General and Comparative Endocrinology 157 (2008) 186–195

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Cloning and retinal expression of melatonin receptors in the European sea bass, *Dicentrarchus labrax*

Sandrine Sauzet [a,b], Laurence Besseau [a,b], Patricia Herrera Perez [c], Denis Covès [d], Béatrice Chatain [d], Elodie Peyric [a,b], Gilles Boeuf [a,b], José Antonio Muñoz-Cueto [c], Jack Falcón [a,b,*]

[a] *Université Pierre et Marie Curie-Paris6, UMR7628, Laboratoire Aragó, Avenue Fontaulé, BP44, F-66651 Banyuls-sur-Mer, Cedex, France*
[b] *CNRS UMR7628 et GDR2821, Modèles en Biologie cellulaire et évolutive, Avenue Fontaulé, BP44, F-66651 Banyuls-sur-Mer, Cedex, France*
[c] *Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, E-11510 Puerto Real, Spain*
[d] *Station d'Aquaculture Expérimentale, IFREMER-CNRS, GDR2821, Laboratoire Aquaculture Languedoc-Roussillon, F-34250 Palavas, France*

ARTICLE INFO

Article history:
Received 14 February 2008
Revised 28 March 2008
Accepted 8 April 2008
Available online 22 April 2008

Keywords:
Sea bass
Melatonin receptors
Retina

ABSTRACT

Melatonin contributes to synchronizing behaviors and physiological functions to daily and seasonal rhythm in fish. However, no coherent vision emerges because the effects vary with the species, sex, age, moment of the year or sexual cycle. And, scarce information is available concerning the melatonin receptors, which is crucial to our understanding of the role melatonin plays. We report here the full length cloning of three different melatonin receptor subtypes in the sea bass *Dicentrarchus labrax*, belonging, respectively, to the MT1, MT2 and Mel1c subtypes. MT1, the most abundantly expressed, was detected in the central nervous system, retina, and gills. MT2 was detected in the pituitary gland, blood cells and, to a lesser extend, in the optic tectum, diencephalon, liver and retina. Mel1c was mainly expressed in the skin; traces were found in the retina. The cellular sites of MT1 and MT2 expressions were investigated by *in situ* hybridization in the retina of pigmented and albino fish. The strongest signals were obtained with the MT1 riboprobes. Expression was seen in cells also known to express the enzymes of the melatonin biosynthesis, i.e., in the photoreceptor, inner nuclear and ganglion cell layers. MT1 receptor mRNAs were also abundant in the retinal pigment epithelium. The results are consistent with the idea that melatonin is an autocrine (neural retina) and paracrine (retinal pigment epithelium) regulator of retinal function. The molecular tools provided here will be of valuable interest to further investigate the targets and role of melatonin in nervous and peripheral tissues of fish.

© 2008 Published by Elsevier Inc.

1. Introduction

Melatonin is one hormonal output of the vertebrates' circadian clocks, which contributes to synchronizing behaviors and neuroendocrine regulations to the daily and annual variations of photoperiod. In fish, melatonin is produced by the retina and pineal organ, two organs with photosensitive and circadian properties (Falcón et al., 2007a). In most species investigated, the variations in plasma melatonin content result from the rhythmic production by the pineal organ. Early physiological studies indicated that the pineal organ and melatonin contribute to controlling daily and annual behavioral and physiological rhythms (e.g., locomotor activity/rest, food intake, migration, shoaling, skin pigmentation, osmoregulation, smoltification, growth and reproduction (Falcón et al., 2007b). However, there is as yet no clear-cut picture on the exact roles the hormone plays in fish because of an apparent inconsistency in the results obtained. This is because most of the studies performed to date report on the effects of pinealectomy and/or melatonin administration, and the responses to these treatments depend on too many factors (for extensive Section 4 see Ekström and Meissl, 1997; Falcón et al., 2007b; Mayer et al., 1997).

The effects of melatonin are mediated through low and high affinity receptors. The low affinity melatonin receptor (MT3) identified in mammals corresponds to 'quinone reductase-2', a cytosolic enzyme that might be involved in detoxification processes (Mailliet et al., 2005). Three high affinity receptor subtypes have been identified to date, all belonging to the family of the seven transmembrane (TM) domains G-protein coupled receptors (GPCR) (Brydon et al., 1999; Falcón et al., 2007a). The MT1 and MT2 subtypes are found in all vertebrates investigated so far whereas the Mel1c subtype is found only in nonmammalian vertebrates. In comparison with the huge literature concerning mammals, very few studies report on the cloning of melatonin receptors in fish. A few partial sequences have been obtained from zebrafish (*Danio rerio*), pike (*Esox lucius*) and trout (*Oncorhynchus mykiss* (Mazurais et al., 1999)), and only three full length sequences are available to date for trout MT1 (AF156262), pike MT2 (Gaildrat and Falcón,

* Corresponding author. Address: Université Pierre et Marie Curie-Paris6, UMR7628, Laboratoire Aragó, Avenue Fontaulé, BP44, F-66651 Banyuls-sur-Mer, Cedex, France. Fax: +33/0 468 88 73 98.
E-mail address: falcon@obs-banyuls.fr (J. Falcón).

0016-6480/$ - see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.ygcen.2008.04.008

2000; Park et al., 2007a,b), and rabbitfish (*Siganus guttatus*) MT1 and Mel1c (Park et al., 2007a,b). Melatonin receptors display a wide distribution in fish. Several binding studies, using ^{125}IMel (Ekström and Meissl, 1997; Falcón et al., 2007b) and one *in situ* hybridization study (Mazurais et al., 1999) indicated the receptors are associated with areas that receive or integrate information from sensory organs (olfactive bulbs, telencephalon, diencephalon, optic tectum and cerebellum), including light, chemo- and mechano-reception. Melatonin receptors are also expressed in areas involved in neuroendocrine regulations, including the preoptic area and the pituitary gland (Falcón et al., 2007b). In peripheral tissues, melatonin binding sites have been detected in gills, intestine and kidney (Kulczykowska et al., 2006). Altogether, very little is known on the effects that are mediated by melatonin binding to its receptors in fish; only two studies report on a direct modulation of hormones release by cultured fish pituitary glands (Falcón et al., 2003; Khan and Thomas, 1996). One key element in the understanding of melatonin role in fish is a comprehensive identification and characterization of its receptors, and further identification of their sites of expression and modes of regulation. No clear-cut picture arises from the studies in fish, in great part because an exhaustive investigation of the receptors is lacking among species or within the same species. For this reason we decided to study the different melatonin receptor subtypes in a fish of both basic and economic interest, the sea bass, *Dicentrarchus labrax*, L., in keeping with the idea that cloning the different subtypes is a necessary and indispensable step in the more general task of investigating their daily and seasonal localization, regulation and role in nervous and peripheral tissues. We report here the cloning of three different melatonin receptor subtypes in the sea bass, respectively, MT1, MT2 and Mel1c. We also provide evidence that the former two are differentially expressed in the retina. We focused attention on the retina because it is as a closed nervous system, which synthesizes melatonin in different cell types (Besseau et al., 2006), in order to get insights into the paracrine and autocrine functions of melatonin in this organ.

2. Material and methods

2.1. Animals

Pigmented (*D. labrax*, L.) were obtained from "Méditerranée Pisciculture" (Salses, France). Animals (250 g b.w.) were maintained under natural conditions of photoperiod and temperature. Albino fish were from a natural mutant line reared at the Station Ifremer (Palavas les Flots, France). Albino fish were used in order to better detect labeled areas that could be masked by the retinal pigments. At this stage, all fish used were immature males. All samples were collected between 11:00 and 12:00 a.m. In all cases fish were killed by decapitation. All experiments were performed according to the European Union regulations concerning the protection of experimental animals.

2.2. Tissue processing

The tissues used for the cloning and PCR studies were collected and either dipped into Trizol (Invitrogen; Cergy Pontoise, France) and stored at +4 °C, or frozen in liquid nitrogen and stored at −80 °C until they were processed. Blood cells were prepared after centrifuging the blood for 10 min at 2500 rpm at +4 °C; the supernatant was discarded and the pellet containing all the cells was processed as indicated above for the other tissues sampled. Alternatively, tissues to be used for *in situ* hybridization studies were fixed overnight at +4 °C in freshly prepared 4% paraformaldehyde in phosphate buffer saline (PBS). After fixation, they were washed in PBS buffer containing, successively, 4% sucrose (5 min), 5% glycerol/10% sucrose (30 min), 10% glycerol/15% sucrose (1 h); they were then placed overnight in 10% glycerol/20% sucrose in PBS. The samples were then embedded in tissue freezing medium (Leica Microsystems; Rueil–Malmaison, France) and frozen at −48 °C.

2.3. Cloning strategy

Total RNA was extracted using the Trizol method (Invitrogen; Cergy Pontoise, France). Messenger RNA was isolated using oligo(dT)-magnetic beads (Dynal; Oslo, Norway) and used as a template to synthesize a bank of first strand cDNAs on beads (SMART RACE cDNA amplification kit: Clontech; Palo Alto, CA) according to the manufacturer's instructions. Extracts from retina, optic tectum and skin were used to clone the MT1, MT2 and Mel1c receptor subtypes, respectively. Degenerated primers were designed from peptide sequences located in the 3rd and 7th transmembrane domains, which are highly conserved among the melatonin receptors available from the data bases. Primer sequences were as indicated in Table 1. The polymerase chain reaction (PCR) was performed in a total volume of 50 µl as follows: 95 °C (1 min) followed by 10 cycles of denaturation at 94 °C (20 s), annealing at 37 °C (1 min) and extension at 68 °C (30 s), and by another 30 cycles of denaturation at 94 °C (10 s), annealing at 42 °C (1 min) and extension at 68 °C (30 s). Polymerase was Clontech Advantage (Clontech; Mountain View, CA) and template was cDNA from the selected extracts. The PCR products were then purified from an agarose gel using a gel extraction kit and sub-cloned into pGEM-T Easy (Promega; Charbonnières, France). Several positive clones were obtained from DH5α competent bacteria transformed by electroporation; sequencing was by Genome Express (Meylan, France). This allowed designing primers (Table 1) for further extension by 5′,3′-rapid amplification of cDNA ends (RACE; SMART RACE cDNA amplification kit: Clontech; Palo Alto, CA). The products of the 5′,3′-RACE were submitted to a second round of PCR using nested primers (Table 1), sub-cloned and sequenced.

2.4. Sequence analysis

The deduce amino acid sequences were obtained using the ExPASy Translate Tool (http://www.expasy.ch/tools/dna.html). Sequence comparison was made using the BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis was performed using the ClustalW program (http://www.ebi.ac.uk/clustalw/) and the BioEdit Sequence Aligment Editor (Hall, 1999). The phylogenetic tree was made using TreeView (Page, 1996).

Table 1
Primers used in this study

First round of RT-PCR	
MT1	
Forward	cggtactgctryathtgyca
Reverse	cgccggacctggatcacnarnaycca
MT2	
Forward	gatgcgtagataacagtaggtaaccactgc
Reverse	gacccacgagtttactcctgcacctttt
Mel1c	
Forward	gstaytgctacatctgccacag
Reverse	accacaaacatdgtcrgaaatt
5′,3′-RACE	
MT1	
5′ extension	tgtctggtttgacccgtctcctcacc
5′ nested	aaaggtgcaggagtaaactcgtgggtc
5′ end extension	agagggtacggatagatggccaccacaa
5′ end nested	gtctgccactgccaggctcaccacaaag
3′ extension	gacccacgagtttactcctgcacc
3′ nested	cgcatttggatactggtcatacaggtgagg
MT2	
5′ extension	gatgcgtagataacagtaggtaaccactgc
5′ nested	tgccactgtgtaggaactgctgacattctg
5′ end extension	gatgcgtagataacagtaggtaaccactgc
5′ end nested	tgccactgtgtaggaactgctgacattctg
3′ extension	gacccacgagtttactcctgcacctttt
3′ nested	cgcatttggatactggtcatacaggtgagg
Mel1c	
5′ extension	gaaggcttactcttcagtccctctgtggc
5′ nested	gcgttgaggcagctgttgaagtacgcc
5′ end extension	accagaggataggggtacaaagccaccacc
5′ end nested	tacagacaaactcaccacgaagatgttgcc
3′ extension	cctgtacagcctgaggaacacctgctgcta
3′ nested	accgccatcgccacagtgcccaacttcttt
Amplification from different tissues	
MT1	
Forward	ctctgtctgctatgtgatgctaatctgggc
Reverse	gtttctaacgtcatgcggcgttagcttggg
MT2	
Forward	ccacgagtttactcctgcacctttgcccag
Reverse	gttctttacagctgatggcatgctaacggg
Mel1c	
Forward	accgccatcgccacagtgcccaacttcttt
Reverse	cagtttggtcctttgctccggtttaacccg

```
rattus          -------------MKGNVS-ELLNASQQAPGGGE-EIRSRPSWLASTLAFILIFTIVVDI 45
phodopus        -------------MKGNGS-TLLNASQQAPGVGE-GGGPRPSWLASTLAFILIFTIVVDI 45
ovis            MAGRLWGSPGGT-PKGNGSSALLNVSQAAPGAGD-GVRPRPSWLAATLASILIFTIVVDI 58
homo            MQ-------------GNGS-ALPNASQPVLRGD--GAR--PSWLASALACVLIFTIVVDI 42
macaca          MP-------------GNGS-ALPNASQPGPGGD--GARPQPSWLASALACVLIFTIVVDV 44
canis           MAGPWGAAGGPPKGNGSGS-ALLNASQRAAGGGEGAAGPRPPWVACTLAVVLIFTIVVDV 59
siganus         -------------MVINGS--LLNSSAPD---PSDAVLSRPPWVTTTLGCFLIFTIIVDI 42
gallus          -------------MRANGS--ELNGTVLPRDPPAEGSPRRPPWVTSTLATILIFTIVVDL 45
taeniopygia     -------------MRVNES--ELNSSVLPRDPPAEGAPRRQPWVTSTLAAILIFTIAVDL 45
dicentrarchus   -------------MITNGS--HLNSSSPD---PADAVLNRPPWVTTTLGCFLIFTIVVDI 42
                                . *    * :               .*:: :*. .***** **:

rattus          LGNLLVILSVYRNKKLRNAGNIFVVSLAVADLVVAIYPFPLALTSILNNGWNLGYLHCQV 105
phodopus        LGNLLVILSVYRNKKLRNAGNIFVVSLAIADLVVAIYPYPLVLTSIFNNGWNLGYLHCQI 105
ovis            VGNLLVVLSVYRNKKLRNAGNVFVVSLAVADLLVAVYPYPLALASIVNNGWSLSSLHCQL 118
homo            LGNLLVILSVYRNKKLRNAGNIFVVSLAVADLVVAIYPYPLVLMSIFNNGWNLGYLHCQV 102
macaca          LGNLLVILSVYRNKKLRNAGNIFVVSLAVADLVVAVYPYPLVLTSIFNNGWNLGYLHCQI 104
canis           LGSLLVILSVYRNKKLRNAGNIFVVSLAVADLVVAVYPYPLVLTSIFNNGWNLGYLHCQI 119
siganus         LGNLLVIFSVYRNKKLRNAGNIFVVSLAVADLVVAIYPYPLVLSSIFHNGWNLGYVHCQI 102
gallus          LGNLLVILSVYRNKKLRNAGNIFVVSLAIADLVVAIYPYPLVLTSVFHNGWNLGYLHCQI 105
taeniopygia     LGNLLVILSVYRNKKLRNAGNVFVVSLAVADLIVAIYPYPLVLTSVFHNGWKLGYLHCQI 105
dicentrarchus   LGNLLVIFSVYRNKKLRNAGNIFVVSLAVADLVVAIYPYPLVLTSIFHNGWNLGYVHCQI 102
                :*.***::********:*:**:******:***:**:**:**.* *:.:.**.*. :***:

rattus          SAFLMGLSVIGSVFNITGIAMNRYCYICHSLKYDRIYSNKNSLCYVFLIWTLTLIAIMPN 165
phodopus        SAFLMGLSVIGSIFNITGIAINRYCYICHSLKYDRLYSNKNSLCYVFLIWVLTLVAIMPN 165
ovis            SGFLMGLSVIGSVFSITGIAINRYCCICHSLRYGKLYSGTNSLCYVFLIWTLTLVAIVPN 178
homo            SGFLMGLSVIGSIFNITGIAINRYCYICHSLKYDKLYSSKNSLCYVLLIWLLTLAAVLPN 162
macaca          SGFLMGLSVIGSIFNITGIAINRYCYICHSLKYDKLYSSKNSFCYVLLIWLLTLVAVLPN 164
canis           SGFVMGLSVIGSIFNITGIAINRYCYICHSLKYDKLYSNKNSLCYVFLIWMLTLVAVMPN 179
siganus         SGFLMGVSVIGSIFNITGIAINRYCYICHSLKYDKLYSDKNSVCYVMLIWALTVVAIVPN 162
gallus          SGFLMGLSVIGSIFNITGIAINRYCYICHSLKYDKLYSDKNSLCYVGLIWVLTVVAIVPN 165
taeniopygia     SGFLMGLSVIGSIFNITGIAINRYCYICHSLKYDKLYSDRNSLCYIVLIWLLTFVAIVPN 165
dicentrarchus   SGFLMGVSVIGSIFNITGIAINRYCYICHSLKYDKLYSDKNSVCYVMLIWALTVVAIVPN 162
                *.*:**:*****:*.*****:**** *****:*.::**. **.**: *** **. *::**

rattus          LQTGTLQYDPRIYSCTFTQSVSSAYTIALVVFHFVVPMIIVTFCYLRIWILVLQVRRRVK 225
phodopus        LQTGTLQYDPRIYSCTFTQSVSSAYTIAVVVFHFIVPMIIVIFCYLRIWILVLQVRRRVK 225
ovis            LCVGTLQYDPRIYSCTFTQSVSSAYTIAVVVFHFIVPMLVVVFCYLRIWALVLQVRWKVK 238
homo            LRAGTLQYDPRIYSCTFAQSVSSAYTIAVVVFHFLVPMIIVIFCYLRIWILVLQVRQRVK 222
macaca          LRAGTLQYDPRIYSCTFAQSVSSAYTIAVVVFHFLVPMMIVIFCYLRIWILVLQVRQRVK 224
canis           LRTGTLQYDPRIYSCTFAQSISSAYTIAVVVFHFIVPMTIVIFCYLRIWILVLQVRRRVK 239
siganus         LFVGSLQYDPRVYSCTFEQSASSAYTIAVVFFHFILPIMIVTYCYLRIWILVIQVRRRVK 222
gallus          LFVGSLQYDPRIYSCTFAQSVSSAYTIAVVFFHFILPIAIVTYCYLRIWILVIQVRRRVK 225
taeniopygia     LFVGSLQYDPRIYSCTFAQSVSSAYTIAVVFFHFLLPIAVVTFCYLRIWILVIQVRRRVK 225
dicentrarchus   LFVGSLQYDPRVYSSTFEQSASSAYTIAVVFFHFILPIMIVTYCYLRIWILVIQVRRRVK 222
                * .*:******:**.** ** *******:*.***::*: :* :****** **:*** :**

rattus          PDSKPKLKPQDFRNFVTMFVVFVLFALCWAPLNFIGLIVASDPAAMAPRIPEWLFVASYY 285
phodopus        PDSKPRLKPQDFRNFVTMFVVFVLFAICWAPLNFIGLIVASDPATMAPRIPEWLFVASYY 285
ovis            PDNKPKLKPQDFRNFVTMFVVFVLFAICWAPLNFIGLVVASDPDSMAPRIPEWLFVASYY 298
homo            PDRKPKLKPQDFRNFVTMFVVFVLFAICWAPLNFIGLAVASDPASMVPRIPEWLFVASYY 282
macaca          PDRKPRLKPQDFRNFVTMFVVFVLFAICWAPLNFIGLAVASDPASMVPRIPEWLFVASYY 284
canis           PDSKPKMKPQDFRNFVTMFVVFVLFAICWAPLNFIGLAVASNPDSMGPRIPEWLFVASYY 299
siganus         PDNRPKITPHDVRNFVTMFVVFVLFAVCWAPLNFIGLAVAIKPEVVVPLIPEWLFVSSYF 282
gallus          PDNNPRLKPHDFRNFVTMFVVFVLFAVCWAPLNFIGLAVAVDPETIIPRIPEWLFVSSYY 285
taeniopygia     PDNNPRLKPHDFRNFVTMFVVFVLFAVCWAPLNFIGIAVAVNPKTVIPRIPEWLFVSSYY 285
dicentrarchus   PDNRPKLTPHDVRNFVTMFVVFVLFAVCWAPLNFIGLAVAIKPEVVVPLIPEWLFVASYF 282
                ** .*::.*:*.**************:******:**: ** .*  : * *******:**:

rattus          LAYFNSCLNAIIYGLLNQNFRKEYKRIIISLCTAKMFFVDSSNDAADKIKCKPSPLITNN 345
phodopus        MAYFNSCLNAIIYGLLNQNFRQEYKRILVSLFTAKMCFVDSSNDPADKIKCKPAPLIANN 345
ovis            MAYFNSCLNAIIYGLLNQNFRQEYRKIIVSLCTTKMFFVDSSNHVADRIKRKPSPLIANR 358
homo            MAYFNSCLNAIIYGLLNQNFRKEYRRIIVSLCTARVFFVDSSNDVADRVKWKPSPLMTNN 342
macaca          MAYFNSCLNAIIYGLLNQNFRKEYRRIIVSLCTARMFFVDSSNDVADRVKCKPSPLTTKN 344
canis           MAYFNSCLNAIIYGLLNQNFRKEYRRIIVSLCTARMFFVDSSNDVAHRVNCKPSPLMTNN 359
siganus         MAYFNSCLNAIVYGVLNQNFRREYKRIVVSVCTARIFFQDSSNDAGERLKSKPSPLMANN 342
gallus          MAYFNSCLNAIIYGLLNQNFRREYKKIVVSFCTAKAFFQDSSNDAADRIRSKPSPLITNN 345
taeniopygia     MSYFNSCLNAIVYGLLNQNFRREYKRIIVNFCTAKVFFQDSSNDAGDRMRSKPSPLITNN 345
dicentrarchus   MSYFNSCLNAIVYGVLNQNFRREYKRIVVSVCTARIFFQDSSNDAGERLKSKPSPLMTNN 342
                :**********:**:******:**::*::.. *::  * :***.  .::. **:** .:.

rattus          NLIKVDSV- 353
phodopus        NLIKVDSV- 353
ovis            NLVKVDSV- 366
homo            NVVKVDSV- 350
macaca          NLVKVDSV- 352
canis           NLIKVDSV- 367
siganus         NQVKVDSV- 350
gallus          NQVKVDSV- 353
taeniopygia     NQVKVDSV- 353
dicentrarchus   NQVKVDSV- 350
                * :*****
```

Fig. 1. Deduced amino acid sequence of *D. labrax* MT1 melatonin receptor and alignment with MT1 from other vertebrate species. The sea bass sequence is the last listed. The transmembrane domains are underlined (sequentially from I to VII). Amino acids known to be important for the proper function of mammalian MT1 receptor are in bold on a gray background. The dotted box shows the conserved NRY motif just after transmembrane domain III. *Canis familiaris*: XP_540019.2; *D. labrax*: EU_378918; *Gallus gallus*: NP_990693.1; *Homo sapiens*: NP_005949.1; *Macaca mulatta*: XP_001090972.1; *Ovis aries*: AAC_02699.1; *Phodopus sungorus*: AAB_17722.1; *Rattus norvegicus*: AF_130341.1; *S. guttatus*: ABG_77572.1; *Taeniopygia guttata*: ABG_37785.1.

Table 2
Percent of identity/similarity between the sea bass melatonin receptors and the melatonin receptors from other species

	% Identity/similarity		
Sea bass /	Fish	Frogs/birds	Mammals
dlMT1/MT1	92–97/98	82/90	72–80/84–90
dlMT1/MT2	69/81	69/83	60/78
dlMT1/Mel1c	71/84	72/85	n.a.
dlMT2/MT1	67/79	67/80	62–69/76–81
dlMT2/MT2	76/85	71/85	64/79
dlMT2/Mel1c	69/83	65/82	n.a.
dlMel1c/MT1	70/84	70/86	67/82
dlMel1c/MT2	71/83	74/87	64/81
dlMel1c/Mel1c	97/98	77/90	n.a.

The species were those mentioned in Figs. 1–3. n.a. = not applicable.

2.5. Amplification from different tissues

Total RNA from the different tissues tested was extracted as described above, and 1 µg was incubated with 1 U of DNAse I (Roche; Meylan, France) for 20 min at 37 °C. DNAse inactivation (10 min at 65 °C) was followed by reverse transcription using Powerscript Reverse Transcriptase (Clontech; Mountain View, CA). For each tissue, PCR amplification was performed using a set of specific forward (F) and reverse (R) primers designed from the cloned receptors (Table 1), using similar volumes of cDNA obtained from the same retrotranscription reaction. The conditions were : 95 °C (1 min), then 10 cycles of 94 °C (20 s), 67 °C (MT1), 65 °C (MT2) or 70 °C (Mel1c) (1 min), 68 °C (1 min), followed by another 20 cycles of 94 °C (15 s), 62 °C (MT1), 60 °C (MT2) or 65 °C (Mel1c) (1 min), 68 °C (1 min), and terminated with 7 min at 68 °C. In the controls, the template was replaced by either water or RNA. The PCR products were loaded in an agarose gel, in the presence of DNA size markers (DNA/Hinf I marker: Promega; Charbonnières, France). Fragments of the expected size were extracted, sub-cloned in pGEM-T Easy and sequenced as indicated above, to verify that it did correspond to the sequence corresponding to the gene under investigation. All experiments were duplicated using a different set of animals.

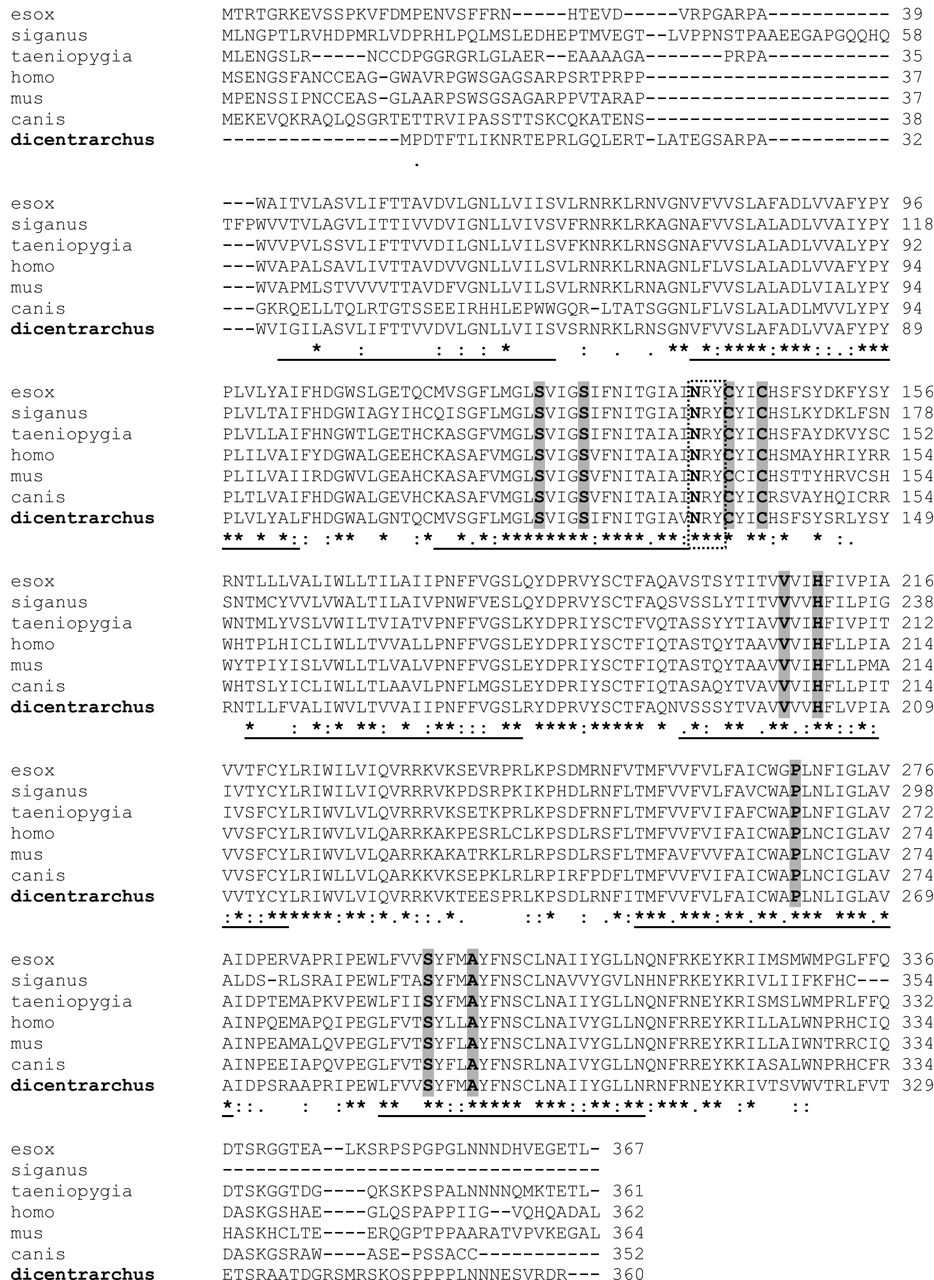

Fig. 2. Deduced amino acid sequence of *D. labrax* MT2 melatonin receptor and alignment with MT2 from other vertebrate species. The sea bass sequence is the last listed. The transmembrane domains are underlined (sequentially from I to VII). Amino acids known to be important for the proper function of mammalian MT1 receptor are in bold on a gray background. The dotted box shows the conserved NRY motif just after transmembrane domain III. *C. familiaris*: XP_849722.1; *D. labrax*: EU_378919; *E. lucius* AAG_17109.1; *H. sapiens*: NP_005950.1; *Mus musculus*: AI_04326.1; *S. guttatus*: ABF67976.1; *T. guttata*: NP_001041723.1.

2.6. In situ hybridization

In situ hybridization was done on 10 μm cryo-sections mounted on 2% 3-amino-propyltriethoxysilane (Sigma; Saint Quentin Fallavier, France) coated slides. Sense and anti-sense digoxigenin-labeled riboprobes probes were made using the kit from Roche (Meylan, France) according to the manufacturer's instructions. The probes were generated using cDNA fragments of, respectively, 480 (MT1: bp 800–1280), and 575 (MT2: bp 1010–1585) bp. The hybridization process was as detailed elsewhere (Besseau et al., 2006). Briefly, the sections were rehydrated and treated with proteinase K (Sigma; 5 μg/ml for 10 min at 37 °C). After post-fixation with 4% paraformaldehyde the sections were hybridized overnight at 55 °C using a probe concentration of 1 μg/ml in hybridization buffer (50% formamide, 5× SSC, 9.2 mM citric acid, 0.1% Tween 20®, 50 μg/ml heparin). After blocking (2% sheep serum in PBS Tween), digoxigenin was immunodetected using a commercially available kit (Roche, Meylan, France). All experiments were triplicated using different animals.

3. Results

*3.1. Cloning of **D. labrax** MT1, MT2 and Mel1c melatonin receptors*

The strategy used in this study allowed obtaining three different nucleotide sequences. The first sequence is 1279 nucleotides (nt) in length. This sequence appears to encode a protein of 350 amino acids, leaving a 172 nt 5′-UTR and a 54 nt 3′-UTR. Sequence comparison indicated it displays high homology with receptors from the MT1 subtype (Fig. 1). Amino acid identity with other MT1 sequences was >90% (fish), 81–83 (frogs, birds) and <80% (mammals); identity with other melatonin receptor subtypes was <80% (Table 2). The second sequence is 1584 nucleotides (nt) in length. The deduced peptide sequence is made of 360 amino acids; there are 501 nt in the 5′-UTR. Sequence comparison indicated it displays high homology with receptors from the MT2 subtype (Fig. 2). Identity is of 76% with the pike MT2 receptor; amino acid identity with other melatonin receptor sequences was less than 70% (Table 2). The third sequence is 1218 nt in length; the deduce peptide sequence is made of 353 amino acid, with 39 and 114 nt left in the 5′- and 3′-UTR regions, respectively. The peptide sequence displays 97% (fish) and 76–78% (frogs, birds) identity with peptide sequences of the Mel1c receptor subtype (Fig. 3). Identity with other melatonin receptor amino acid sequences is 70% or below (Table 2).

The three deduced amino acid sequences displayed the 7 TM motifs profile as well as amino acid known to be crucial for the function of the receptors in mammals (see Section 4). The phylogenetic tree built after a comparative analysis of sequences further confirmed that the three clones isolated were each representative of one high affinity melatonin receptor subtype (Fig. 4), and were therefore tentatively named dlMT1 (EU378918), dlMT2 (EU378919), and dlMel1c (EU378920), respectively.

3.2. Expression of D. labrax melatonin receptors in different tissues

The cloning of the melatonin receptors allowed searching for the tissue specific expression of each subtype. At the time of year investigated (February) the MT1 subtype displayed the largest distribution. In nervous tissues, expression was evident in the optic tectum and, to a lower extent, in the cerebellum, telencephalon and diencephalon (Fig. 5); MT1 was also expressed in the retina. In peripheral tissues expression was detected in the gills, and weak

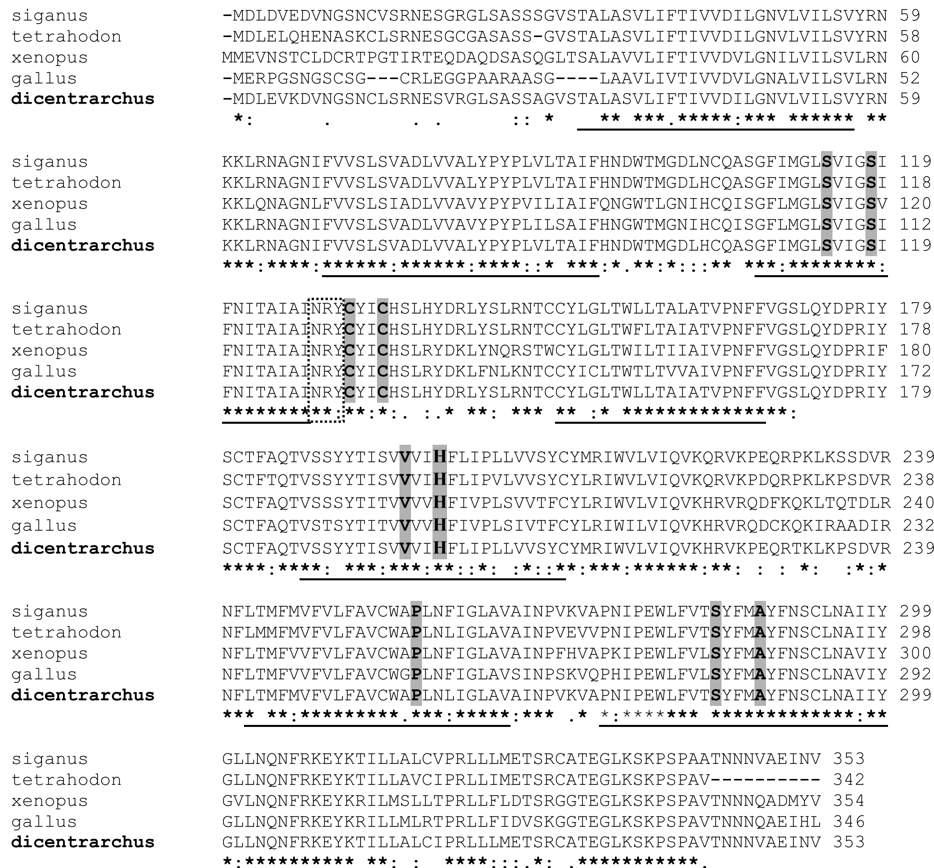

Fig. 3. Deduced amino acid sequence of *D. labrax* Mel1c melatonin receptor and alignment with Mel1c from other vertebrate species. The sea bass sequence is the last listed. The transmembrane domains are underlined (sequentially from I to VII). Amino acids known to be important for the proper function of mammalian MT1 receptor are in bold. The dotted box shows the conserved NRY motif just after transmembrane domain III. *D. labrax*: EU_378920; *G. gallus*: NP_990692.1; *S. guttatus*: ABG_77573.1; *Xenopus laevis*: AAB_48391.1.

expression was seen in the muscles (Fig. 5). In contrast to MT1, MT2 expression was strong in pituitary extracts; it was weak in retinal extracts and low (optic tectum, diencephalon) or even absent (cerebellum) in extracts from the central nervous system (Fig. 5). No expression was detected in peripheral tissues except the liver and the blood cells. Mel1c expression was only detected in extracts from the skin and traces were also detected in retina (not shown).

3.3. In situ localization of MT1 and MT2 melatonin receptor expression in the retina

We investigated the localization of MT1 and MT2 expression in the retina, using *in situ* hybridization. With the anti-sense probes, the MT1 hybridization signal was seen in all photoreceptor cells of the outer nuclear layer (ONL); it seemed more intense at the level of the outer limiting membrane (Fig. 6). By their position in the inner nuclear layer (INL), the cell bodies that express the MT1 could belong to either bipolar or amacrine or interstitial cells (Fig. 6). Most of the cell bodies in the ganglion cell layer (GCL) were also labeled. The general pattern was maintained with the MT2 probes with, however, a lower intensity as expected from the RT-PCR studies (Fig. 6). The differences in intensity were mainly seen in the ONL and INL. In the later, the number of labeled cells was less than with the MT1 probe; by their position in the INL, these MT2 expressing cells would correspond to amacrine cells. In the albino fish the pattern was quite different than the one described above (Fig. 6). Only the cells of the pigment epithelium cells layer were intensely labeled with the MT1 probe. A weaker labeling was seen in the ONL and GCL. In contrast, the pigment epithelium cells were not labeled with the MT2 probe; MT2 expression was mainly observed in the photoreceptor cells layer. No labeling was detected in the control sections treated with the sense probes (Fig. 6).

4. Discussion

This study in the sea bass reports the cloning of one representative of each of the three high affinity melatonin receptor subtypes known in vertebrates. Their identification was supported by the comparative analysis of sequences available in the data bases, and the family tree that was subsequently drawn. In the tree, the sea bass receptors appeared linked to their respective fish relatives. This tree also showed that the sea bass (this study) and pike (Gaildrat et al., 2002) MT2 receptors constitute a distinct subgroup among the MT2 receptor family, bringing support to a previous hypothesis. This subgroup did not include the rabbitfish melatonin receptor previously reported as an MT2 (Park et al., 2006). It is questioned whether the *S. guttatus* melatonin receptor identifies

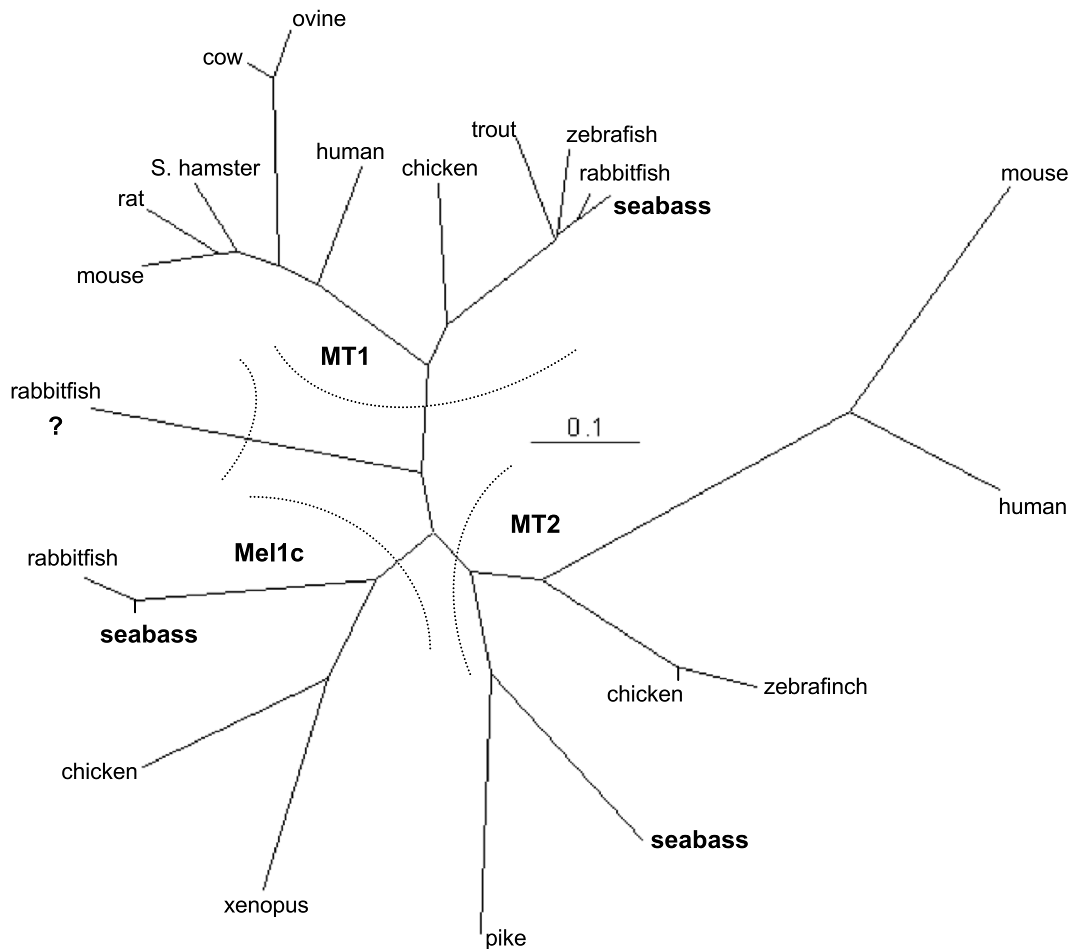

Fig. 4. PROTDIST Fitch phylogenetic unrooted tree. The tree shows the interrelationships of the different melatonin receptor subtypes. Each of the sea bass cloned receptors fits into one category. In this tree, the rabbitfish melatonin receptor initially classified as a MT2 (?) does not fit into any of the three melatonin receptor families. In all cases the sea bass melatonin receptors cloned are closely linked to those of the other fish species available. MT1: chicken: NP_990693.1; cow: XP_614283.2; human: NP_005949.1; mouse: NP_032665.1; ovine: AAC_02699.1; sea bass: EU_378918; Syrian hamster: AAB_17722.1; rat: AF_130341.1; rabbitfish: ABG_77572.1; trout: AAF00191.1; zebrafish: NP_571468.1; MT2: chicken: XP_417201.2; human: NP_005950.1; mouse: AI_04326.1; pike: AAG_17109.1; sea bass: EU_378919; zebrafinch: NP_001041723.1. Mel1c: chicken: NP_990692.1; rabbitfish: ABG_77573.1; sea bass: EU_378920; *Xenopus*: AAB_48391.1.

a new family of receptors in fish. Indeed, although it displayed high similarity in the TM domains regions of the other two fish MT2 receptors cloned to date, it had a longer 5′-end and a shorter 3′-end. Because of this uncertainty, the following discussion includes no reference to this receptor subtype. All three sea bass melatonin receptors possess the structural motifs consisting of 7 TM domains typically found in the GPCR family, and connected by a series of intra and extra-cellular loops. They also possess conserved amino acid known to be important for the function of the mammalian MT1 receptor (Figs 1–3) (Kokkola et al., 2003, 2005; Witt-Enderby et al., 2003). These include the two serine residues in TM domain 3, 2 cysteine residues of the 4th loop domain and the adjacent NRY motif, the valine and histidine residues in TM domain 4, a proline and a serine residues in TM domains 5 and 6, respectively.

With the sequences in hands, it was possible to design specific primers to search for each subtype in the different tissues of the sea bass. The observation that MT1 and MT2 were expressed in distinct brain areas and in the retina is in general agreement with the results from previous studies on both melatonin receptor expression (Mazurais et al., 1999) and ^{125}IMel binding (Davies et al., 1994; Ekström and Vanecek, 1992; Gaildrat et al., 2002; Martinoli et al., 1991). Although no quantitative study was done, we found some differences in the respective levels of expression of one subtype *vs.* another; the MT1 seemed more widely distributed and more strongly expressed than the other subtypes in the sea bass brain and retina. Differences were also found between sea bass and other fish species concerning the tissue distribution of the different subtypes. For example, we found no expression of either receptor subtype in the sea bass kidney and intestine, whereas MT1 expression or ^{125}IMel binding were found in other fish species (Kulczykowska et al., 2006; Park et al., 2006). Similarly, in our hands expression of Mel1c subtype was restricted to the skin and, to a much lesser degree, to the retina, whereas another study reports low levels of expression in the brain (Park et al., 2006). Several reasons may account for these discrepancies, which include technical aspects (e.g., number of PCR cycles), reproductive status, differences in the time of day or year at which the experiments were done, or species related differences. Our future investigations will aim at elucidating to which extend daytime and calendar time affect the expression of the receptors under investigation in the sea bass. In addition to these general considerations, some interesting characteristics deserve attention. First, a strong MT2 expression was found in extracts from sea bass pituitaries. The issue concerning the detection of melatonin receptors in the fish pituitary had been a matter of contradictory discussions in the past (Davies et al., 1994; Ekström and Vanecek, 1992; Falcón et al., 2003; Gaildrat et al., 2002; Mazurais et al., 1999). Our results bring strong support to the idea that melatonin controls fish neuroendocrine functions through, at least, a direct action on the pituitary, mediated by MT2 receptors (Falcón et al., 2003; Gaildrat et al., 2002). Second, MT2 melatonin receptors appeared expressed in fish blood cells. This observation might relate with previous data showing *in vitro* uptake of [^{3}H]-melatonin by one third of the red blood cells population in chicken and pike (Falcón and Collin, 1985; Voisin et al., 1983). Nevertheless, the nature of these cells in sea bass and the functional significance of this finding remain to be investigated. Interestingly, melatonin receptors and melatonin effects on gene expression have been described in human peripheral blood mononuclear cells (Ha et al., 2006; Pozo et al., 2004). Third, there was a conspicuous MT1 expression in the sea bass gills. This complements previous studies that showed specific ^{125}IMel binding in rainbow trout, flounder and seabream gills (Kulczykowska et al., 2006). The gill is a richly vascularized organ; however, MT1 was not expressed in blood cells, thereby indicating that the expression found in gills is probably tissue specific. It suggests that melatonin may modulate electrolyte balance through a direct control of gills function, in addition to its pituitary effects on growth hormone and prolactin secretions (Falcón et al., 2003).

Before going deeper into a discussion on the role melatonin plays in the different organs where receptor expression has been detected, it is necessary to more precisely identify the cell types that express these receptors. As a first step in this task, we focused attention on the retina, which is an active site of melatonin synthesis (Iuvone et al., 2005); considering that in fish, retinal melatonin is usually not released into the blood, but rather acts locally (Falcón et al., 2007a). Retinal melatonin has been for a long time involved in the control of a number of retinal functions, including melanosome aggregation in the pigment epithelium, rod outer segment shedding, cone retinomotor movements and modulation of neurotransmitters release (Lundmark et al., 2006; O'Brien and Klein, 1986; Pautler and Hall, 1987). The mechanisms through which melatonin acts are far from being understood, particularly in fish. Here we bring the first demonstration that the MT1 and MT2 melatonin receptors were expressed in the three nuclear layers of the neural fish retina as well as in the retinal pigment epithelium. At the time point investigated, the labeling was more intense with the MT1 than with the MT2 probe. In the sea bass retina, the cells expressing the melatonin receptors were the photoreceptor and ganglion cells as well as yet unidentified cells located in the most inner part of the INL.

The demonstration that melatonin receptors are expressed in the three different layers of the sea bass retina extends to fish previous findings obtained in frog, chicken, rodent and human retinas (Fujieda et al., 1999; Natesan and Cassone, 2002; Savaskan et al., 2002). The evidence that the whole ONL of the sea bass retina expressed MT1 receptors indicates melatonin as an autocrine regula-

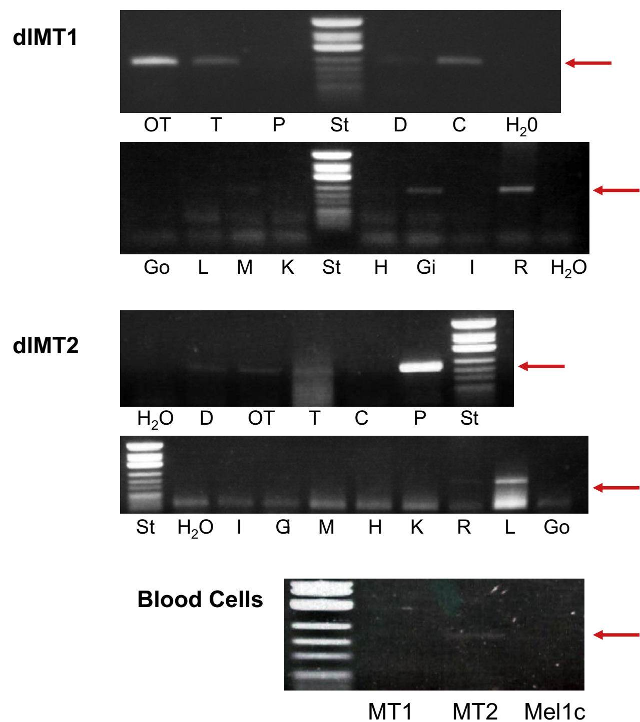

Fig. 5. Tissue specific distribution of the melatonin receptors mRNA assessed by RT-PCR. The RT-PCR conditions were as described in Section 2. The organs were sampled in February. The identity of the fragments of interest was verified after extraction, sub-cloning and sequencing. No signal is seen in the controls where the template was replaced by water (H_20) or nontranscribed mRNA (not shown). C: cerebellum; D: diencephalon; Gi: gills; Go: gonads (testis); H: heart; I: intestine; K: kidney; L: liver; M: muscle; OT: optic tectum; P: pituitary; R: retina; T: telencephalon; st: molecular weight standards.

tor of rod and cone function, including its own biosynthesis (Falcón et al., 2007a), electrical activity (ERG; (Peters and Cassone, 2005; Pierce and Besharse, 1985), disc shedding and photoreceptor movements (Peters and Cassone, 2005; Pierce and Besharse, 1985), and synchronization of circadian clocks units (Cahill and Besharse, 1993; Chaurasia et al., 2006; Yu et al., 2007). MT1 and MT2 receptors were also expressed in yet unidentified cells of the sea bass INL retina (in bipolar and/or amacrine and/or Müller cells) as well as in the ganglion cells. The results are consistent with the demonstration that melatonin modulates dopamine release by A-II amacrine cells in the INL of fish and other vertebrates (Ribelayga et al., 2004), as part of a loop in which dopamine feeds back on the melatonin biosynthesis and circadian activity of the photoreceptor cells (Stella and Thoreson, 2000; Yu et al., 2007). The large distribution of MT1 receptors in the INL and GCL could reflect functions of melatonin related to control of neurotransmitter release (Fujieda et al., 2000; Mitchell and Redburn, 1991), or modulation of the electroretinogram and Purkinje shift (Peters and Cassone, 2005).

It is generally believed that melatonin is produced by the photoreceptor cells in a circadian manner and that it acts as an autocrine and paracrine modulator of retinal function (Green and Besharse, 2004; Iuvone et al., 2005; Iigo et al., 2007). However, we have recently demonstrated that cells from the INL and GCL also expressed the enzymes of the melatonin synthesizing pathway, the arylalkylamine *N*-acetyltransferase (AANAT) and hydroxyindole-*O*-methyltransferase (HIOMT) in trout (Besseau et al., 2006) and sea bass (unpublished) retinas. And, in both species the melatonin synthesizing cells occupied the same position in the retinal epithelium as those shown here to express the melatonin receptors. This would suggest that melatonin is also an autocrine modulator in the inner fish retina; i.e., it acts locally where ever it is produced. As an output of the circadian clocks, melatonin is thought to act as a synchronizer of rhythmic functions (Falcón et al., 2007b). In fish, there is indication that light entrained circadian clocks are located in the retina and pineal as well as in extraocular and extra-pineal tissues (Whitmore et al., 2000); and, nonvisual photopigment molecules have been identified in the inner layers of the neural retina (Bellingham et al., 2006; Foster and Bellingham, 2004). The question raises therefore to know whether the different neuronal cells that express the melatonin receptors in the INL and GCL of the sea bass are photoperiod entrained circadian oscillators, and what role melatonin plays in this picture?

It is interesting that the intensity of the labeling was considerably reduced in the albino retinas when compared to the pigmented retinas processed simultaneously. Further investigations are necessary in order to determine the reasons for these discrepancies. Our main interest in using albino fish was that it allowed visualizing a strong MT1 expression in the retinal pigment epithelium (RPE) cells. This is the first demonstration that melatonin receptors are expressed in the fish RPE, supporting previous similar findings in the African clawed frog (Wiechmann et al., 1999). The expression of melatonin receptor RNA in the sea bass RPE is in accordance with previous studies involving melatonin in the con-

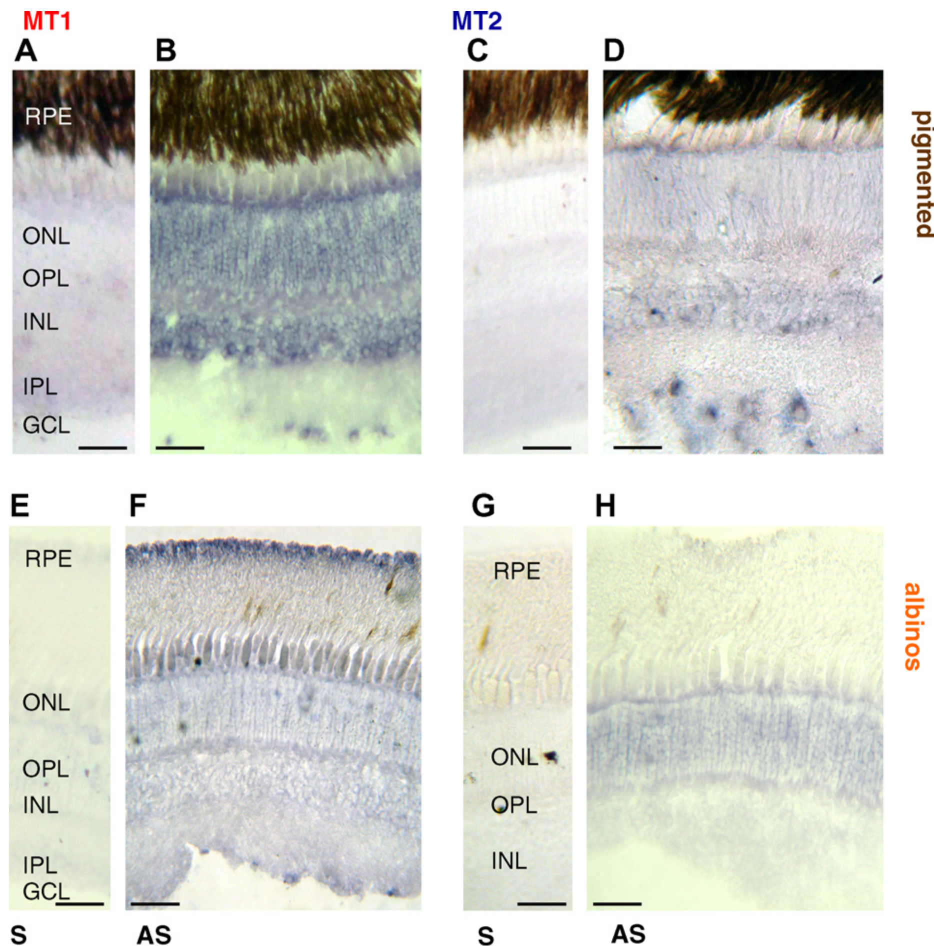

Fig. 6. Retinal localization of MT1 (B, D) and MT2 (F, H) mRNA by *in situ* hybridization. Retinal sections from pigmented (A–D) and albino (E–F) fish were treated with the anti-sense (AS: B, D, F, H) or sense (S: A, C, E, G) probes. See text for details. GCL: ganglion cell layer; INL: inner nuclear layer; IPL: inner plexiform layer; ONL: outer nuclear layer; OPL: outer plexiform layer; RPE: retinal pigmented epithelium. Bars = 50 μm.

trol of RPE chemotactic cellular movements, pigment migration and phagocytosis of photoreceptor outer segment membranes (Shirakawa and Ogino, 1987; Zawilska, 1992; Zawilska and Nowak, 1992).

In conclusion, this study reports the cloning of three melatonin receptor subtypes in sea bass, adding to the very short list of melatonin receptors cloned to date in fish. We show that these receptors already display the main features that characterize those found in tetrapods. We were also able to provide information on the tissue specific distribution of each subtype in the sea bass. The demonstration that receptors are present in structures such as the pituitary, gills or blood cells opens interesting lines of investigations that have received yet no or not enough attention. The results of our *in situ* hybridization studies in the retina extend to fish information available from tetrapods only, and we bring anatomical support to previous data involving melatonin in the control of various retinal processes. Interestingly, we found that the retinal distribution of the MT1 receptor and melatonin synthesizing enzymes mRNAs were very similar, highlighting the possibility that fish retinal melatonin is an autocrine modulator of retinal function. Future studies will aim at more precisely identifying the cell types that express the melatonin biosynthesis enzymes and receptors in the inner retina. More generally, this study was a necessary step in our way to more precisely identify the sites of expression of the different melatonin receptors in the fish brain, their regulation and respective roles.

Acknowledgments

This work was supported by the CNRS, the UPMC, the IFREMER and the MEC (Grants #752887/00, #753447/00, #6432 and AGL2001-0593-C03-02).

References

Bellingham, J., Chaurasia, S.S., Melyan, Z., Liu, C., Cameron, M.A., Tarttelin, E.E., Iuvone, P.M., Hankins, M.W., Tosini, G., Lucas, R.J., 2006. Evolution of melanopsin photoreceptors: discovery and characterization of a new melanopsin in nonmammalian vertebrates. PLoS Biol. 4, e254.

Besseau, L., Benyassi, A., Moller, M., Coon, S.L., Weller, J.L., Boeuf, G., Klein, D.C., Falcón, J., 2006. Melatonin pathway: breaking the 'high-at-night' rule in trout retina. Exp. Eye Res. 82, 620–627.

Brydon, L., Roka, F., Petit, L., de Coppet, P., Tissot, M., Barrett, P., Morgan, P.J., Nanoff, C., Strosberg, A.D., Jockers, R., 1999. Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins. Mol. Endocrinol. 13, 2025–2038.

Cahill, G.M., Besharse, J.C., 1993. Circadian clock functions localized in *xenopus* retinal photoreceptors. Neuron 10, 573–577.

Chaurasia, S.S., Pozdeyev, N., Haque, R., Visser, A., Ivanova, T.N., Iuvone, P.M., 2006. Circadian clockwork machinery in neural retina: evidence for the presence of functional clock components in photoreceptor-enriched chick retinal cell cultures. Mol. Vis. 12, 215–223.

Davies, B., Hannah, L.T., Randall, C.F., Bromage, N., Williams, L.M., 1994. Central melatonin binding sites in rainbow trout (*Onchorhynchus mykiss*). Gen. Comp. Endocrinol. 96, 19–26.

Ekström, P., Meissl, H., 1997. The pineal organ of teleost fishes. Rev. Fish Biol. Fisheries 7, 199–284.

Ekström, P., Vanecek, J., 1992. Localization of 2-[^{125}I]iodomelatonin binding sites in the brain of the Atlantic salmon, *Salmo salar* L. Neuroendocrinology 55, 529–537.

Falcón, J., Besseau, L., Fazzari, D., Attia, J., Gaildrat, P., Beauchaud, M., Boeuf, G., 2003. Melatonin modulates secretion of growth hormone and prolactin by trout pituitary glands and cells in culture. Endocrinology 144, 4648–4658.

Falcón, J., Besseau, L., Sauzet, S., Boeuf, G., 2007a. Melatonin effects on the hypothalamo-pituitary axis in fish. Trends Endocrinol. Metab. 18, 81–88.

Falcón, J., Besseau, L., Sauzet, S., Boeuf, G., 2007b. Mélatonine et régulations neuroendocrines chez le poisson. J. Soc. Biol. 201, 21–29.

Falcón, J., Collin, J.P., 1985. *In vitro* uptake and metabolism of [^{3}H]indole compounds in the pineal organ of the pike. II. A radioautographic study. J. Pineal Res. 2, 357–373.

Foster, R.G., Bellingham, J., 2004. Inner retinal photoreceptors (IRPs) in mammals and teleost fish. Photochem. Photobiol. Sci. 3, 617–627.

Fujieda, H., Hamadanizadeh, S.A., Wankiewicz, E., Pang, S.F., Brown, G.M., 1999. Expression of MT1 melatonin receptor in rat retina: evidence for multiple cell targets for melatonin. Neuroscience 93, 793–799.

Fujieda, H., Scher, J., Hamadanizadeh, S.A., Wankiewicz, E., Pang, S.F., Brown, G.M., 2000. Dopaminergic and GABAergic amacrine cells are direct targets of melatonin: immunocytochemical study of mt1 melatonin receptor in guinea pig retina. Vis. Neurosci. 17, 63–70.

Gaildrat, P., Becq, F., Falcón, J., 2002. First cloning and functional characterization of a melatonin receptor in fish brain: a novel one? J. Pineal Res. 32, 74–84.

Gaildrat, P., Falcón, J., 2000. Melatonin receptors in the pituitary of a teleost fish: mRNA expression, 2-[^{125}I]iodomelatonin binding and cyclic AMP response. Neuroendocrinology 72, 57–66.

Green, C.B., Besharse, J.C., 2004. Retinal circadian clocks and control of retinal physiology. J. Biol. Rhythms 19, 91–102.

Ha, E., Han, E., Park, H.J., Kim, H.J., Hong, M.S., Hong, S.J., Yoon, K.S., Kang, I., Cho, Y.H., Chung, J.H., Yim, S.V., Baik, H.H., 2006. Microarray analysis of transcription factor gene expression in melatonin-treated human peripheral blood mononuclear cells. J. Pineal Res. 40, 305–311.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Iigo, M., Furukawa, K., Nishi, G., Tabata, M., Aida, K., 2007. Ocular melatonin rhythms in teleost fish. Brain Behav. Evol. 69, 114–121.

Iuvone, P.M., Tosini, G., Pozdeyev, N., Haque, R., Klein, D.C., Chaurasia, S.S., 2005. Circadian clocks, clock networks, arylalkylamine *N*-acetyltransferase, and melatonin in the retina. Prog. Ret. Eye Res. 24, 433–456.

Khan, I.A., Thomas, P., 1996. Melatonin influences gonadotropin II secretion in the Atlantic croaker (*Micropogonias undulatus*). Gen. Comp. Endocrinol. 104, 231–242.

Kokkola, T., Foord, S.M., Watson, M.A., Vakkuri, O., Laitinen, J.T., 2003. Important amino acids for the function of the human MT1 melatonin receptor. Biochem. Pharmacol. 65, 1463–1471.

Kokkola, T., Salo, O.M., Poso, A., Laitinen, J.T., 2005. The functional role of cysteines adjacent to the NRY motif of the human MT1 melatonin receptor. J. Pineal Res. 39, 1–11.

Kulczykowska, E., Kalamarz, H., Warne, J.M., Balment, R.J., 2006. Day-night specific binding of 2-[^{125}I]iodomelatonin and melatonin content in gill, small intestine and kidney of three fish species. J. Comp. Physiol. [B] 176, 277–285.

Lundmark, P.O., Pandi-Perumal, S.R., Srinivasan, V., Cardinali, D.P., 2006. Role of melatonin in the eye and ocular dysfunctions. Vis. Neurosci. 23, 853–862.

Mailliet, F., Ferry, G., Vella, F., Berger, S., Coge, F., Chomarat, P., Mallet, C., Guenin, S.P., Guillaumet, G., Viaud-Massuard, M.C., Yous, S., Delagrange, P., Boutin, J.A., 2005. Characterization of the melatoninergic MT3 binding site on the NRH:quinone oxidoreductase 2 enzyme. Biochem. Pharmacol. 71, 74–88.

Martinoli, M.G., Williams, L.M., Kah, O., Titchener, L.T., Pelletier, G., 1991. Distribution of central melatonin binding sites in the goldfish (*Carassius auratus*). Mol. Cell. Neurosci. 2, 78–85.

Mayer, I., Bornestaf, C., Wetterberg, L., Borg, B., 1997. Melatonin does not prevent long photoperiod stimulation of secondary sexual characters in the male three-spined stickleback *Gasterosteus aculeatus*. Gen. Comp. Endocrinol. 108, 386–394.

Mazurais, D., Brierley, I., Anglade, I., Drew, J., Randall, C., Bromage, N., Michel, D., Kah, O., Williams, L.M., 1999. Central melatonin receptors in the rainbow trout: comparative distribution of ligand binding and gene expression. J. Comp. Neurol. 409, 313–324.

Mitchell, C.K., Redburn, D.A., 1991. Melatonin inhibits ACh release from rabbit retina. Vis. Neurosci. 7, 479–486.

Natesan, A.K., Cassone, V.M., 2002. Melatonin receptor mRNA localization and rhythmicity in the retina of the domestic chick, *Gallus domesticus*. Vis. Neurosci. 19, 265–274.

O'Brien, P.J., Klein, D.C., 1986. Pineal and Retinal Relationships. Academic Press, Orlando, FL.

Page, R.D., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Park, Y.J., Park, J.G., Hiyakawa, N., Lee, Y.D., Kim, S.J., Takemura, A., 2007a. Diurnal and circadian regulation of a melatonin receptor, MT1, in the golden rabbitfish, *Siganus guttatus*. Gen. Comp. Endocrinol. 150, 253–262.

Park, Y.J., Park, J.G., Jeong, H.B., Takeuchi, Y., Kim, S.J., Lee, Y.D., Takemura, A., 2007b. Expression of the melatonin receptor Mel(1c) in neural tissues of the reef fish *Siganus guttatus*. Comp. Biochem. Physiol. A 147, 103–111.

Park, Y.J., Park, J.G., Kim, S.J., Lee, Y.D., Saydur Rahman, M., Takemura, A., 2006. Melatonin receptor of a reef fish with lunar-related rhythmicity: cloning and daily variations. J. Pineal Res. 41, 166–174.

Pautler, E.L., Hall, F.L., 1987. Movement of melatonin across the retinal pigment epithelium. Exp. Eye Res. 45, 351–355.

Peters, J.L., Cassone, V.M., 2005. Melatonin regulates circadian electroretinogram rhythms in a dose- and time-dependent fashion. J. Pineal Res. 38, 209–215.

Pierce, M.E., Besharse, J.C., 1985. Circadian regulation of retinomotor movements. I. Interaction of melatonin and dopamine in the control of cone length. J. Gen. Physiol. 86, 671–689.

Pozo, D., Garcia-Maurino, S., Guerrero, J.M., Calvo, J.R., 2004. mRNA expression of nuclear receptor RZR/RORalpha, melatonin membrane receptor MT, and hydroxindole-*O*-methyltransferase in different populations of human immune cells. J. Pineal Res. 37, 48–54.

Ribelayga, C., Wang, Y., Mangel, S.C., 2004. A circadian clock in the fish retina regulates dopamine release via activation of melatonin receptors. J. Physiol. 554, 467–482.

Savaskan, E., Wirz-Justice, A., Olivieri, G., Pache, M., Krauchi, K., Brydon, L., Jockers, R., Muller-Spahn, F., Meyer, P., 2002. Distribution of melatonin MT1 receptor immunoreactivity in human retina. J. Histochem. Cytochem. 50, 519–526.

Shirakawa, H., Ogino, N., 1987. Novel activity of melatonin. Its chemotactic effect on retinal pigment epithelial cells. Ophthalmic Res. 19, 226–229.

Stella Jr., S.L., Thoreson, W.B., 2000. Differential modulation of rod and cone calcium currents in tiger salamander retina by D2 dopamine receptors and cAMP. Eur. J. Neurosci. 12, 3537–3548.

Voisin, P., Juillard, M.T., Collin, J.P., 1983. Indole metabolism in the pineal organ of the pigeon with special reference to melatonin-synthesizing cells. *In vitro* study combining thin layer chromatography, autoradiography and pharmacological treatment. Cell Tissue Res. 230, 155–169.

Whitmore, D., Cermakian, N., Crosio, C., Foulkes, N.S., Pando, M.P., Travnickova, Z., Sassone-Corsi, P., 2000. A clockwork organ. Biol. Chem. 381, 793–800.

Wiechmann, A.F., Campbell, L.D., Defoe, D.M., 1999. Melatonin receptor RNA expression in *Xenopus* retina. Brain Res. Mol. Brain Res. 63, 297–303.

Witt-Enderby, P.A., Bennett, J., Jarzynka, M.J., Firestine, S., Melan, M.A., 2003. Melatonin receptors and their regulation: biochemical and structural mechanisms. Life Sci. 72, 2183–2198.

Yu, C.J., Gao, Y., Li, P., Li, L., 2007. Synchronizing multiphasic circadian rhythms of rhodopsin promoter expression in rod photoreceptor cells. J. Exp. Biol. 210, 676–684.

Zawilska, J.B., 1992. Melatonin in vertebrate retina: biosynthesis, receptors and functions. Pol. J. Pharmacol. Pharm. 44, 627–654.

Zawilska, J.B., Nowak, J.Z., 1992. Regulatory mechanisms in melatonin biosynthesis in retina. Neurochem. Int. 20, 23–36.