Melatonin receptors in Atlantic salmon stimulate cAMP levels in heterologous cell lines and show season-dependent daily variations in pituitary expression levels

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Abstract
The hormone melatonin connects environmental cues, such as photoperiod and temperature, with a number of physiological and behavioural processes, including seasonal reproduction, through binding to their cognate receptors. This study reports the structural, functional and physiological characterization of five high-affinity melatonin receptors (Mtnr1aaα, Mtnr1aaβ, Mtnr1ab, Mtnr1al, Mtnr1b) in Atlantic salmon. Phylogenetic analysis clustered salmon melatonin receptors into three monophyletic groups, Mtnr1A, Mtnr1Al and Mtnr1B, but no functional representative of the Mtnr1C group. Contrary to previous studies in vertebrates, pharmacological characterization of four receptors in COS-7, CHO and SH-SY5Y cell lines (Mtnr1Aα, Mtnr1Aβ, Mtnr1Ab, Mtnr1B) showed induction of intracellular cAMP levels following 2-iodomelatonin or melatonin exposure. No consistent response was measured after N-acetyl-serotonin or serotonin exposure. Melatonin receptor genes were expressed at all levels of the hypothalamo-pituitary-gonad axis, with three genes (mtnr1aaβ, mtnr1ab and mtnr1b) detected in the pituitary. Pituitary receptors displayed daily fluctuations in mRNA levels during spring, prior to the onset of gonadal maturation, but not in autumn, strongly implying a direct involvement of melatonin in seasonal processes regulated by the pituitary. To the best of our knowledge, this is the first report of cAMP induction mediated via melatonin receptors in a teleost species.

Key words
daily expression, melatonin receptors, pharmacology, phylogeny, pituitary, sexual maturation, signalling pathway

Romain Fontaine and Gersende Maugars contributed equally to this paper.

Nomenclature: We use the following nomenclature: “Mtnr” for protein names and “mtnr” for gene names.
1 | INTRODUCTION

Melatonin is a highly conserved neurohormone produced in vertebrates by the pineal gland and retina, as well as a number of peripheral tissues, notably the gastrointestinal tract. In all organisms, circulating melatonin levels show a pronounced diurnal rhythm, being high during the night and low during the day; in teleost fishes, the nocturnal rise in plasma melatonin is clearly a function of pineal production. Melatonin is considered the primary hormone that mediates photoperiodic information to an organism. Changes in the rhythmic cycle of melatonin release confer photoperiodic information for the control and timing of both circadian and circannual rhythms, including growth and development, and seasonal migration and reproduction (for review, see. Most, if not all, of these processes are ultimately controlled by altered output from endocrine cells in the pituitary. Although direct effects of melatonin on pituitary cells have been shown both in mammals and reproduction (for review, see.4,5 Most, if not all, of these paralogs has not been determined.

The effects of circulating melatonin are mediated through specific melatonin receptors (Mtnr) belonging to the G-protein coupled receptor superfamily. Three sub-groups of Mtnr have been characterized in vertebrates: Mtnr1A (Mel1a or MT1), Mtnr1B (Mel1b or MT2) and Mtnr1C (Mel1c or GPR50). In some teleost species, an additional Mtnr1A has been reported, but the origin of the two teleost Mtnr1A paralogs has not been determined.

Mtnr activates different intracellular signalling pathways, including the cAMP/PKA pathway, via G proteins that inhibit adenylyl cyclase and subsequently cAMP formation (Mtnr1A and Mtnr1B), the PLC/PKC pathway via Gq proteins (Mtnr1A and Mtnr1C) and the cGMP pathway (Mtnr1B). In therian mammals, Mtnr1C has lost the ability to respond to melatonin.

Melatonin has widespread effects, as evidenced by the broad distribution of Mtnr in vertebrate nervous and peripheral tissues. Of special interest to the present paper is the pituitary, where Mtnr expression has been detected in the pituitary pars tuberalis of mammals. The presence of melatonin binding sites has also been detected in the pituitary of teleosts, including the salmonids chum salmon, Oncorhynchus keta and rainbow trout, Oncorhynchus mykiss, but not, to date, in Atlantic salmon, Salmo salar. Whether the effects of melatonin on reproduction in teleosts result from direct effects on pituitary gonadotropes is not clear, but melatonin has been shown to modulate gonadotropin levels in vivo in some teleost species.

In the Senegalese sole (Solea senegalensis), mtnr1a and mtnr1b display circannual fluctuations in pituitary expression levels, with higher expression towards the end of the spawning season in June than in the rest of the season. Other studies have reported circadian fluctuations in Mtnr gene expression, peaking either during the daytime or during the night depending on tissue, species and gene. In the Siberian and Syrian hamster (Cricetidae) pituitary, levels of mtnr1a mRNA are higher during the daytime and lower at the end of the night. In teleosts, most of the research reporting circadian fluctuations in Mtnr have been conducted in brain. At the pituitary level, mtnr1a mRNA levels increased during daytime in Chum salmon parr, while no circadian fluctuations were reported in the pituitary of Senegalese sole.

To understand further the effect of melatonin on pituitary cells, particularly the gonadotrope cells controlling gonadal maturation, we have characterized five functional Mtnr genes in Atlantic salmon; three paralogs of the Mtnr1A sub-group and single genes of the Mtnr1Al and Mtnr1B sub-groups. Phylogenetic analyses reveal that teleost Mtnr1A paralogs belong to two separate sub-groups. Pharmacological analyses show that, contrary to results reported from other species, Atlantic salmon Mtnr modulates the cAMP/PKA pathway by induction of intracellular cAMP in response to melatonin exposure. Furthermore, the three Mtnr genes expressed in the salmon pituitary (mtnr1aαβ, mtnr1b and mtnr1b), all display daily fluctuations in expression levels in springtime, just prior to initiation of gonadal maturation, but not in the autumn.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

This study was performed on 1-year-old Atlantic salmon (Salmo salar) male parr from wild-caught broodstock (Figge stock) at the Norwegian Institute for Nature Research (NINA) at Ims, Norway (58°54′N, 5°57′E), reared under natural conditions regarding photoperiod and temperature (yearly range: 5-21°C). All experiments were performed according to EU regulations concerning the protection of experimental animals (Directive 2010/63/EU). Appropriate measures were taken to minimize pain and discomfort (FOTS application ID12523).

2.2 | Mtnr phylogenetic analysis

Mtnr sequences from 14 vertebrate representatives (Table S1) were retrieved from GenBank, including Atlantic salmon, rainbow trout and northern pike (Esox lucius), the latter belonging to a sister group of the salmonids that diverged before the salmonid-specific genome duplication. Deduced amino acid sequences were aligned using CLC Bio Main Workbench (Qiagen Bioinformatic) and manually adjusted. Phylogenetic trees were inferred by maximum-likelihood algorithm and the AIC model selection using PhyML 3.0 on ATGC Bioinformatic browser. A consensus tree was generated using the SPR algorithm, and robustness of
the topology was assessed by bootstrapping 1000 replicates. Lancelet Mtnr was used to root the tree. Mtnr nomenclature was based on HUGO, GenBank and ZFin nomenclatures.

### 2.3 Mtnr pharmacology

All receptor-activation experiments were performed first in COS-7 cells and thereafter verified in CHO and SH-SY5Y (human neuroblastoma) cell lines. Atlantic salmon mtnr<sub>1aaα</sub>, mtnr<sub>1aaβ</sub>, mtnr<sub>1ab</sub> and mtnr<sub>1b</sub> inserted into pcDNA3.1 (Invitrogen) were obtained from GenScript Biotech based on sequence information retrieved from GenBank and verified by cloning and sequencing (see PCR primers in Table S2). The procedures for transient transfection of the different cell lines and receptor stimulation were according to Ref.28 In brief, COS-7 cells were transfected with luciferase reporter plasmid (3 µg) together with one of the mtnr constructs (3 µg). After 48 hours, cells were stimulated with increasing concentrations (0, 0.24, 0.98, 3.91, 15.63, 62.50, 250, 1000 nmol/L, each in triplicate) of four possible activators: melatonin, N-acetylserotonin, serotonin (all Sigma-Aldrich) or 2-iodomelatonin (Santa Cruz Biotechnology,), either alone or in combination with 20 µmol/L forskolin (used as positive control for cAMP production; Sigma-Aldrich). Additionally, luzindole, a known inhibitor of Mtnr (Sigma-Aldrich), was tested at increasing doses (0, 0.01, 0.1, 1, 10, 100, 1000 nmol/L) in combination with either melatonin (100 nmol/L) alone or with melatonin (100 nmol/L) together with forskolin (20 µmol/L). Six hours after stimulation, cells were analysed using GloMax-multidetection system (Promega). As negative control, COS-7 cells transfected with CRE-LUC reporter only were exposed to 2-iodomelatonin or melatonin, either alone or in combination with 20 µmol/L forskolin. In addition, the human Mtnr1A was tested in both COS7 and SH-SY5Y cell lines. All exposures were performed in at least three independent experiments.

### 2.4 Sampling procedure for mtnr gene expression analyses

Three experiments were conducted to measure gene expression: “experiment 1”—tissue distribution of mtnr expression; “experiment 2”—measurement of mtnr expression in the pituitary gland during early gonadal maturation; and “experiment 3” quantification of daily mtnr expression in the pituitary gland during spring and autumn.

In experiment 1, the following tissues were collected from five male salmon parr on 4 July 2017: telencephalon, optic nerves, optic tectum, cerebellum, medulla oblongata together with diencephalon, pituitary, eye, testis and skin.
In experiment 2, individual pituitaries from maturing and nonmaturing males were collected every two weeks from May to August 2016 (N = 6 per group). Fish biometry was recorded and gonadosomatic index (GSI = gonad weight/body weight × 100) calculated to discriminate between maturing and nonmaturing fish.

In experiment 3, individual pituitaries were collected every 4 hours over a 24-hour cycle in autumn 2017 (October 23; Sunrise 08.33; Sunset 18.08; N = 6 per time point) and in spring 2018 (April 13; Sunrise 06.29; Sunset 20.47; N = 10 per time point). During night dissections, we used a dim red light to avoid cessation of melatonin synthesis and release (Figure S1).

In all experiments, fish were treated with an overdose of MS222 (Pharmaq,Overhalla, Norway) and euthanized by decapitation. Pituitaries were collected and stored in TRIzol reagent (Invitrogen), and other tissues were collected in RNAlater (Sigma‐Aldrich). All samples were stored overnight at 4°C and then frozen at −20°C until RNA extraction.

2.5 | RNA extraction and cDNA synthesis

For qPCR analyses, total RNA was isolated using TRIzol reagent and DNasel (Ambion) according to the manufacturer’s instructions. RNA was quantified using NanoDrop (Thermo Scientific) or Qubit (Invitrogen), while RNA quality was checked using Bioanalyzer 2100 (Agilent). One microgram (experiment 2) or 170 ng (experiments 1 and 3) of total RNA was reverse-transcribed using SuperScriptIII and 2.5 μmol/L random hexamers (Invitrogen).

2.6 | Quantification PCR

qPCR primers (Table 1) for salmon mtnr were designed using Primer‐Blast (Ye et al, 2012). mtnr transcript levels were measured using SYBR Green I (Roche, Basel, Switzerland) on Light Cycler 96 (Roche). Thermal conditions were 10 minutes at 95°C followed by 40 cycles at 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 8 seconds. Specificity was verified by melting curve analysis and sequencing. Each sample was run in duplicate using 3 μL cDNA diluted 1:10. Each plate contained triplicates of nontemplate control and calibrator. Relative expression was determined using GenEx software using algorithms from Vandesompele et al. rna18s and efla were validated as reference genes using RefFinder and used for data normalization.

2.7 | Statistical analysis

qPCR results were expressed as mean ± SEM. Statistical differences were determined by two-way (experiment 1) or one-way ANOVA (experiments 2 and 3, and receptor-activation experiments), followed by Tukey’s HSD test. When necessary, data were log-transformed to meet test criteria. Significance was imparted at P < 0.05 level. All statistical analyses were performed using JMP pro V.13.0 SAS. Half-maximal effective concentrations (EC50) were calculated from dose-response curves by nonlinear curve fitting (GraphPad Prism 7.04).

3 | RESULTS

3.1 | Characterization of Atlantic salmon Mtnr

The Atlantic salmon GenBank reference genome contains eight annotated mtnr loci: four mtnra, two mtnrb and two mtnrc. Among the mtnra paralogs, one is located on chromosome ssa04, a second on ssa08 and two on ssa09. All four mtnra paralogs are encoded by two exons. Among the mtnrb paralogs, one is located on ssa09 and the other on ssa20. Both paralogs are encoded by three exons, although the one on ssa20 is split, with exon 1 located 6 Mbp apart from the other. Among the two mtnrc paralogs, one is located on ssa04 and the other on ssa13. Both paralogs are encoded by two exons, although exon 2 is only partial and includes many frameshifts. The two mtnrc paralogs and the mtnrlb paralog on chromosome ssa20 that has a first exon too distant from exon 2 and 3 to be transcribed are considered pseudogenes and were not further included in our analysis.

3.2 | Mtnr phylogenetic analysis

Phylogenetic analysis clustered the receptors into four monophyletic groups, each containing tetrapod and actinopterygian sequences: Mtnr1A, Mtnr1A-like (Mtnr1Al), Mtnr1B and Mtnr1C (Figure 1). Teleost Mtnr1A divided into two clades, Mtnr1Aa and Mtnr1Ab, each clade comprising a salmonid cluster branching from pike. Two Atlantic salmon Mtnr1As branched within salmonid Mtnr1Aa and a single sequence within the salmonid Mtnr1Ab. The Mtnr1A paralogs resulting from the teleost-specific third whole-genome duplication (3R) were named Mtnr1Aa and Mtnr1Ab, and the Mtnr1Aa paralogs resulting from the salmon-specific whole-genome duplication (4R) were named Mtnr1Aα and Mtnr1Aβ. Atlantic salmon Mtnr1Al clustered with trout Mtnr1Al, again branching from pike Mtnr1Al. Atlantic salmon Mtnr1B clustered with trout Mtnr1B and pike Mtnr1B within the teleost MtnrB clade.

3.3 | Mtnr molecular structure

Comparison of deduced amino acid sequences with the human Mtnr1A and Mtnr1B reveals that the five putatively functional salmon Mtnr possesses the characteristic features of melatonin receptors, including seven transmembrane domains (TM) with the typical NRY and NAXXY motifs and conserved residues interacting with G-protein in the TM3 (Figures S2-S4). All the salmon receptors have conserved residues predicted to form the ligand-binding pocket in different human Mtnr1A and Mtnr1B 3D models (for review,
These include the two cysteine residues that form an extracellular stabilizing disulphide bridge. The 3D structure of salmon Mtnr confirms the presence of the extracellular, intracellular and seven transmembrane domains, together with the ligand-binding pocket and the G-protein interacting site (Figure 2).

3.4 | Mtnr pharmacological characterization

Four of the five functional Mtnr genes in Atlantic salmon were pharmacologically characterized in transfected cell lines (COS-7, CHO and SH-SY5Y). Melatonin and 2-iodomelatonin induced concentration-dependent increases in CRE-LUC activity with all tested receptors: Mtnr1Aαα, Mtnr1Aαβ, Mtnr1Ab and Mtnr1B (Figure 3A-D). Exposure to N-acetyl-serotonin or serotonin gave no consistent response (data not shown). Luzindole, an Mtnr inhibitor, decreased melatonin-induced CRE-LUC activity (Figure 3E-H) but had no effect when administered alone (Figure 3I-N). The different Mtnr showed an EC50 to melatonin, ranging from 1.58 nmol/L (Mtnr1Aαβ) to 372.03 nmol/L (Mtnr1Aαα). While the EC50 for two iodo melatonin ranged from 1.3 (Mtnr1Aαβ) to 30.23 nmol/L (Mtnr1Aαα). EC50 values for luzindole ranged from 7.77 nmol/L (Mtnr1Aαα) to 24.08 nmol/L (Mtnr1Aαβ), leading to partial inhibition (50% to 80%) in Mtnr1Aαα Mtnr1Aαβ and Mtnr1Ab, and complete inhibition (up to 100%) in Mtnr1B. Results are summarized in Table 2. No concentration-dependent increases in CRE-LUC activity were observed in negative controls (Figure S5). Exposure to forskolin induced cAMP over basal levels and

![Phylogenetic relationship between melatonin receptors (Mtnr). Tree topology was inferred by maximum likelihood from an amino acid sequence alignment using PhyML 3.0 combined with the substitution model selection (SMS) algorithms. Node support was estimated by bootstrapping from 1000 replicates and is indicated as per cent. *Branchiostoma belcheri* Mtnr-like was assigned as tree root. Different colour backgrounds indicate the four main Mtnr clades: MtnrA, MtnrAl, MtnrB and MtnrC/Gpr50. Salmonid Mtnr are surrounded by dotted lines. Teleost Mtnr paralogs from the teleost whole-genome duplication (3R) are indicated by suffixes a and b, and salmonid Mtnr paralogs from the salmonid whole-genome duplication (4R) are indicated by suffixes α and β. Mtnr references are given in Table S1. 

![Phylogenetic tree](image-url)
amplified melatonin-induced cAMP stimulation (Figure S6). Dopamine D2 receptor—that served as a positive control—decreased cAMP levels after exposure to quinpirole together with forskolin (Figure S7). The fact that salmon Mtnr1Aaα, Mtnr1Aaβ, Mtnr1Ab and Mtnr1B increased cAMP levels after melatonin exposure was also confirmed in CHO (Figure S8) and SH-SY5Y (Figure S9) cell lines. The human Mtnr1A decreased cAMP after melatonin exposure in COS7 and increased it in SH-SY5Y cell lines (Figure S10-S11).

3.5 | mtnr tissue distribution

mtnr1ab and mtnr1b showed a broad tissue distribution, being found in all tissues studied (Figure 4). The
4R-paralogs, mtnr1aaα and mtnr1aaβ, showed differential distribution: mtnr1aaβ was expressed in all brain parts, pituitary and skin, whereas mtnr1aaα was expressed only in some brain parts (optic nerves, optic tectum, cerebellum) and in eye, testis, skin. mtnr1al was expressed in all brain parts and in eye.

### 3.6 Pituitary mtnr expression—sexual maturation

The three mtnr expressed in the pituitary of male Atlantic salmon parr (mtnr1aaβ, mtnr1ab, mtnr1b) showed no significant differences in transcript levels between maturing and nonmaturing salmon during the initial stages of sexual maturation (Figure 5A-C; Figure S12).

### 3.7 Pituitary mtnr expression—daily rhythms

Major differences were seen between daily expression of mtnr in the pituitary in the spring and autumn. In autumn, mtnr1aaβ and mtnr1b expression remained stable and low during the course of 24 hours, while mtnr1ab showed a 5-fold increase between 04.00 and 12.00 (Figure 6). In spring, all receptors displayed strong sinusoidal expression patterns, with highest levels at 08.00 and lowest levels at 16.00 or 20.00.
**FIGURE 5** Relative expression of pituitary mtnr in male Atlantic salmon parr during gonad maturation. Relative abundance of mtnr1aaβ (A), mtnr1ab (B) and mtnr1b (C) mRNA in maturing (orange line, N = 6 per point), nonmaturing (blue line, N = 6 per point) in spring 2016. mRNA levels were normalized against rna18s and ef1a. Error bars indicate mean ± SEM. Values graphically expressed as fold change to the lowest point (set as value 1). Different letters denote statistically significant differences among groups (P < 0.05), analysed using two-way ANOVA, followed by Tukey multiple comparison test.

**FIGURE 6** Daily pituitary expression of mtnr in male Atlantic salmon parr in spring and autumn. Relative abundance of mtnr1aaβ (A, B), mtnr1ab (C, D) and mtnr1b (E, F) mRNA in pituitaries of male parr over a 24-h cycle in autumn (A, C, E; 23 October 2017; N = 6 per point) and in spring (B, D, F; 13 April 2018; N = 10 per point). Samplings were performed every four hours (04.00, 08.00, 12.00, 16.00, 20.00, 24.00). Grey column represents dark hours between sunset and sunrise. mRNA levels are normalized to rna18s and ef1a. Error bars indicate mean ± SEM. Values are graphically expressed as fold change to the lowest point (set as value 1). Different letters denote statistically significant differences among groups (P < 0.05), analysed using one-way ANOVA followed by Tukey multiple comparison test.
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Tsim, & Wong showed that enylyl cyclase and increase cAMP levels. For example, Yung, demonstrated that, under specific conditions, Mtnr may activate ad-

The validity of our assay is confirmed by latipes the ability of tilapia dopamine D2 receptor, which transduce and (c) absence of response in cells not transfected with mtnr (a) the specificity of the response to relevant agonists, (b) the 

It is well established that melatonin modulates reproduction in seasonal breeders. In mammals and birds, melatonin seems to modulate reproduction via activation of Mtnr1A in the pituitary pars tuberalis. In the rat, melatonin inhibits GnRH-induced LH release via activation of Mtnr1A. In salmonids, melatonin influences both gonad maturation and smoltification. Melatonin was reported to directly regulate growth hormone and prolactin in rainbow salmon Mtnr using adenylyl cyclase and G-proteins endo-

DISCUSSION

This study reports the structural, pharmacological and physiological characterization of melatonin receptors (Mtnr) in Atlantic salmon, with particular focus on pituitary expression in relation to gonadal maturation. An in silico search identified five genes encoding putative functional Mtnr. Phylogenetic analysis shows conservation of three GPCR of subtype 1A (mtnr1aaα, mtnr1aaβ, mtnr1ab) in Atlantic salmon, one of subtype 1Al (mtnr1al) and one of subtype 1B (mtnr1b). Although up to four paralogs for each receptor subtype could be expected from the teleost 3R and salmonid 4R, only subtype 1A shows a high number of functional paralogs, indicating higher functional dependence on this subtype. In contrast, no functional gene of subtype 1C is conserved in salmonids. In silico comparison of primary and tertiary structures of the five Atlantic salmon Mtnr reveals high conservation of key features known to be involved in receptor binding and activation in mammalian Mtnr (for review, see.13,33,34

Receptor-activation experiments showed that Mtnr1Aαα, Mtnr1Aαβ, Mtnr1Aβ and Mtnr1B were all activated in a dose-dependent manner by melatonin and 2-iodomelatonin and inhibited by luzindole. However, the efficacy of luzindole differed slightly between type 1A and type 1B receptors, with a partial inhibition in the former group and a complete inhibition in the latter, probably resulting from the different primary structures of the two groups. The receptor pharmacology provides in vitro confirmation of the functionality of four salmon Mtnr. Further, activation of the four salmon Mtnr resulted in increased intracellular cAMP levels in both COS-7, CHO and SH-SY5Y cell lines. This result contrasts with the findings from previous in vitro studies, in which activation of Mtnr in different vertebrate species led to decreased cAMP production; human Mtnr1A and Mtnr1B; chicken Mtnr1A and Mtnr1C; pike Mtnr1B; and medaka (Oryzias latipes) Mtnr1B. The validity of our assay is confirmed by (a) the specificity of the response to relevant agonists, (b) the absence of response in cells not transfected with mtnr and (c) the ability of tilapia dopamine D2 receptor, which transduce its signal through Gi protein and human Mtnr1A, both used as assay positive controls, to decrease cAMP production at the same conditions. Interestingly, some studies have demonstrated that, under specific conditions, Mtnr may activate adenylyl cyclase and increase cAMP levels. For example, Yung, Tsim, & Wong showed that Xenopus Mtnr1C increased cAMP levels in HEK293 cells co-transfected with type II adenylyl cyclase and α subunit. Furthermore, mouse Mtnr1A in COS-7 cells co-transfected with adenylyl cyclase VI and Gs protein increased intracellular cAMP levels in response to 2-iodomelatonin. In both those studies, receptor activation decreased cAMP when using, respectively, Xenopus or mouse intracellular signalling proteins. In contrast, our results showed increased cAMP levels upon activation of salmon Mtnr using adenylyl cyclase and G-proteins endogenous to the cell lines in use. This suggests that functional coupling of xenopus and mouse Mtnr may occur with both Gi and Gs proteins, with a much stronger affinity with the former; salmon Mtnr, on the other hand, may transduce their signal via Gs proteins, resulting in overall induction of adenylyl cyclase activity and increased cAMP levels. Alternatively, the measured cAMP induction may result from activation of AC by a Ca2+/calmodulin pathway coupled with Gq11 proteins as showed from Schuster and colleagues. These authors demonstrated that human Mtnr1A stimulates cAMP synthesis in human neuroblastoma cell line (SH-SY5Y). As an additional control, the same response was reported in the present study. This proves that the result obtained via heterologous cell lines may not reflect real in vivo conditions. Therefore, to validate the actual in vivo response of salmon Mtnr to melatonin exposure, future studies should be performed in salmon cell lines or tissue cultures.

Distribution analysis showed that the five receptors are expressed in the eyes and different brain regions, but only mtnr1aαα, mtnr1lab and mtnr1b are expressed in the salmon pituitary. In humans, Mtnr1A and Mtnr1B are found in the brain, eyes, pituitary, testis and skin. The presence of different mtnr in the pituitary has been observed in several teleost fish: mtnr1a, mtnr1al and mtnr1b in goldfish, Carassius auratus, mtnr1b in European seabass, Dicentrarchus labrax, mtnr1a, mtnr1b and mtnr1c in the Senegalese sole. For salmonids, mtnr1a and mtnr1b have been detected by PCR in the pituitary of chum salmon and pike, and melatonin binding sites have been observed in trout pituitary, but neither PCR nor autoradiography has previously detected any pituitary Mtnr in Atlantic salmon. The wide and specific tissue distribution of salmon mtnr may indicate the array of processes controlled by melatonin. The different distribution patterns of the three subtype 1A receptors (mtnr1aαα, mtnr1aαβ, mtnr1lab indicate functional divergence that may reflect cases of sub-functionalization). In contrast, and differing from other teleosts such as rabbitfish, Siganus guttatus, and European sea bass, no functional subtype 1C receptors are conserved in salmon or trout, indicating a pseudogenization or fractionation process in the salmonid lineage.
trout\textsuperscript{19} and to stimulate Lh release in the Atlantic croaker, \textit{Micropogonias undulatus}.\textsuperscript{21} This suggests that different pituitary cell types could express one or more \textit{mtnr}. We report the expression of three \textit{mtnr} in the salmon pituitary, two belonging to subtype 1A (\textit{mtnr1aaβ, mtnr1ab}) and one to the subtype 1B (\textit{mtnr1b}). However, there is no difference in expression between maturing and nonmaturing males. In addition, our results show clear daily variations in expression of the three receptors between seasons: expression levels remained low and stable during the 24-hour cycle in the autumn, but showed strong fluctuation in the spring, just around the time when gonad maturation begins. This might explain why Ekström & Vaněček \textsuperscript{20} could not identify melatonin binding sites in Atlantic salmon pituitary in December. Daily fluctuations in melatonin binding sites were detected in the brain of Masu salmon under natural photoperiod in July,\textsuperscript{54} but not under artificial photoperiod (LL, DD and LD) conditions.\textsuperscript{55}; Salmonids lack of circadian clock system regulating melatonin production, which is produced in an on/off manner in response to dark/light cycles.\textsuperscript{56} Interestingly, the variation in \textit{mtnr} mRNA, reported in the present study, appeared out of phase compared to light and melatonin cycles. A circadian regulation of melatonin binding sites has been detected in pike\textsuperscript{57} and goldfish\textsuperscript{58}; however, this remains to be shown in salmon under artificial photoperiod (LL, DD and LD) conditions. Differences in pituitary receptivity to melatonin may be involved in determining whether male salmon parr will initiate early sexual (precocious) maturation or not. Further studies are needed to confirm which cell types express which \textit{mtnr}, but this suggests that, in salmon, melatonin can regulate endocrine functions through a direct action at the pituitary level. In addition, these results underline the importance of considering time of day in interpretation of gene expression profiles.

In conclusion, our data add to our understanding of the function and regulation of vertebrate Mtnr. The presence of five functional genes belonging to four distinct phylogenetic clusters, in combination with the wide tissue distribution, is in accordance with the array of processes influenced by melatonin. Pharmacological characterization of salmon Mtnr proved, for the first time in a teleost species, the ability of Mtnr to increase intracellular cAMP levels in response to melatonin exposure. Finally, the identification of Mtnr expression in the salmon pituitary and their clear daily fluctuation in spring suggests the involvement of melatonin in neuroendocrine functions through a direct action on the pituitary.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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