Vertebrates originally possess four functional subtypes of G protein-coupled melatonin receptor

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Melatonin receptors (MTNRs) belonging to the G protein-coupled receptor family are considered to consist of three subtypes in vertebrates: MTNR1a, MTNR1b and MTNR1c. Additionally, MTNR1a-like genes have been identified in teleostean species as a fish-specific subtype of MTNR1a. However, similar molecules to this MTNR1a-like gene can be found in some reptiles upon searching the DNA database. We hypothesized that a vertebrate can essentially have four functional subtypes of MTNR as ohnologs. Thus, in the present study we examined the molecular phylogeny, expression patterns and pharmacological profile(s) using the teleost medaka (Oryzias latipes). The four conserved subtypes of MTNR (MTNR1a, MTNR1b, MTNR1c and MTNR1a-like) in vertebrates were classified based on synteny and phylogenetic analysis. The fourth MTNR, termed MTNR1a-like, could be classified as MTNR1d. It was observed by using RT-qPCR that expression patterns differed amongst these subtypes. Moreover, mtnr1a, mtnr1c and mtnr1a-like/mtnr1d expression was elevated during short days compared to long days in diencephalons. All the subtypes were activated by melatonin and transduced signals into the Gi pathway, to perform a CAMP-responsive reporter gene assay. It was shown that MTNR originally consisted of four subtypes: MTNR1a, MTNR1b, MTNR1c and MTNR1d. These subtypes were functional, at least in fish, although some organisms, including mammals, have lost one or two subtypes.

Melatonin (N-acetyl-5-methoxytryptamine) is primarily synthesized in the pineal gland and retina in all vertebrate species analyzed, and is considered a potent regulator of circadian and seasonal rhythms. Melatonin exists in almost all living organisms, and its actions are mediated by binding to G protein-coupled receptors (GPCR), nuclear receptors, or a low affinity protein (quinone reductase 2; QR2). However, the observation concerning nuclear receptors has been challenged, and the homolog in the medaka did not display efficiently promoted transcriptional activity by melatonin. The GPCR types are named MTNRs and are divided into three subtypes, MTNR1a (MT1/Mel1a), MTNR1b (MT2/Mel1b) and MTNR1c (Mel1c). MTNR1a and MTNR1b were identified in almost vertebrate species, whereas MTNR1c was only found in non-mammalian species. Additionally, MTNR1a (MTNR1a1/MTNR1a1.7) and MTNR1a-like (MTNR1a2/MTNR1a1.4) have been identified as two distinct subtypes of MTNR1a in teleostean species. Many gene families in vertebrates originated during two rounds of whole genome duplication occurring early in the evolution of the Chordata. An additional fish-specific whole genome duplication (FSGD) occurred in the teleostean lineage. Applying MTNR genes to this scenario, the four paralogs MTNR1a, MTNR1b, MTNR1c and ‘MTNR1d’ were thought to have been generated after the second genome duplication event. Because mammals possess only MTNR1a and MTNR1b, they must have lost MTNR1c and MTNR1d. In non-mammalians, MTNR1d was lost and MTNR1a, MTNR1b and MTNR1c remained. Interestingly, there are four subtypes, as within teleosts and some reptiles, which did not undergo teleost-specific whole genome duplication. It is possible that the MTNR1a-like gene is not a fish-specific paralog derived from FSGD, but is equivalent to MTNR1d. Because the above-mentioned classification for the MTNR subtypes appears contradictory, we attempted to investigate MTNR evolutionary history using molecular phylogenetic and syntenic relationships analyses in the current study.

Thus, a new question arises, if the MTNR1a-like (MTNR1a2/MTNR1a1.4) is the ortholog corresponding to the lost MTNR1d in mammals and birds, does MTNR1d remain as the receptor for melatonin? Divergence of genes often leads to neo-functionalization or non-functionalization. There are no reports on whether MTNR1d (MTNR1a-like/MTNR1a2/MTNR1a1.4) can work as a GPCR following melatonin binding. Even MTNR1c...
exhibits no evidence indicating that it is the functional receptor in teleosts. Therefore, it is necessary to clarify that MTNRs are able to transduce melatonin signals.

Here, we try to investigate for the existence of four ‘functional’ subtypes in MTNR and their relationship(s).

**Results**

**Synteny and phylogenetic analysis.** Phylogenetic and synteny analyses were performed to make inferences regarding the occurrence of the MTNR paralogs conserved in vertebrates. In many vertebrates the fat1 and cyp4v genes are situated adjacent to mtnr1a; however, the spotted gar has lost mtnr1a between these genes (Fig. 1a). The cluster of genes surrounding mtnr1b differs between teleosts and other vertebrates (Fig. 1b). However, the location of the hephl1 gene is to the same in both teleosts and tetrapods. Although synteny of the mtnr1c gene also shows differences between teleosts and other vertebrates, vma21 was located next to mtnr1c in many species (Fig. 1c). The gene residing next to vma21 was not mtnr1c, but gpr50 in humans. The pattern of synteny in genes neighboring the fourth subtype of the mtnr gene, named mtnr1a-like, was conserved in a form sandwiched between fat2 and slc36a1 (Fig. 1d). This form was similar in the spotted gar, softshell turtle and anole lizard. Chickens and humans do not have this fourth mtnr homolog, even though its synteny shares a close similarity to these holostean and reptiles.

By using phylogenetic analysis it was determined that the four groups of MTNR formed monophyletic clades (Fig. 2). These clades correspond with MTNR1a (MT1/Mel1a), MTNR1b (MT2/Mel1b), MTNR1c (Mel1c) and the fourth subtype, called MTNR1a-like (MTNR1a2/MTNR1a1.4). It is conceivable that these are ohnologs. Two groups, the ancestral subtype of MTNR1a and MTNR1a-like as well as the ancestral subtype of MTNR1b and MTNR1c, branched off from a common ancestor. Subsequently, four subtypes were derived from these. The MTNR of four teleostean fishes, the soft-shelled turtle and anole lizard presented in each group the four subtypes, and the two MTNR1a’s of the sea turtle MTNR belonged to the group of MTNR1a or MTNR1a-like, respectively.

**Melatonin-dependent regulation for cAMP-response reporter gene by MTNRs.** All MTNRs expressed in cells are reduced cAMP-responsive reporter activity in a dose-dependent manner via melatonin (Fig. 3); however, their sensitivities differ. MTNR1c was the most sensitive for melatonin with an EC50 = 0.062 ± 0.006 nM. The second was MTNR1a with an EC50 of 0.48 ± 0.2 nM. The third and final were MTNR1b (EC50 = 1.9 ± 0.49 nM) and MTNR1d (EC50 = 22 ± 6.3 nM), respectively (Table 1).

**Pharmacological characterization of MTNRs.** Pharmacological characterization of the MTNRs was performed with different concentrations of three compounds, known ligands for MTNR1a and/or MTNR1b in mammals. The responses of these analogs varied among the subtypes (Fig. 3, Table 1). While 4P-PDOT and luzindole are known antagonists for MTNRs in mammals, they also exhibited agonistic activity in medaka MTNR1a and MTNR1d. In MTNR1a-expressing cells, the EC50 values for 2-Iodomelatonin, 4P-PDOT and luzindole were 0.027, 4.7 and 330 nM, respectively. Their efficacies were weaker than melatonin, as their RAA values were less...
than 100%. In contrast, 2-Iodomelatonin was potent (EC$_{50}$ = 0.039 nM) against MTNR1b, whereas 4P-PDOT and luzindole had no antagonistic effects. The agonistic activity of 2-Iodomelatonin was equivalent to that of melatonin for MTNR1b. Concerning MTNR1c, the EC$_{50}$ values for 2-Iodomelatonin and luzindole were 0.013 and greater than 1000 nM, respectively, whereas 4P-PDOT displayed no agonistic effects. Luminescence was decreased in MTNR1c cells treated with 2-Iodomelatonin and luzindole compared with that of melatonin-treated cells, amounting to 112% and 118%, respectively. When MTNR1d was transfected in cells, 2-Iodomelatonin, 4P-PDOT and luzindole responded as agonists and their EC$_{50}$ values were 0.40, 120 and $>$1000 nM, respectively. The maximum inhibition of luciferase transcription of these ligands compared with melatonin were 69.3%, 68.7% and 83.1%, respectively.

The melatonin-induced inhibition of luciferase transcription activity was antagonized by 4P-PDOT in MTNR1b or MTNR1c expression in cells with 68.3% or 59.8% inhibition, respectively, but luzindole did not exhibit inhibition against melatonin action in any MTNR-expressing cells (Fig. 4, Table 2).

Temporal changes in expression of mtnr genes in the brain and eyes. The diurnal expression profile for the four mtnr genes was examined in the pituitary gland, diencephalon, optic tectum and eyes of the medaka using qPCR. Overwhelmingly, mtnr1a was detected at the highest levels in all tested tissues (Figs 5–8). By contrast, the transcript of mtnr1c had the lowest expression level of all the mtnr genes.

In the pituitary gland, mtnr1a mRNA levels fluctuated during the night period; a significantly higher number of transcripts was detected at Zeitgeber time (ZT) 23 versus ZT 11, 19 during 14 h light and 10 h dark cycle (14L10D) (Fig. 5a). No statistically significant difference was observed for mtnr1b for either photoperiod (Fig. 5b). Furthermore, mtnr1c transcripts were detected at significantly higher levels at ZT 3, 15 and 23 compared to that...
of ZT 11, 19 during 14L10D (Fig. 5c). In terms of the mtnr1a-like gene it was also observed that its mRNA levels fluctuated during nighttime in the pituitary gland during 14L10D, similar to mtnr1a and mtnr1c (Fig. 5d).

In diencephalons, mtnr1a, mtnr1c and mtnr1a-like expression was elevated during 10 h light and 14 h dark cycle (10L14D) compared to 14L10D (Fig. 6). During 10L14D, the mtnr1a mRNA level was significantly higher at ZT 19 than that of ZT 7, 11, and 23 (Fig. 6a). The mtnr1c and mtnr1d transcripts were also significantly increased at ZT 19 than that of ZT 7 (Fig. 6c,d). No statistically significant difference was observed for mtnr1b during either photoperiod (Fig. 6b).

In the optic tectum, mtnr1a exhibited a daily variation with a single peak at ZT 19 vs. ZT 3, 7 and 23 during 10L14D, while the expression level at ZT 23 was higher than that of ZT 3, 7, 11 and 19 during 14L10D (Fig. 7a). Significantly higher levels of mtnr1b expression were observed at ZT 19, 23 compared to ZT 7 during 10L14D (Fig. 7b). The expression of mtnr1b showed daily variation with significantly upregulated levels at ZT 15 and ZT 23 vs. ZT 19 during 14L10D (Fig. 5b). In terms of the mtnr1a-like gene, the transcripts were detected at significantly higher levels at ZT 23 compared to that of ZT 3, 7, 11 and 19 during long days (14L10D), whereas the mRNA levels significantly decreased at ZT 7 compared to that of ZT 11, 15 and 19 during short days (10L14D; Fig. 7d). No statistically significant difference was observed for mtnr1c during 10L14D, while during the 10L14D mtnr1c expression levels fell below measurable limits (Fig. 7c).

In the eyes, a large amount of mtnr1a expression was detected during 10L14D compared with 14L10D. The expression level of mtnr1a showed a significant change in the eyes with one peak at ZT19 during 14L10D (Fig. 8a). Moreover, mtnr1a exhibited a daily variation with high expression at ZT 19 vs. ZT 7, 11 and 23 during 10L14D. Transcription of the mtnr1b gene was the second highest after mtnr1a, and its expression level at ZT19 was significantly increased compared to ZT 7 and ZT 11 during 10L14D, although the mtnr1b mRNA was higher at ZT 7 versus ZT 11 and ZT 15 during 14L10D (Fig. 8b). The mRNA level of mtnr1c was significantly elevated at ZT 7, 19 compared to that of ZT 11 during 14L10D (Fig. 8c). In the 10L14D condition, a significantly higher number of mtnr1a-like transcripts were detected at ZT 15 and 19 in comparison to ZT 7, 11 and 23 (Fig. 8d).

Discussion
The present study investigated the following: (1) whether the MTNR1a-like gene is the fourth subtype of MTNR1, which could be termed MTNR1d, (2) if the four subtypes are functional, and (3) if they are actually expressed in cells. In response to the first question, yes, it is popularly considered that MTNR1a and MTNR1b are present in mammals, and other vertebrates have MTNR1a, MTNR1b and MTNR1c. Additionally, a second MTNR1a
gene, referred to as MTNR1a-like, MTNR1a2 or MTNR1a1.4, was identified in teleostean fish\textsuperscript{9,11,16}. During recent years, the sequence that shares homology with the MTNR1a-like was identified in several reptiles (softshell turtle: ENPSIG00000008066; anole lizard: ENSACAG00000010003; sea turtle: KB598657.1). Our synteny analysis, which included these MTNR subtypes, indicated that some genes located close to the MTNR paralogs are conserved among vertebrates and, thus, it would appear that the occurrence of MTNR1a and MTNR1a-like was

| Ligand                | MTNR1a Potency (M) | RAA (%) | MTNR1b Potency (M) | RAA (%) | MTNR1c Potency (M) | RAA (%) | MTNR1a-like Potency (M) | RAA (%) |
|-----------------------|-------------------|---------|-------------------|---------|-------------------|---------|-------------------------|---------|
| Melatonin             | 4.8 ± 2.0 × 10\(^{-10}\) | 100 ± 3.8 | 1.9 ± 0.49 × 10\(^{-4}\) | 100 ± 4.4 | 6.2 ± 0.61 × 10\(^{-11}\) | 100 ± 1.8 | 2.2 ± 0.63 × 10\(^{-8}\) | 100 ± 2.9 |
| 2-Iodo melatonin      | 2.7 ± 1.7 × 10\(^{-11}\) | 95.8 ± 6.9 | 3.9 ± 0.32 × 10\(^{-11}\) | 101.4 ± 2.8 | 1.3 ± 0.39 × 10\(^{-11}\) | 111.8 ± 3.2 | 4.0 ± 1.1 × 10\(^{-11}\) | 69.3 ± 6.8 |
| 4P-PDOT               | 4.7 ± 0.63 × 10\(^{-9}\) | 67.4 ± 6.1 | NC                | ND      | NC                | ND      | 1.2 ± 0.24 × 10\(^{-7}\) | 68.7 ± 6.4 |
| Luzindole             | 3.3 ± 0.21 × 10\(^{-7}\) | 68.2 ± 3.8 | NC                | ND      | NC                | ND      | 117.6 ± 11.5            | 83.1 ± 13.4 |

Table 1. Potency and relative agonistic activity (RAA) of typical MTNR ligands in mammals for medaka MTNRs. NC: not calculable (low activity, but its concentration-response curve did not reach maximum levels). ND: not detected.

| Ligand                | MTNR1a Potency (M) | AI (%) | MTNR1b Potency (M) | AI (%) | MTNR1c Potency (M) | AI (%) | MTNR1a-like Potency (M) | AI (%) |
|-----------------------|-------------------|--------|-------------------|--------|-------------------|--------|-------------------------|--------|
| 4P-PDOT               | NC                | ND     | 1.9 ± 0.22 × 10\(^{-7}\) | 68.3 ± 8.0 | NC                | 59.8 ± 4.9 | NC                | ND     |
| Luzindole             | NC                | ND     | NC                | ND     | NC                | ND     | NC                     | ND     |

Table 2. Potency and antagonistic index (AI) of typical MTNR antagonist in mammals for medaka MTNRs with EC\(_{90}\) melatonin. NC: not calculable (low activity, but its concentration-response curve did not reach maximum levels). ND: not detected.

Figure 4. Concentration-response curve of typical MTNR antagonist for medaka MTNRs with EC\(_{90}\) melatonin. Y-axis shows antagonistic index (AI) and the x-axis shows the concentration of reagents used for cell treatments. The upper gray box signifies the mean ± standard error of the mean (S.E.M.) value of forskolin (FSK) and the lower gray box represents the average (=S.E.M.) value of the 90% effective concentration of melatonin (MTN). Each point represents the mean of triplicate calculations, and vertical bars represent the S.E.M.
not teleost-specific whole genome duplication, but instead a second whole genome duplication. The nodes of a phylogenetic tree exhibiting the divergence between the \( MTNR1a \)/\( MTNR1a\)-like and \( MTNR1b \)/\( MTNR1c \) groups would suggest that these two groups were divided by the first whole genome duplication and was then bifurcated into four \( MTNR \)s. Therefore, we would like to emphasize that the fourth \( MTNR \), currently called \( MTNR1a\)-like, should be recognized as \( MTNR1d \).

Second, we showed that all four subtypes have the potential to be a receptor for melatonin. In the present study, luminescence in response to the amount of cAMP was decreased by melatonin in a concentration-dependent manner in Hepa-E1 cells transfected with each subtype. This result is consistent with previous reports indicating that the fish melatonin receptor activates the inhibitory G protein, thus leading to decreases in cAMP production by inhibiting the adenylate cyclase\(^{17,18} \). This is also evidence for the four subtypes of \( MTNR \) in the medaka being functional.

Because comparison of the responses to reagents showed different pharmacological profiles between subtypes, we would like to discuss the relevant points. The order of potency for melatonin was as follows: \( MTNR1c \) > \( MTNR1a \) > \( MTNR1b \) > \( MTNR1d/MTNR1a\)-like. \( MTNR1c \) has the highest potency with more than three hundred-fold effectiveness compared with \( MTNR1d/MTNR1a\)-like. It is possible that these might play different roles depending on the amount of melatonin.

The 2-Iodomelatonin is known as a potent melatonin analog. In a binding assay using 2-[\( \text{I}^{125} \)I]iodomelatonin, the pharmacological characteristics of \( MTNR \) showed that ligand selectivity varies among melatonin receptor subtypes in many vertebrates\(^{17,18,21} \). In the medaka, a high potency of this synthetic melatonin-related substance agrees with these previous reports.

Interestingly, luzindole and 4P-PDOT, chemical compounds using as antagonists of the melatonin receptor\(^{22-24} \), stimulated both \( MTNR1a \) and \( MTNR1d/MTNR1a\)-like within the medaka as agonists. In contrast, 4P-PDOT suppressed melatonin-induced inhibition within transcriptional activity of the luciferase gene only in \( MTNR1b \). Thus, 4P-PDOT probably provides an \( MTNR1b\)-specific inhibitory effect in the medaka at an organism level.

Figure 5. Diurnal expressions of medaka \( mtnr1s \) in the pituitary under short and long photoperiods as assessed by quantititative real-time (RT)-PCR. The temporal changes in expression of \( mtnr1a \) (a), \( mtnr1b \) (b), \( mtnr1c \) (c) and \( mtnr1a\)-like (d). The sample collection time is indicated as ZT. The white and black bars above each graph represent light and dark periods during 14L10D (upper) and 10L14D (lower). Y-axes represent \( mtnr1 \) expression as copies/\( \mu \)g of total RNA. Data are expressed as mean ± standard error of the mean (S.E.M.) (\( n = 6 \)). Different letters on the columns indicate group means that are differ statistically when analyzed using one-way ANOVA followed by Tukey’s Multiple Comparison Test (\( P < 0.05 \)).
It was an unexpected result that luzindole did not inhibit any of the medaka MTNR subtypes, although 4P-PDOT effected MTNR1b as an antagonist. Luzindole has been used for inhibition of melatonin receptors in various teleosts \(^{25-28}\). Therefore, it is logical that this agent has been selected as an antagonist for piscine MTNRs, as it is a ‘proven’ agent in mammals. To investigate the function of medaka MTNR subtypes and perhaps teleostean MTNRs, it is necessary to develop a new antagonist that specifically binds to each MTNR subtype and inhibits the effect of melatonin for a sufficient period of time.

Last, we showed that the four subtypes were detected by an adequate amount of transcripts and that these have physiologically changed in conditions of light. To demonstrate that these are functional, it is necessary to show their existence as well as potency. In the medaka, there have been no previous reports concerning MTNR expression patterns. In the present study, the melatonin receptor gene transcripts exhibited diurnal variations. Such day-night variations in melatonin receptor expression was also reported in the Senegalese sole (Solea senegalensis) \(^{20}\). It is possible that expression diurnation in mtnrs reflects the roles for entrainment of circadian rhythms to light/dark cycles. Thus, these four MTNR subtypes seem to be functional, and not the products of pseudogenes. However, these transcript levels sometimes remain steady, depending on the tissue, such as the brain, under long-day conditions in our study, which is consistent with a previous report showing significant changes in the expression of mtnr1a and mtnr1b genes, not in optic tectum but in the retina of goldfish \(^{1}\).

Fluctuation in the expression of mtnrs in accordance with the photoperiod was also reported in relation to differences in other fish \(^{1,29}\). Changes in the expression of mtnrs subtypes were observed in association with a photoperiod in medaka, which held its rhythm in the present study. MTNRs may mediate melatonin function in proportion to the amount of hormones since this change was dependent on the length of dark periods. In other words, it is possible that MTNRs in the eyes and diencephalon of the medaka are needed to transduce light information under short-day conditions. Moreover, elevated expression of mtnr during short (10L14D) compared with long days (14L10D) provides evidence that the four subtypes of MTNR within the medaka are functional. Even though mtnr1a mRNA was abundant in the eyes as well as brain areas analyzed, other MTNR subtype genes may be expressed locally. Further studies demonstrating tissue localization of MTNRs in more detail are necessary to elucidate the role of each of the melatonin receptors.

**Figure 6.** Diurnal expression of medaka mtnrs within diencephalons under short and long photoperiods as assessed by quantitative real-time (RT)-PCR. Temporal changes in expression of mtnr1a (a), mtnr1b (b), mtnr1c (c) and mtnr1a-like (d). The sample collection time is indicated as ZT. The white and black bars above each graph represent light and dark periods during 14L10D (upper) and 10L14D (lower). Y-axes represent mtnr expression as copies/μg of total RNA. Data are expressed as mean ± standard error of the mean (S.E.M.) (n = 6). Different letters on the columns indicate group means that are differ statistically when analyzed using a one-way ANOVA followed by Tukey’s Multiple Comparison Test (P < 0.05).
Conclusions

This report showed that vertebrates have four original subtypes of MTNR, and that they are functional as the receptors for melatonin via analysis of molecular phylogeny, pharmacological profiles and tissue-dependent expression patterns. The synteny of the MTNR1a-like gene was conserved in vertebrates, including within some reptiles, suggesting that the MTNR1a and MTNR1a-like genes have diverged not in teleost-specific whole genome duplication but instead second whole genome duplication. Thus, MTNR1a-like should be recognized as a missing MTNR1d in mammals. Pharmacological analysis using a reporter gene assay indicated that the four MTNR subtypes of the medaka are functional, with differing potencies for melatonin. All the subtypes were expressed to some level in the brain and eyes. MTNR1a mRNA was highly abundant in the medaka brain and eyes, with diurnal rhythmicity increasing at night. Moreover, the expression level of mtnr1a was more enhanced during the non-reproductive photoperiod versus the reproductive photoperiod. These results suggest that the roles of MTNRs are associated with photoperiod-dependent physiological action in teleost fish. Our results also indicate that vertebrates have essentially four functional MTNR subtypes, although some organisms have lost one or two subtypes, including mammals.

Materials and Methods

Assessments of conserved synteny. The MTNR genes of 11 vertebrate species were compared to syntenic regions (Table 3) near each medaka subtype using Genomicus, including: six teleosts, the medaka (Oryzias latipes), amazon molly (Poecilia formosa), platyfish (Xiphophorus maculatus), tilapia (Oreochromis niloticus), stickleback (Gasterosteus aculeatus), and tetraodon (Tetraodon nigroviridis); one holostean fish, the spotted gar (Lepisosteus oculatus); and four tetrapods, the soft-shelled turtle (Pelodiscus sinensis), anole lizard (Anolis carolinensis), chicken (Gallus gallus) and human (Homo sapiens). When the mtnr gene was not found within the syntenic region of a species, we also used the program Genscan to confirm whether the mtnr was lost or unannotated.

Phylogenetic analysis. The protein sequences of the MTNRs were retrieved from the Ensembl database and National Center for Biotechnology Information (NCBI). Amino acid sequence IDs were
as follows: human 1a: NP_005949.1, 1b: NP_005950.1; chicken 1a: NP_990693.1, 1b: NP_001280032.1, 1c: NP_990692.1; sea turtle (*Chelonia mydas*) 1a1: EMP24711.1, 1a3: EMP33109.1; soft-shelled turtle 1a: ENSPSIT0000001974.1, 1b: ENSPSIT0000001684.1, 1c: ENSPSIT000000237.1, 1a-like:

Table 3. Chromosomes with *mtnr* genes.

| *mtnr* | medaka | amazon molly | platyfish | tilapia |
|--------|---------|--------------|------------|---------|
| *mtnr1a* | Chr:1 | Chr:K1519642.1 | Chr:5 | Chr:GL831212.1 |
| *mtnr1b* | Chr:14 | Chr:K1519613.1 | Chr:11 | Chr:GL831183.1 |
| *mtnr1c* | Chr:10 | Chr:K1519738.1 | Chr:23 | Chr:GL831174.1 |
| *mtnr1d* | Chr:10 | Chr:K1519678.1 | Chr:23 | Chr:GL831190.1 |

| *mtnr* | spotted gar | stikleback | tetraodon | softshell turtle |
|--------|-------------|------------|-----------|-----------------|
| *mtnr1a* | Chr:LG4 | Chr:groupIX | Chr:7 | Chr:JH210278.1 |
| *mtnr1b* | Chr:LG3 | Chr:groupVII | Chr:7 | Chr:JH205573.1 |
| *mtnr1c* | Chr:LG7 | Chr:groupIV | Chr:18 | Chr:JH224646.1 |
| *mtnr1d* | Chr:LG6 | Chr:groupIV | Chr:1 | Chr:JH224652.1 |

| *mtnr* | anole lizard | chicken | human |
|--------|--------------|---------|-------|
| *mtnr1a* | Chr:5 | Chr:4 | Chr:4 |
| *mtnr1b* | Chr:3 | Chr:1 | Chr:11 |
| *mtnr1c* | Chr:GL343310.1 | Chr:4 | Chr:X |
| *mtnr1d* | Chr:2 | Chr:13 | Chr:5 |

Figure 8. Diurnal expressions of medaka *mtnr1s* in the eyes under short and long photoperiods as assessed by quantitative real-time (RT)-PCR. Temporal changes in expression of *mtnr1a* (a), *mtnr1b* (b), *mtnr1c* (c) and *mtnr1a-like* (d). The sample collection time is indicated as ZT. The white and black bars above each graph represent light and dark periods during 14L10D (upper) and 10L14D (lower). Y-axes represent *mtnr* expression as copies/μg total RNA. Data are expressed as mean ± standard error of the mean (S.E.M.) (n = 6). Different letters on the columns indicate group means that are statistically different when analyzed using a one-way ANOVA followed by Tukey’s Multiple Comparison Test (P < 0.05).
Little was known about the transcriptional regulation of medaka mtnr genes. We firstly constructed transfection vector for medaka mtnr genes, (pcDNA-OIMTNR1a, pcDNA-OIMTNR1b, pcDNA-OIMTNR1c and pcDNA-OIMTNR1a-like) were constructed by PCR amplification of the entire protein coding region, using primers which introduced in-frame Kozak sequence \(^{32}\) at the 5′ end. A cyclic adenosine monophosphate (cAMP)-regulated reporter vector containing the luciferase gene under the control of a cAMP response element upstream of the TATA-like promoter region from the herpes simplex virus thymidine kinase promoter, named pCRE-luc, originated from the Pathway Profiling Luciferase System (Clontech). Hepa-E1 cells, i.e. epithelial-like Nile tilapia hepatocytes, were obtained from the Institute of Physical and Chemical Research (RIKEN) cell bank (Tsukuba, Japan). For MTNR1a, MTNR1b and MTNR1d, stable transfections were performed as described below. Hepa-E1 cells were seeded in 9 cm dishes prior to transfection. Both plasmids, pCRE-luc as well as expression vectors for medaka mtnrs were linearized by PCR and transfected with X-tremeGENE 9 (Roche) according to the manufacturer's instructions. Stably transfected cells were selected with G418. After selection, each colony was collected by pipetting. Sensitivity for cAMP in each clone was tested by forskolin treatment after seeding into 96-well plates with E-RDF medium (Kyokuto, Tokyo, Japan) and supplemented with 5% dextran-charcoal treated fetal bovine serum. After 24 h the cells were collected, and cellular luciferase activities were measured by luminescence assay with the Steady-Glo Assay System Kit (Promega) according to the manufacturer's instructions. Forskolin-sensitive cell lines derived from co-transfection of pCRE-luc and pcDNA3.1(+) were developed as control lines and named null-CRE-luc. Forskolin-sensitive cell lines derived from co-transfection of pCRE-luc and each pcDNA-OIMTNR1 were selected for by sensitivity for melatonin. For MTNR1c, transient transfection was performed as outlined below. The MTNR1c experiment was performed using Hepa-E1 cells in 9 cm dishes, which were transiently transfected by pcDNA-OIMTNR1c (500 ng) and pCRE-luc (5000 ng) by Transit-X2 Dynamic Delivery System (Mirus) and incubated 24 h before seeding, following the manufacturer's instructions. Cells derived from a chosen clone or MRNR1c-transfected cells were seeded at 10° cells per well in 96-well luminometer plates. Then, cells were treated with test chemicals in triplicate at four-fold serial dilutions in the presence or absence of melatonin with 6.25 \times 10^{-7} M forskolin. For testing the antagonistic effect, reagents were mixed with melatonin at 90% effective concentration (EC\(_{90}\)). Control wells were dosed with media plus 0.1% ethanol. Positive control wells were dosed with 1 \mu M melatonin. After 24 h, luciferase activities were measured as described above. Concentration-response curves were fitted and the EC\(_{50}\) or IC\(_{50}\) were calculated using sigmoidal fit in GraphPad Prism. The relative agonistic activity (RAA) of each reagent was expressed as a percentage of the maximal activity for the reporter induction to that of melatonin. The antagonistic index (AI) of each reagent was expressed as a percentage of the maximal inhibition of the reagent. Maximal inhibition was calculated as the quotient of the subtraction of the luminescence for melatonin from the maximal luminescence for the reagent divided by the maximal inhibition of melatonin. When the reagent inhibits the transactivity to the same level of that of the vehicle control (forskolin), the AI equals 100%.

### Quantitative Real-time PCR

Total RNA from each tissue was extracted using Sepasol RNA I Super G (Nacalai Tesque) according to the manufacturer's instruction. Total RNA was reverse-transcribed and genomic DNA was removed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). Quantitative Real-time PCR was conducted with SsoAdvanced Universal SYBR Green Supermix (BIO-RAD) using the MiniOpticon real-time PCR system (BIO-RAD). The primer set for mtnr1a (XM_004065955.3) was 5′-AGCGTTATACATCGCAAGTGTTG-3′ (forward) and 5′-TTGTGCGTGTGTTGTTTGG-3′ (reverse). Primers for mtnr1b (LC032111.1) were 5′-CGCGTAAAGTAAAGACTGAAGAAA-3′ (forward) and 5′-TTGGTCGGTTGTCTGGTTTG-3′ (reverse). Primers for mtnr1c (LC033812.1) were 5′-GCTGCTCTAATCCCTTCATCATA-3′ (forward) and 5′-CGCGCACGCACAGACAA-3′ (reverse). Primers for mtnr1a-like (XM_004073612.3) were 5′-GGGACACTTTCCGCAACTGA-3′ (forward) and 5′-GCTGCTCGTGGAGTTGTTG-3′ (reverse). These primers were designed using Primer3 ver.0.4.0 (1, 2). The conditions for PCR were as follows: initial denaturation at 98 °C for 2 min, 40 amplification cycles with denaturation at 98 °C for 2 s, and annealing at 60 °C for 5 s. Standards for the mtnr1a plasmid were constructed with a
Data Availability
The data supporting the conclusions of this article are included within the article and its additional files.

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Additional Information

Competing Interests: The authors declare no competing interests.

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