. The reproductive status of each female fish was evaluated based on the GSI and oocyte diameter.

A similar approach was used for the dose response experiment. Female fish were intraperitoneally injected with saline (control) or melatonin at doses of 0, 50, 500, and 5000 ng/g body weight. 1 h

sampling time was selected based on the results of the time course experiment and so, plasma samples were collected 1 h following injection for the quantification of DHP. The method for the quantification of DHP in plasma is described in Section 2.11.

2.10. Effect of melatonin on DHP production in ovarian tissue in vitro

Around 12:00, fully-grown follicles were collected from mature females that were deeply anesthetized with 0.01% MS222 (Sigma–Aldrich) and humanely decapitated. Freshly removed ovaries were rinsed several times with ice-cold D-Hank's solution and cut into fragments of 2–3 mm in each dimension in a culture medium. The culture medium consisted of 60% 15 g/L Leibovitz L-15 (GIBCO), 10 mm Hepes, 0.5% w/v bovine serum albumin fraction V, 0.4 mg/L amphotericine B and 200,000 U/L penicillin/streptomycin with pH adjusted to 7.4. Each ovarian fragment was cultured in 1 ml medium containing different concentrations of melatonin (0 as control, 100 and 500 pg/ml) in a 24-well flat-bottom plate (NEST, China) at 25 °C for 24 h in a humidified incubator. The ovarian fragments were weighed at the end of incubation. The medium was removed and heated at 80 °C for 1 h. Following centrifugation (30 min, 16,000*g*), the supernatant was collected and stored at −20 °C until the quantification of DHP (see Section 2.11).

2.11. Measurement of steroid hormones

DHP levels in plasma or culture medium samples were determined using a previous protocol (Wang et al., 2008) and an EIA kit purchased from the Caymen Chemical Company (Ann Arbor, Michigan, USA).

2.12. Statistical analysis

All data, presented as means ± standard error of the mean (SEM), were analyzed using one-way ANOVA followed by Tukey's post hoc test to assess statistical differences among the individual groups. The analyses were performed using the GraphPad Prism4 software package (San Diego, CA, USA).

3. Results

3.1. Cloning and phylogenetic analyses of melatonin receptor subtypes

All four melatonin receptor subtypes were cloned and their sequences were deposited into GenBank (accession numbers: *mtnr1a1.4* KC622029, *mtnr1a1.7* KC622030, *mtnr1b* KC622031, and *mtnr1c* KC622032). All four melatonin receptors had seven transmembrane domains, characteristics of G protein-coupled receptors (Fig. 2). Importantly, these receptors had motifs typical for melatonin receptors including an NRY motif immediately downstream of the transmembrane domain III, and an NAXXY (where X is any amino acid except praline) motif in the transmembrane domain VII. All four melatonin receptor subtypes also contained amino acids known to be crucial for the functioning of the receptors in vertebrates (see Fig. 2 for details). Phylogenetic analysis classified these melatonin receptors into four distinct subtypes (Fig. 3).

3.2. Melatonin receptors expressed in the brain and peripheral tissues including the ovary

All four melatonin receptor subtypes were expressed abundantly in the neural tissues, i.e., the retina and whole brain (Fig. 4). The transcripts of *mtnr1a1.4* and *mtnr1a1.7* could be detected in most of the peripheral tissues except muscle (*mtnr1a 1.4* and *mtnr1a1.7*) and liver (*mtnr1a1.7*). In contrast, the expression of the *mtnr1b* and *mtnr1c* transcripts were considerably restricted and were undetectable in ovary (*mtnr1c*), skin (*mtnr1b* and *mtnr1c*), liver (*mtnr1b* and *mtnr1c*), muscle (*mtnr1b*), and spleen (*mtnr1b* and *mtnr1c*). All four melatonin receptor subtypes were also clearly detected in different regions of the brain (Fig. 5). In particular, the expression of *mtnr1a1.4*, *mtnr1a1.7* and *mtnr1b* genes in the optic tectum and the mesencephalon were significantly higher than those in other areas of the brain ($p < 0.05$). On the HPG axis, all four melatonin receptor transcripts were expressed in diencephalon and pituitary, but only three subtypes (*mtnr1a1.4*, *mtnr1a1.7*, and *mtnr1b*) were detectable in ovarian tissues.

3.3. Expression of the melatonin receptor's transcripts showed distinct diurnal changes during the daily cycle in the brain

The expression of *mtnr1a1.4* mRNA was significantly high in the dark phase, and was low in the light phase (Fig. 6; e.g. 06:00, $p < 0.05$) in the brain, but no significant diurnal difference was observed in the retina. In contrast, both *mtnr1a1.7* and *mtnr1b* mRNA were significantly high in the afternoon (e.g. 15:00, $p < 0.05$), and significantly low in the dark phase and in the morning in both the brain and the retina. Interestingly, the expression of *mtnr1c* transcripts was different in the brain, with significantly high levels in the late half of the dark phase and first half of the light phase. Intriguingly, no significant daily fluctuation of *mtnr1c* mRNA in the retina was observed (Fig. 6), while the level of *mtnr1c* mRNA in the retina was 12-fold higher than that in brain (Fig. 4).

3.4. Distinct expression changes of melatonin receptor mRNAs in the diencephalon of female fish within one lunar month during the spawning season

The expression of *mtnr1a1.4* mRNA in the diencephalon showed two distinct cycles corresponding to the lunar phase (Fig. 7). The first peak was observed on 26 May (two days before the first lunar quarter), and the expression levels declined significantly afterwards ($p < 0.05$), reaching their minimum value on 1 June. Thereafter, they increased gradually and reached the second peak on 13 June (three days after the last lunar quarter). Then they decreased significantly ($p < 0.01$) again to the basal level on 19 June.

The *mtnr1a1.7* mRNA levels in the diencephalon also exhibited obvious variations. Relatively low levels of *mtnr1a1.7* mRNA were found at the beginning of the spawning season (20 May), but the levels continued to rise to a maximum with statistical significance on 26 May. Then they diminished significantly ($p < 0.05$) on 1 June, but rose precipitously to the second peak on 4 June. Thereafter, the expression level of *mtnr1a1.7* fell to its lowest value on 19 June.

The *mtnr1b* mRNA amounts in the diencephalon were high between the new moon and the first lunar quarter. However, down-regulation of *mtnr1b* mRNA began towards the beginning of the first lunar quarter, decreasing to minimum values on 1 June, with a slight, but statistically not significant, rise observed on 4 June. For the remainder of the study, the *mtnr1b* expression was maintained at low levels.

The *mtnr1c* mRNA expression in the diencephalon of female fish varied slightly throughout the study period, but displayed a distinct peak on 23 May when compared to the low values detected on 29 May and 19 June.

3.5. Distinct expression changes of melatonin receptor mRNAs in the pituitary of female fish within one lunar month during the spawning season

In the pituitary, the expression pattern of the four subtypes exhibited obvious periodicity (Fig. 7). However, the pattern did not correspond with the semilunar cycle. The *mtnr1a1.4* mRNA

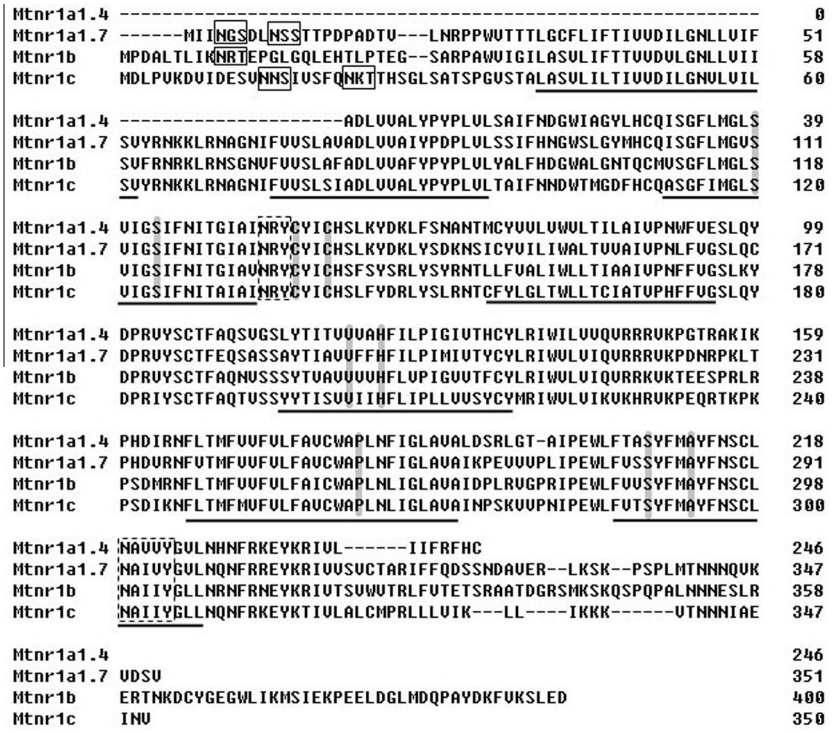

Fig. 2. Alignment of mudskipper melatonin receptor subtypes. The transmembrane domains are underlined and sequentially numbered from I to VII. Amino acids known to be important for the proper function of the mammalian Mtnr1a receptor are highlighted in a gray background. The dotted boxes show the conserved NRY motif just after transmembrane domain III and the NAXXY motif in the seventh transmembrane. The consensus sites for asparagine-linked glycosylation (solid frame) are indicated. GenBank accession numbers are: KC622029 for mudskipper *mtnr1a 1.4*, KC622030 for mudskipper *mtnr1a 1.7*, KC622031 for mudskipper *mtnr1b* and KC622032 for mudskipper *mtnr1c*.

expression decreased ~1-fold from 17 to 23 May and remained at relatively low levels until 29 May. A partial increase was recorded for the period from 1 to 10 June, before the peak was reached on 13 June. Following this significantly highest value ($p < 0.05$), the *mtnr1a1.4* mRNA expression levels decreased gradually. With regard to changes in expression of *mtnr1a1.7* mRNA, the expression pattern was similar to *mtnr1a1.4*, but there was no significant increase on 13 June. Changes of *mtnr1b* mRNA levels exhibited the same periodic pattern as those of *mtnr1a1.4* mRNA, but the statistically significant peak ($p < 0.05$) shifted from 13 to 16 June. Unlike the others, the expression pattern of *mtnr1c* showed two distinct platforms. The lower one was in May, while the higher one, which was about 2.5-fold and significantly higher ($p < 0.05$), was in June.

3.6. Distinct expression changes of melatonin receptors (*mtnr1a1.4*, *mtnr1a1.7*, and *mtnr1b*) in the ovary within one lunar month during the spawning season

We determined the expression levels of *mtnr1a1.4*, *mtnr1a1.7*, and *mtnr1b* mRNA in ovaries, but not *mtnr1c* due to its absence from the ovary, during the spawning season from 17 May to 19 June (Fig. 8). The transcript levels of *mtnr1a1.4* mRNA reached its peak at the end of the lunar month (17 May and 16 June). For the remainder of the period, the levels of *mtnr1a1.4* mRNA were low and did not change significantly.

The mRNA levels of *mtnr1a1.7* in the ovary were relatively low at the beginning of the lunar month, and increased significantly ($p < 0.05$) to the first peak around the first lunar quarter (on 26 May). Then they decreased significantly ($p < 0.01$), and reached the lowest level around the full moon (on 1 June). Thereafter, *mtnr1a1.7* mRNA content gradually increased again, reaching the second high peak around the last lunar quarter on 13 June, followed a significant decrease ($p < 0.01$) to the basal level.

The expression pattern of *mtnr1b* mRNA also exhibited obvious periodicity. The first peak appeared at the beginning of the lunar month (on 20 May), and the expression levels decreased after that. Then they increased to the highest peak ($p < 0.05$) around the full moon (on 1 June). Thereafter, the expression levels came down in waves, and reached the lowest levels on 16 June.

3.7. Expression of mtnr1a 1.7 in the fully-grown follicular cells and denuded oocytes

Only *mtnr1a1.7* mRNA was detected in both the isolated follicle layers and denuded oocytes obtained from fully-grown follicles (Fig. 9). Since the *mtnr1a1.4* and *mtnr1b* mRNA were expressed in the ovary (Fig. 4) but were not detectable in the fully-grown follicles, it was likely that these two melatonin receptors were expressed in early stages of the oocytes, which co-existed with fully grown oocytes in this species (Wang et al., 2008). Clean separation of follicular cells and oocytes was confirmed by the presence and absence of specific markers for the follicular cells and oocytes (Fig. 9): the transcript of *cpy19a*, a follicle cell specific marker, was expressing in the isolated follicle layers but not in the denuded oocytes. At the same time, the oocyte specific markers, *vasa* and *gdf9* mRNAs, were detected only in the denuded oocytes, but not in the isolated follicle layers.

3.8. Melatonin stimulates the production of MIH from ovaries both in vivo and in vitro

We further investigated the potential effects of melatonin on the production of DHP, an MIH in teleosts.

Plasma DHP level increased significantly ($p < 0.05$) to 145.01 ± 19.91 pg/ml at 1 h post intraperitoneal injection of melatonin (50 ng/g body weight), and decreased gradually thereafter

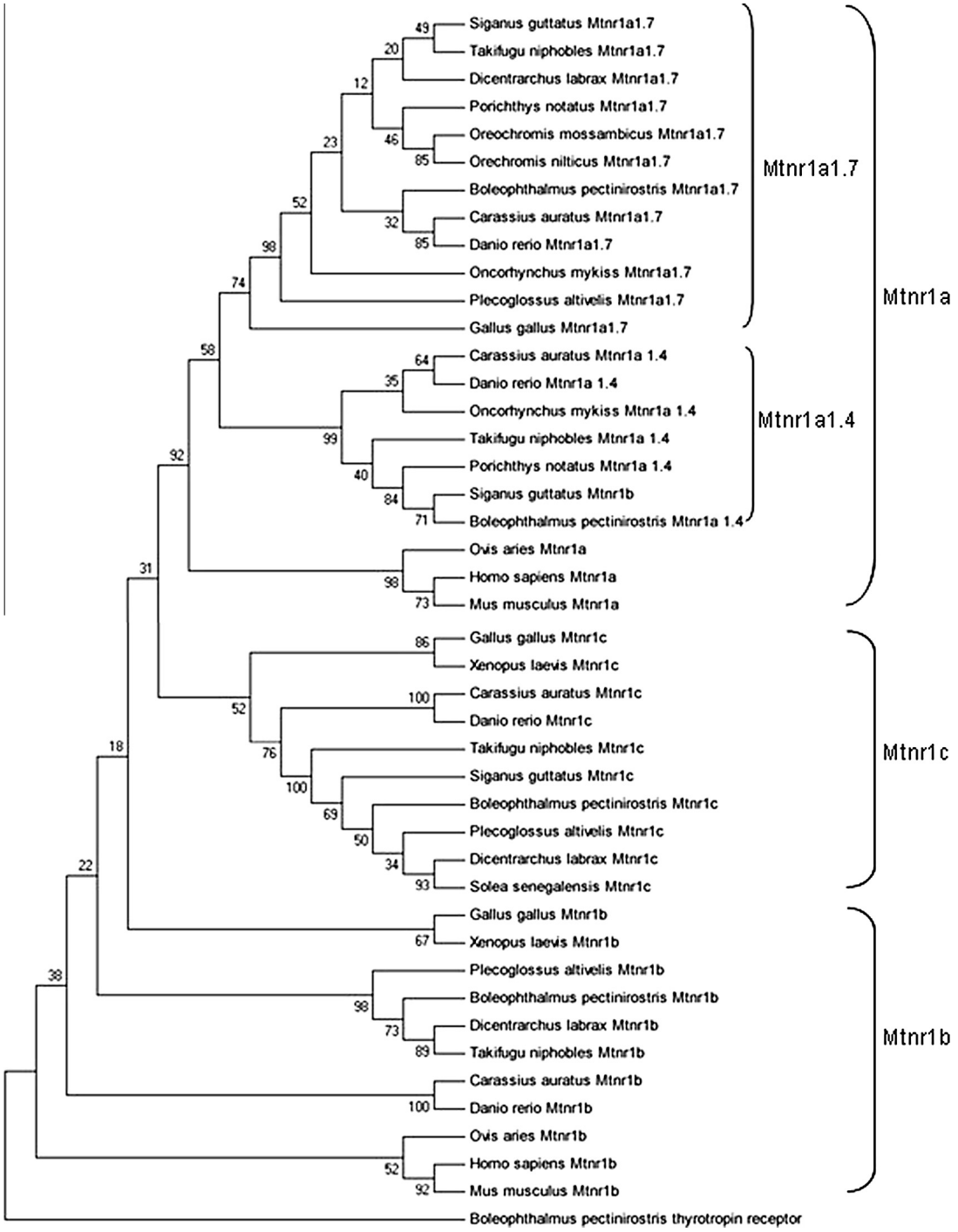

Fig. 3. Phylogenetic analysis of mudskipper melatonin receptor subtypes. Multiple species' amino acid sequences of the receptors were aligned using Clustal W and analyzed using the neighbor-joining method. The numbers on the branches are the results of bootstrap analysis. Mudskipper thyrotropin receptor (GenBank accession number KC686694) was used as an outgroup for the tree. GenBank accession numbers for sequence data analyzed are: *Homo sapiens* Mtnr1a, NP_005949.1; *Mus musculus* Mtnr1a, NP_032665.1; *Ovis aries* Mtnr1a, NP_001009725.1; *Gallus gallus* Mtnr1a1.7 NP_990693.1; *Danio rerio* Mtnr1a1.7, NP_571468.1; *Carassius auratus* Mtnr1a1.7, BAI65862.1; *Dicentrarchus labrax* Mtnr1a1.7, ACB13280.1; *Oncorhynchus mykiss* Mtnr1a1.7, AAF00191.1; *Oreochromis niloticus* Mtnr1a1.7, XP_003449745.1; *Oreochromis mossambicus* Mtnr1a1.7, ACL78767.1; *Porichthys notatus* Mtnr1a1.7, ADU15834.1; *Plecoglossus altivelis* Mtnr1a1.7, BAI65858.1; *Siganus guttatus* Mtnr1a1.7, ABG77572.1; *Takifugu niphobles* Mtnr1a1.7, BAI39598.1; *Boleophthalmus pectinirostris* Mtnr1a1.7, KC622030; *Carassius auratus* Mtnr1a1.4, BAI65861.1; *Porichthys notatus* Mtnr1a1.4, ADU15835.1; *Takifugu niphobles* Mtnr1a1.4, BAI39597.1; *Oncorhynchus mykiss* Mtnr1a1.4, AAD54384.1; *Danio rerio* Mtnr1a1.4, NP_001153381.1; *Boleophthalmus pectinirostris* Mtnr1a1.4, KC622029; *Homo sapiens* Mtnr1b, NP_005950.1; *Mus musculus* Mtnr1b, NP_663758.2; *Ovis aries* Mtnr1b, NP_001124410.1; *Gallus gallus* Mtnr1b, AAA85098.1; *Xenopus laevis* Mtnr1b, P51051.1; *Danio rerio* Mtnr1b, NP_571470.1; *Carassius auratus* Mtnr1b, BAI65864.1; *Plecoglossus altivelis* Mtnr1b, BAI65859.1; *Dicentrarchus labrax* Mtnr1b, ACB13281.1;*Siganus guttatus* Mtnr1b, ABF67976.1; *Takifugu niphobles* Mtnr1b, BAI39599.1; *Boleophthalmus pectinirostris* Mtnr1b, KC622031; *Gallus gallus* Mtnr1c, NP_990692.1; *Danio rerio* Mtnr1c, NP_001154956.1; *Carassius auratus* Mtnr1c, BAI65865.1; *Dicentrarchus labrax* Mtnr1c, ACB13282.1; *Plecoglossus altivelis* Mtnr1c, BAI65860.1; *Siganus guttatus* Mtnr1c, ABG77573.1; *Solea senegalensis* Mtnr1c, CAR82594.1; *Takifugu niphobles* Mtnr1c, BAI39600.1; *Xenopus laevis* Mtnr1c, NP_001081388; *Boleophthalmus pectinirostris* Mtnr1c, KC622032.

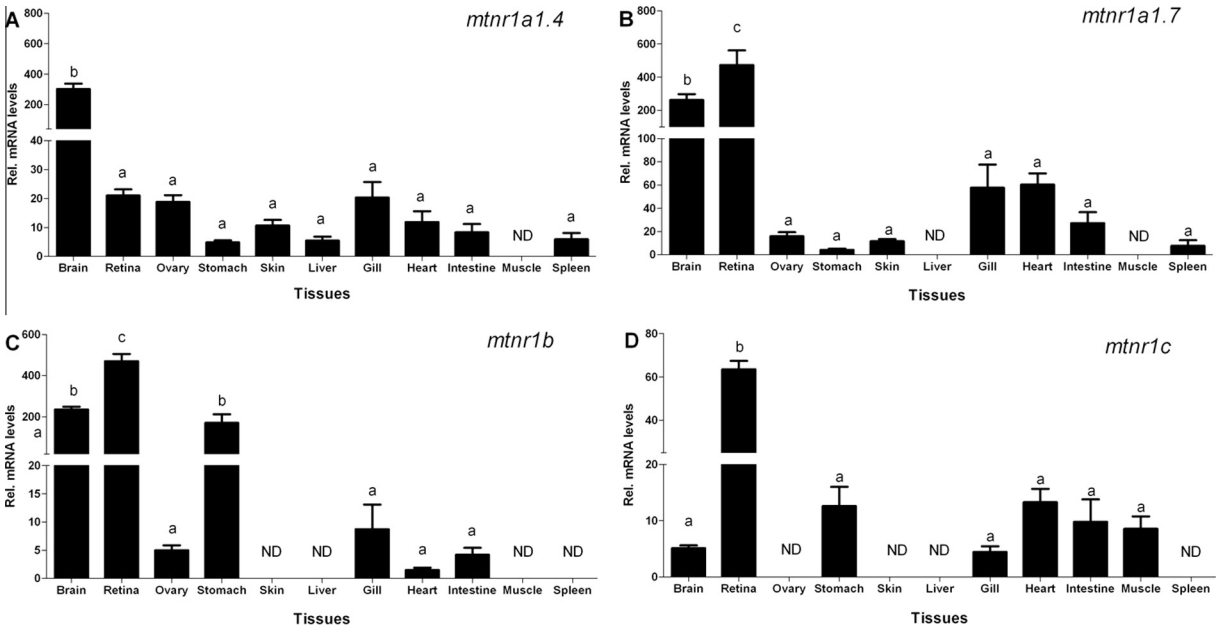

Fig. 4. Expression of melatonin receptor subtypes in the neural tissues (retina and whole brain) and the peripheral tissues (ovary, stomach, skin, liver, gill, heart, intestine, muscle, and spleen) of adult female mudskipper during the spawning season. (A) *mtnr1a1.4*, (B) *mtnr1a1.7*, (C) *mtnr1b*, (D) *mtnr1c*. Tissues were collected at midnight (00:00). The levels of the respective mRNAs were determined using qPCR and normalized to the internal housekeeping gene (*β-actin*). Data are expressed as the mean ± SEM ($n = 6$) relative to the respective transcript levels measured in tissue with the lowest value. Bars marked with different letters are significantly different from each other ($p < 0.05$). ND, not detectable.

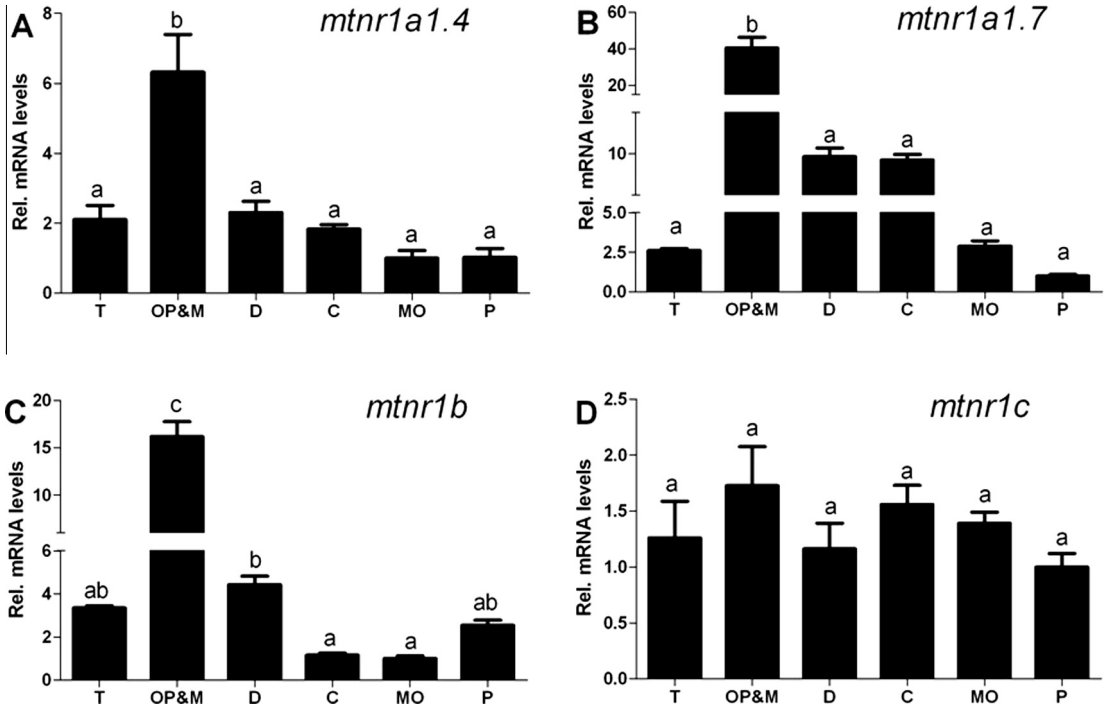

Fig. 5. Expression of four melatonin receptor subtypes in the different brain regions and the pituitary of adult female mudskipper. (A) *mtnr1a1.4*, (B) *mtnr1a1.7*, (C) *mtnr1b*, (D) *mtnr1c*. T: Telencephalon; OP&M: Optic tectum and Mesencephalon; D: Diencephalon; C: Cerebellum; MO: Medulla oblongata; P: Pituitary. Tissues were collected at midnight (00:00). The levels of the respective mRNAs were determined using qPCR and normalized to the house keeping gene (*β-actin*). Data are expressed as the mean ± SEM ($n = 6$) relative to the respective transcript levels measured in the brain region with the lowest value. Bars marked with different letters are significantly different from each other ($p < 0.05$).

(Fig. 10). Therefore, a 1 h sampling interval was selected for the dose response experiment. Plasma DHP level (84.22 ± 10.70 pg/ml) was significantly higher ($p < 0.05$) in fish injected with melatonin at a dose of 50 ng/g body weight than those of the control (38.22 ± 5.22 pg/ml), and two higher doses (500 ng melatonin/g body weight, DHP = 65.17 ± 11.11 pg/ml; 5 μg melatonin/g body weight, DHP = 49.88 ± 8.96 pg/ml, Fig. 10).

To examine whether melatonin acted directly at the local level to stimulate DHP production, an *in vitro* culture of ovaries was utilized. The highest production of DHP (1.43 ± 0.13 ng/g) was

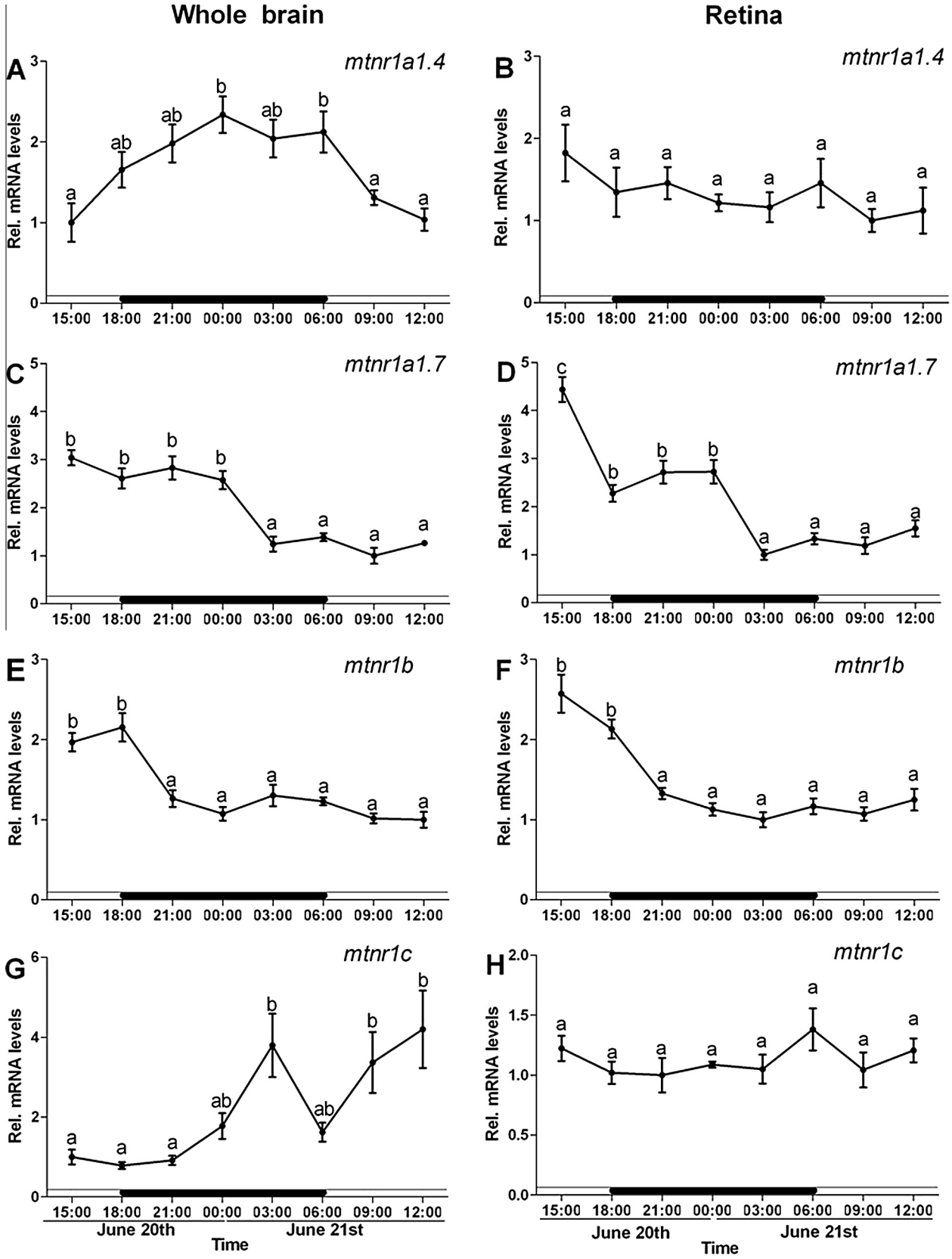

Fig. 6. Expression of four melatonin receptor subtypes in the whole brain (A,C,E,G) and retina (B,D,F,H) of female mudskipper during the daily cycle. Solid and open bars along the X-axis represent the dark phase and the light phase, respectively. The levels of respective mRNAs were determined using qPCR and normalized to the internal control gene (*β-actin*). Data are expressed as the mean ± SEM ($n = 6$) relative to the respective transcript levels of time point with the lowest value. Values marked with different letters are significantly different from each other ($p < 0.05$).

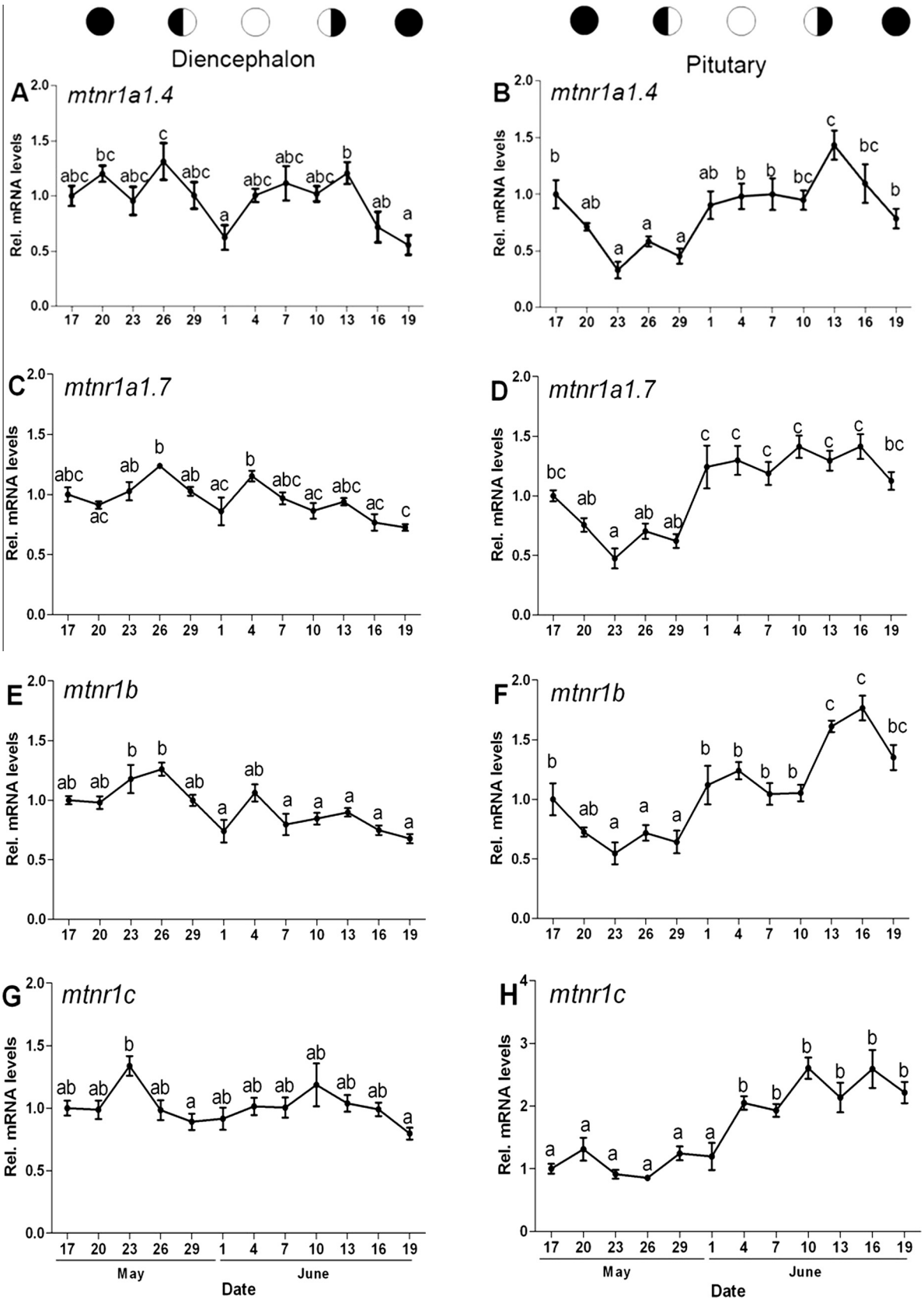

Fig. 7. Expression of melatonin receptor subtypes in the diencephalon (A,C,E,G) and the pituitary (B,D,F,H) of female mudskipper within the lunar cycle during the spawning season. The levels of respective mRNAs were determined using qPCR and normalized to the internal control gene (*β-actin*). Data are expressed as the mean ± SEM ($n = 6$) relative to the respective transcript levels of samples collected on 17 May. Lunar phases are indicated using the following symbols: ● new moon; ◐ first lunar quarter; ○ full moon; and ◑ last lunar quarter. Values marked with different letters are significantly different from each other ($p < 0.05$).

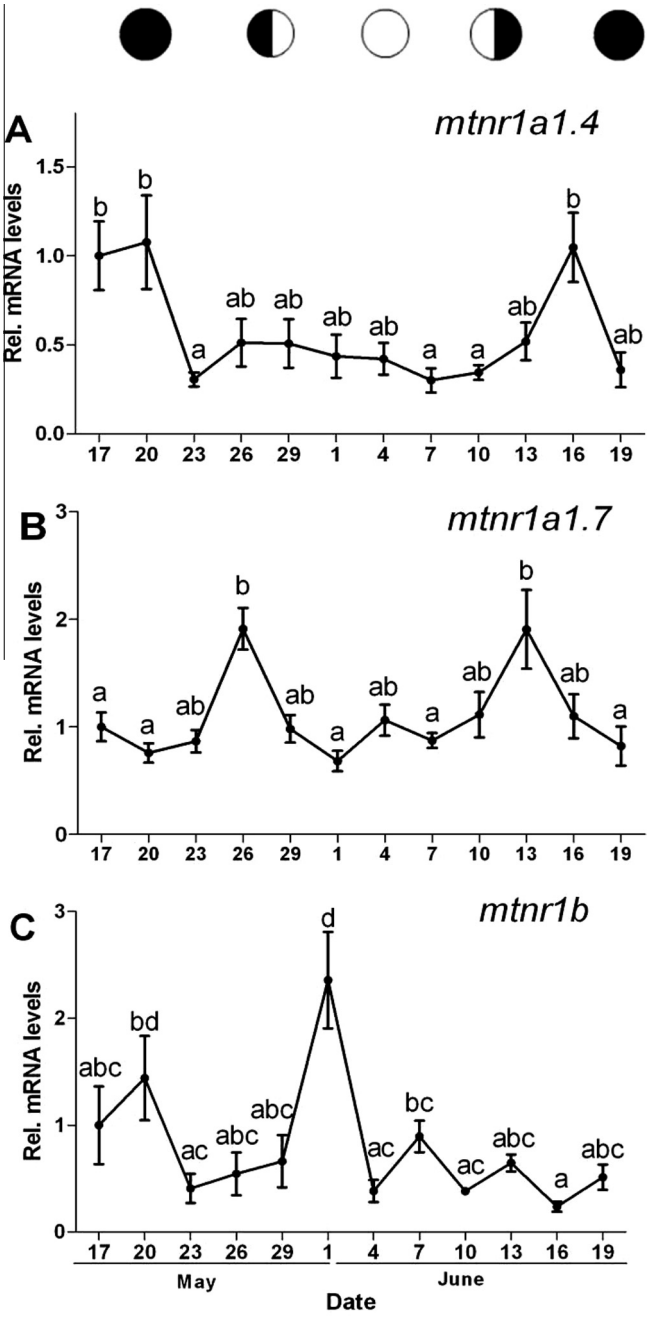

Fig. 8. Expression of *mtnr1a1.4*(A), *mtnr1a1.7*(B), and *mtnr1b* in the ovaries of female mudskipper within one lunar month during the spawning season. The levels of respective mRNAs were determined using qPCR and normalized to the internal control gene (*β-actin*). In each panel, data are expressed as the mean ± SEM ($n = 6$) relative to the respective transcript levels of samples collected on 17 May. Lunar phases are indicated using following symbols: ● new moon; ◐ first lunar quarter; ○ full moon; and ◑ last lunar quarter. Values marked with different letters are significantly different from each other ($p < 0.05$).

observed when ovarian fragments were treated with melatonin at a concentration of 100 pg/ml, which was significantly ($p < 0.001$) higher than those exposed to a higher dose of the melatonin at 500 pg/ml (0.65 ± 0.03 ng/g) or the control group (0.51 ± 0.08 ng/g) (Fig. 11).

4. Discussion

In this study, we provided the evidence that melatonin could synchronize the semilunar spawning cycle in the female

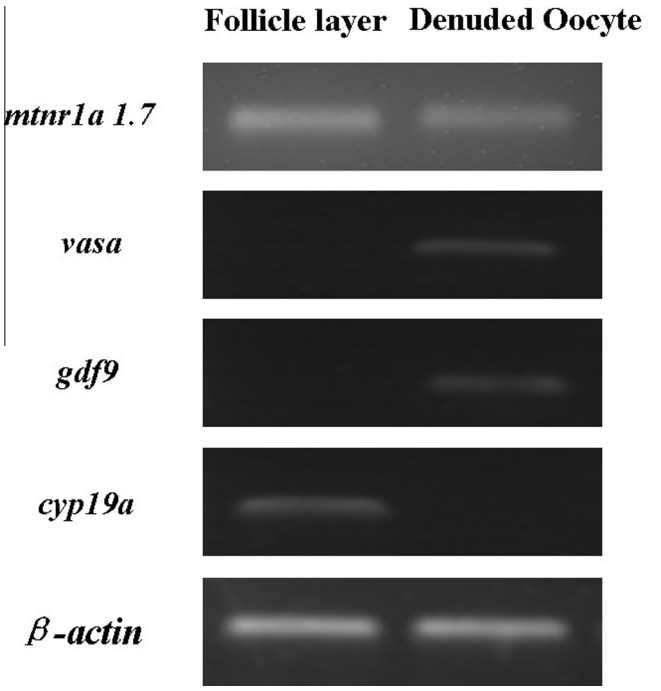

Fig. 9. RT-PCR analysis of *mtnr1a 1.7*, *vasa*, *gdf9*, *cyp19a* and *β-actin* expression in the follicle layers and denuded oocytes obtained from fully-grown follicles of mudskipper during the spawning season. Similar results were obtained from three repeated experiments (only one set of data is shown).

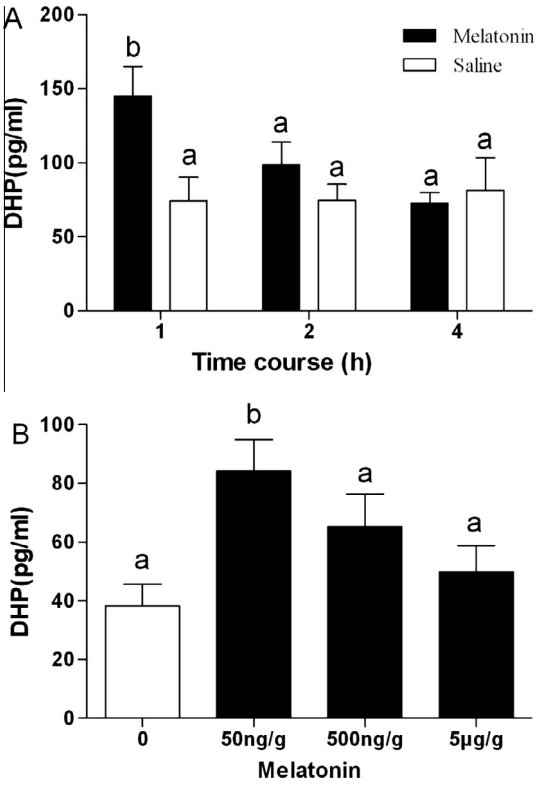

Fig. 10. Effects of melatonin administration on plasma DHP levels in mudskipper with fully-grown follicles during the spawning season. A dose of 50 ng/g body weight melatonin was injected at the selected optimal sampling time point in a time course experiment (panel A). Thereafter, optimal sampling time point (1 h) was selected in a dose response experiment (panel B). Data were expressed as mean ± SEM ($n = 6$) of the amount of DHP per milliliter plasma. Bars marked with different letters are significantly different from each other ($p < 0.05$).

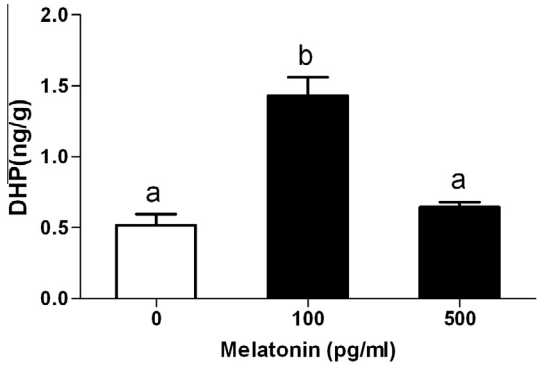

Fig. 11. Effects of melatonin on DHP production in fully-grown follicles *in vitro*. DHP was quantified in incubation medium collected following a 24 h incubation with different concentrations of melatonin (0 as control, 100 and 500 pg/ml). Data were expressed as mean ± SEM ($n = 4$) of the amount of DHP released into the medium per gram of ovarian fragments incubated. Bars marked with different letters are significantly different from each other ($p < 0.001$).

mudskipper by stimulating the production of an MIH, DHP, via melatonin receptors located centrally in the brain and/or directly at its receptor located in the ovaries. We found that the melatonin receptors were expressed abundantly around the HPG axis. In the ovary, only *mtnr1a1.7* was expressed in the fully grown oocytes and exhibited obvious and significant changes, with one peak at the spawning dates. Importantly, melatonin could stimulate DHP production in the ovary both *in vivo* and *in vitro*.

We cloned all four melatonin receptor subtypes in *B. pectinirostris*, in order to understand the synchronization of reproduction controlled by melatonin and it receptors. All four mudskipper melatonin receptors had seven trans-membrane domains, which is characteristic of the GPCR family (Reppert et al., 1996; Shiu and Pang, 1998). Amino acid motifs known to be important for these functions were also conserved in the mudskipper *B. pectinirostris* (Fig. 2) (Kokkola et al., 2003, 2005; Witt-Enderby et al., 2003). These include the two serine residues in transmembrane domain III, 2 cysteine residues in the 4th loop domain, valine and histidine residues in transmembrane domain IV, and a proline and serine residue in transmembrane domains VI and VII, respectively. Phylogenetic analysis further verified that the four melatonin receptors cloned in *B. pectinirostris* belonged to the Mtnr1a1.4, Mtnr1a1.7, Mtnr1b, and Mtnr1c subtypes. In the phylogenetic trees, each *B. pectinirostris* receptor subtype formed a clade with other piscine subtypes.

Melatonin receptors were expressed in a wide range of peripheral tissues in the mudskipper, which was consistent with previous studies in other teleost species (Confente et al., 2010; Ikegami et al., 2009a,b; Patino et al., 2012). These results suggested that melatonin might regulate a variety of different physiological functions in the mudskipper, such as food intake, skin pigmentation, osmoregulation, growth, and reproduction (De Pedro et al., 2008; Porter et al., 1998; Rubio et al., 2004; Suzuki and Hattori, 2002). All melatonin receptors subtypes, except *Mtnr1c* (found only in non-mammalian species), were expressed in the mudskipper ovary. This result suggested the expression and function conservation of melatonin in vertebrate ovaries. Interestingly, the liver expressed only one type of melatonin receptor (Mtnr1a1.4) among all the peripheral tissues examined. A similar result is reported in Senegalese sole *Solea senegalensis* (Confente et al., 2010). In fish, the egg yolk protein precursors (vitellogenins) are synthesized in the liver, secreted into the plasma and transported into the oocytes for uptake in a process known as vitellogenesis. Recently, it is suggested that melatonin is involved in vitellogenesis in the oocytes of zebrafish and the catfish *Channa punctatus* when the fish are exposed to melatonin added water (Carnevali et al., 2011; Renuka and Joshi, 2010). However, no differences in the expression of vitellogenin genes are found in rainbow trout hepatocytes when they are exposed to melatonin *in vitro* (Mazurais et al., 2000).

The predominant expression of melatonin receptors (except *mtnr1c*) in the optic tectum suggested that these melatonin receptors might participate in processing visual information in the mudskipper. Interestingly, all of these melatonin receptors showed different daily variations in the brain. During the night, *mtnr1a1.4* mRNA expression increased, which was similar to MTNR1 in mammals; whereas *mtnr1b* mRNA expression decreased. Unlike the other three melatonin receptors, *mtnr1c* showed a distinct daily expression pattern in the brain with one peak during the day and another peak at night, which was synchronous with the tidal movement on the sampling date. The differing regulation of melatonin receptors in the brain suggested multiple roles of melatonin in the brain.

In the mudskipper retina, *mtnr1a1.4*, and *mtnr1c* did not show significant changes, whereas the levels of *mtnr1a*1.7 and *mtnr1b* genes showed significant diurnal variations. In the retina of other fish species, it would seem that the daily expression pattern of melatonin receptor genes represents a shared characteristic, displaying maximum expression levels at night (Ikegami et al., 2009a,b; Park et al., 2007a,b, 2006). Although this is somewhat in disagreement with the present study, it is worth noting that a study on the Senegalese sole indicates that the *mtnr1a1.4* mRNA expression level is significantly higher during the day in the winter solstice, whereas it exhibits the opposite day-night expression pattern during the spring equinox (Confente et al., 2010). Further investigations are necessary to examine whether the melatonin receptors exhibit a seasonal-dependent day-night expression pattern in the mudskipper retina.

The major focus of this study was to investigate whether melatonin was involved in the synchronization of the semilunar spawning rhythm in the female mudskipper, and the molecular mechanisms of the effects of melatonin on its reproductive physiology. Studies on a variety of fish species have generated a general hypothesis that melatonin plays a pivotal role in the regulation of reproduction, which depends on the gender, the light regimen, and the reproductive status. The reproductive actions of melatonin in fish are believed to be mediated by a hypothalamo-hypophyseal axis (Popek et al., 1992, 1994), but recent studies suggest that melatonin acts at gonad level (Carnevali et al., 2011; Chattoraj et al., 2005; Maitra et al., 2013). In the present study, we found abundant expression of the melatonin receptors around the HPG axis. In the diencephalon and pituitary, all four melatonin receptor mRNAs were expressed and showed fluctuations, which agreed with previous reports concerning the detection of different melatonin receptor genes (Ikegami et al., 2009a,b; Sauzet et al., 2008) or 2-[^{125}I] iodomelatonin binding sites (Gaildrat and Falcon, 2000) in teleost fish brains. In mammals, the suprachiasmatic nucleus (SCN), a primary circadian oscillator, is located in the hypothalamus and has a high density of melatonin receptors (Williams et al., 1995). Melatonin influences the molecular clock, phasing its circadian activity (Agez et al., 2009). Although it is not known whether the SCN of fish is a circadian oscillator, the potential location of the master clock(s) has been assumed to be the hypothalamus, the eyes, or the pineal organ (Falcon et al., 2007, 2003). Indeed, the hypothalamus contains neurosecretory neurons of peptide hormones involved in the control of reproductive activities. A study in Atlantic croaker *Micropogonias undulates* has suggested that melatonin can influence GtH II secretion by acting in the hypothalamic area and also directly at the pituitary (Khan and Thomas, 1996). A more recent study on the grass puffer, a semilunar spawner, suggests that melatonin regulates the activity of the hypothalamic neuroendocrine center for reproduction (Ando et al., 2013). In the present study, *mtnr1a1.4*, expressed in diencephalon,

exhibited a significant semilunar rhythm, which corresponded with the spawning activities of the mudskipper. We also examined the expression of arylalkylamine N-acetyltransferase 2 (*aanat2*), a rate limiting enzyme in the conversion of serotonin to melatonin in the pineal organ using the same brain samples that we obtained in our current study. Our results indicate that the *aanat2* mRNA levels in the brain correlate well with the expression changes of *mtnr1a1.4* in the diencephalon, and have two semilunar cycles within one lunar period (Hong et al., 2013). This might imply that the hypothalamic area is the primary action target of melatonin in relation to the regulation of semilunar-synchronized spawning activity.

The expression of three melatonin receptor subtype mRNAs in the ovaries suggested that melatonin had multiple local effects on folliculogenesis. Interestingly, we only detected *mtnr1a1.7* mRNA in both the follicular layers and oocytes in fully-grown ovaries. Moreover, only the *mtnr1a1.7* mRNA exhibited a semilunar rhythm in the ovaries. These results strongly suggested that melatonin exerted a direct action on the follicle to control timing of final maturation, a critical step for successful spawning in teleosts. In teleosts, oocyte maturation is believed to be separated into two temporal stages: a priming stage during which oocytes acquire maturational competence, and a maturational stage when the oocytes produce an MIH (Patino and Thomas, 1990). The expression of *mtnr1a1.7* mRNA in mudskipper oocytes suggested that melatonin had a function to increase the maturational competence, which is also reported in several carps, including *Labeo rohita*, *Cyprinus carpio*, and *Catla catla* (Chattoraj et al., 2005, 2009), and zebrafish (Carnevali et al., 2011). Besides the oocytes, the follicle layers expressed *mtnr1a1.7* mRNA, which suggested that melatonin was involved in steroidogenic pathways in the mudskipper ovary. Melatonin-induced progesterone production is demonstrated in granulose cell culture from several mammalian species (Baratta and Tamanini, 1992; Webley and Luck, 1986). In most teleosts, DHP serves as a potent MIH, which initiates the final maturation of the oocytes (Nagahama, 1997). In our study, an increase in plasma DHP levels was observed 1 h post injection and this began to decline after the 2 h sampling time. Moreover, results from both *in vivo* and *in vitro* approaches indicated that melatonin at a low dosage induced DHP production, whereas high dosage was ineffective. This result was in agreement with the results of studies on mammals, and may be due to a negative effect of high doses of melatonin on steroidogenesis (Adriaens et al., 2006). Further studies are necessary to examine whether the effect of melatonin on DHP production is dependent on the reproductive status of the fish.

In conclusion, this study reported the cloning of four different melatonin subtypes, i.e., *mtnr1a1.4*, *mtnr1a1.7*, *mtnr1b*, and *mtnr1c*, in *B. pectinirostris*. Moreover, the mRNAs of the four melatonin receptor subtypes were expressed in the diencephalon and pituitary, whereas only three of the four subtypes (except *mtnr1c*) were present in ovarian tissue. Furthermore, the expression of the melatonin receptor genes had two distinct cycles in the diencephalon and ovary within one lunar month. Melatonin treatment significantly induced DHP production both *in vivo* and *in vitro*. These data suggested that the melatonin receptors were likely to be involved in the synchronization of the semilunar spawning rhythm in female *B. pectinirostris*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.11.004.

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