

Central Melatonin Receptors in the Rainbow Trout: Comparative Distribution of Ligand Binding and Gene Expression

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ABSTRACT

To better define the role of melatonin in fish, we have compared in detail the distribution of 2-[¹²⁵I]iodomelatonin binding sites with gene expression for melatonin receptor subtypes in a widely studied seasonal species, the rainbow trout. Three distinct partial sequences of the melatonin receptor gene were cloned from trout genomic DNA. Two of the sequences corresponded to the Mel1a receptor subtype, and one corresponded to the Mel1b receptor subtype. Analysis of numerous clones failed to find a sequence equivalent to the Mel1c receptor subtype. Comparison of receptor gene expression with 2-[¹²⁵I]iodomelatonin binding distribution indicated dendritic transport of the receptor. Melatonin receptors were associated predominantly with visually related areas of the trout brain, such as the thalamic region, the pretectal area, and the optic tectum. The pituitary was devoid of 2-[¹²⁵I]iodomelatonin binding, and melatonin receptor gene expression was not detectable. It would appear from the results of the present study that melatonin in this species is involved primarily in the processing of visual signals. How melatonin interacts with circannual rhythms of growth and reproduction is unclear, although a direct interaction between melatonin and the hypothalamo-pituitary axis is not clearly indicated. *J. Comp. Neurol.* 409:313–324, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: photoperiod; reproduction; vision; radioautography; in situ hybridization; teleost fish

In all vertebrates, it is believed that the pineal hormone melatonin mediates many of the circadian and seasonal rhythmic activities, although the precise mechanisms underlying its effects are still largely unknown. Melatonin is synthesized from tryptophan, which, after 5-hydroxylation and decarboxylation, forms serotonin and, in turn, is converted into melatonin by the sequential activities of N-acetyl-transferase and hydroxyindole-N-acetyltransferase. In mammals, the synthesis of melatonin depends on a noradrenergic stimulus originating from the superior cervical ganglia and is governed in a circadian fashion by the hypothalamic suprachiasmatic nucleus, which is considered to be the center of the biological clock in mammals (Klein et al., 1991). In all vertebrates, melatonin is liberated during the dark phase, and it is now accepted that melatonin acts through specific membrane receptors with pharmacological characteristics that were studied first by using the ligand 2-[¹²⁵I]iodomelatonin, which also allowed the description of the central distribution of melatonin-

binding (Mel-binding) sites by radioautography. Such binding sites were located mainly in the suprachiasmatic nucleus and the pars tuberalis of the pituitary, although there appears to be a high degree of species variation between mammals (Stankov et al., 1993). More recently, the first melatonin receptor (Mel-R) to be cloned was from *Xenopus* dermal melanophores (Ebisawa et al., 1994), leading to the cloning and characterization of melatonin receptors in a number of mammalian species and in birds (for review, see Reppert et al., 1995). According to these

Grant sponsor: European Union; Grant number: Fair PL96.1410; Grant sponsor: CNRS; Grant sponsor: INRA; Grant sponsor: Fondation Langlois. D. Mazurais and I. Brierley contributed equally to this study.

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Received 25 September 1998; Revised 15 January 1999; Accepted 3 February 1999

studies in different vertebrate groups, a family of high-affinity, G protein-coupled receptors for the pineal hormone melatonin has been cloned from vertebrates (Reppert et al., 1995; Reppert, 1997). Two mammalian melatonin receptor subtypes, Mel1a and Mel1b, have been identified by molecular cloning studies. These recombinant receptors exhibit similar affinity and pharmacological characteristics to one another and to endogenous receptors, as defined with the melatonin agonist 2-[¹²⁵I]iodomelatonin (Reppert et al., 1995). The mammalian Mel1a melatonin receptor is expressed in most sites containing melatonin binding. This includes the hypothalamic suprachiasmatic nucleus and hypophyseal pars tuberalis, presumed sites of the circadian actions and some of the reproductive actions of melatonin, respectively. The mammalian Mel1b melatonin receptor is expressed in retina and brain and may mediate the reported effects of melatonin on retinal physiology in some mammals. A third receptor subtype, the Mel1c melatonin receptor, has been cloned from zebrafish, *Xenopus*, and chicken but not from mammals. In zebrafish, five partial sequences have been obtained, two corresponding to the Mel1a subtype, two corresponding to the Mel1b subtype, and one corresponding to the Mel1c subtype (Reppert et al., 1995, 1996). In addition, a melatonin-related receptor has been cloned from human pituitary (Reppert et al., 1996).

In early vertebrates, such as agnatha, fish, and amphibians, the pineal is a photosensitive organ that possesses true photoreceptive pinealocytes, and, in the course of evolution, these pinealocytes progressively have lost their photoreceptive properties, whereas the retinohypothalamic tract progressively has developed to control melatonin production through the suprachiasmatic nucleus (Collin et al., 1989). Reptiles and birds present an intermediate organization in which both the intracranial route and the retinohypothalamic tract are used to mediate the photoperiodic information to the pineal (Collin et al., 1989). Some fish species, such as salmonids, provide a model in which the pattern of melatonin secretion is influenced directly by the day/night cycle, indicating that these species lack an endogenous rhythm of melatonin secretion (Gern and Greenhouse, 1988). On the contrary, in other species, such as the goldfish (Kezuka et al., 1989), the white sucker (Zachmann et al., 1992), or the pike (Falcon et al., 1989), daily variations of melatonin production are entrained by intrapineal circadian oscillators. In fish, pioneer studies had characterized one class of high-affinity Mel-binding site in the central nervous system of the goldfish with a prominent distribution in the pretectal region, the optic tectum, and the cerebellum, although other regions exhibited binding but to a lower extent (Martinoli et al., 1991). These data have been confirmed in other teleost fishes (Molina-Borja et al., 1994; Vernadakis et al., 1998), in particular in the rainbow trout (Davies et al., 1994), the Atlantic salmon (Ekström and Vanecek, 1992), and other salmonids (Pang et al., 1994). In the trout, central Mel-binding sites have been characterized by using 2-[¹²⁵I]iodomelatonin (Davies et al., 1994). A single class of high-affinity receptor has been demonstrated with a dissociation constant in the low picomolar range, in agreement with other studies in goldfish (Martinoli et al., 1991) and Atlantic salmon (Ekström and Vanecek, 1992), and slightly lower than the range reported for high-affinity melatonin receptors in various mammals (for review, see Stankov et al., 1993). The binding was specific, saturable, and strongly

inhibited by GTP γ S, a guanosine triphosphate analogue (Davies et al., 1994).

Due to the widespread occurrence of Mel-binding sites in the brain of teleosts and to the obvious differences between teleosts and mammals in the distribution of these Mel-binding sites, it was interesting to compare the distribution of these binding sites with that of the Mel-R-expressing cells in the rainbow trout, which has provided a model for the study of seasonality in fish, in which the changing photoperiod entrains a circannual cycle of physiological events (Dunston and Bromage, 1988, 1991). In the present study, partial sequences of three melatonin receptor subtypes have been obtained by genomic polymerase chain reaction (PCR), and the distribution of the corresponding transcripts, as studied by *in situ* hybridization, has been compared with that of the 2-[¹²⁵I]iodomelatonin binding sites.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss*) were supplied by the INRA fish farm (Le Drennec, Finistère, France) or the Institute of Aquaculture (University of Stirling, Stirling, Scotland) and were kept in the laboratory in a recirculating water system at 12–15°C under an artificial light regime mimicking the natural photoperiod. The animals were fed a trout diet *ad libitum*. Mature and immature males and females ($n = 16$) were used for both *in situ* hybridization and binding studies. Experiments were conducted in late spring and early summer. All fish were killed during the middle of the light phase. Animals were treated in agreement with the European Union regulations concerning the protection of experimental animals.

PCR amplification

PCR amplification was performed on trout genomic DNA by using degenerate primers designed to span the most highly conserved regions of the melatonin receptor (Reppert et al., 1995). The forward primer (5-TGYCACA-GCCTYAAGTAYGACAAGCT-3) and the reverse primer (5-ATGAAGTTYAAYGGWGCCAGCAMAC-3) allowed amplification of rainbow trout segment 1.4 (RT1.4) and RT1.7. The forward primer (5-TGYCAYAGCWYGCYTACGR-3) and the reverse primer (5-RTTGAGMGGNGCCARCA-3) were used to amplify RT2.6. Primers were synthesized by Eurogentec (Seraing, Belgium). A third pair of degenerate primers was designed to correspond to transmembrane domains 3 and 7. Each reaction cycle (32 loops) consisted of incubations at 94°C for 30 seconds, 59°C for 1 minute, and 72°C for 1 minute, with Ampli Taq DNA polymerase (Perkin-Elmer, Branchburg, NJ). The PCR products were separated by agarose (1.5%) gel electrophoresis and subcloned in pBluescript.

DNA sequencing

DNA sequencing was carried out by using the ABI Prism Dye-Deoxy terminator sequencing kit (Perkin Elmer), and the samples were run and analyzed by using an Applied Biosystems DNA Sequencer (model 373; Applied Biosystems, Foster City, CA). Sequences were aligned and the phylogenetic tree was derived by using Lazergene (DNA Star Inc., Madison, WI).

Riboprobe synthesis

For riboprobe synthesis, the PCR fragments corresponding to RT1.4, RT1.7, and RT2.6 were inserted into pBlue-script (Stratagene, La Jolla, CA). The plasmids were linearized with either *Hind*III or *Eco*RI, to give rise to the antisense and sense probe, respectively. RNA synthesis was carried out by means of an RNA transcription kit (Stratagene). One microgram of the linearized DNA template was incubated for 1 hour at 37°C in a solution containing transcription buffer ($\times 1$), dithiothreitol (DTT; 30 mM), rATP (0.4 mM), rGTP (0.4 mM), rCTP (0.4 mM), α [³⁵S]UTP (5 μ Ci/ μ l), RNase inhibitor (1.6 U/ μ l), and RNA polymerase (0.4 U/ μ l) T7 (sense) or T3 (antisense). The DNA template was digested with RQ-1 DNase (10 U) for 15 minutes at 37°C. After incubation, 10 μ g yeast tRNA (dissolved in 8% formamide) were added to the sample. Fragment separation was achieved on a Sephadex G50 column equilibrated with 50 μ g yeast tRNA. A solution of 10 mM Tris-HCl, pH 7.5; 1 mM ethylenediamine tetraacetic acid (EDTA); 10 mM DTT; and 0.1% sodium dodecyl sulfate (SDS) was used as loading buffer. The two fractions with the highest amount of radioactivity (average probe size, 100 nucleotides) were pooled and, after adding 5 M potassium acetate, 100% ethanol, and 10 μ g yeast tRNA, precipitated during an overnight incubation at -20°C. After a centrifugation at 4°C (12,000 rpm, 20 minutes), the pellet was rinsed with 80% ethanol. Finally, the dried pellet was dissolved in hybridization mix (50% formamide; 0.3 M NaCl; 20 mM Tris-HCl, pH 8.5; 5 mM EDTA; 10% dextran sulfate; 1 \times Denhardt's solution; 0.5 μ g/ μ l yeast tRNA; and 10 mM DTT) at a final concentration of 2×10^4 cpm/ μ l and stored at -70°C until hybridization.

In situ hybridization

Anaesthetized (2-phenoxyethanol; 0.04% volume/volume) female rainbow trout either were perfused intracardially with 0.85% NaCl and 4% paraformaldehyde in phosphate buffer, and their brains were removed and postfixed overnight at 4°C, or brains were removed and frozen directly, and 20- μ m-thick cryostat sections were cut. In situ hybridization was performed either on paraffin sections (6 μ m) of fixed tissue or on frozen sections. The frozen sections allowed a direct comparison to be made between 2-[¹²⁵I]iodomelatonin binding patterns and melatonin receptor gene expression. Antisense and sense riboprobes were generated by in vitro transcription, as described above. Adjacent sections were treated with either sense or antisense probe. Sections were covered with hybridization buffer containing 2×10^4 cpm probe/ μ l, coverslipped, and incubated overnight at 51°C. Coverslips were then removed, and the slides washed in $5 \times$ standard saline citrate (SSC; 1 \times trisodium citrate 15 mM, NaCl 150 mM, pH 7.0), 10 mM DTT for 30 minutes at 55°C, followed by 50% formamide, $2 \times$ SSC, 10 mM DTT for 45 minutes at 65°C, then digested with RNase (20 μ g/ml) in 10 mM Tris-HCl, 0.5 M NaCl, 5 mM EDTA (NTE) for 45 minutes at 37°C. Slides were then washed in NTE for 15 minutes at room temperature and incubated in 50% formamide, $2 \times$ SSC, 10 mM DTT for 45 minutes at 65°C, then washed in $2 \times$ SSC for 15 minutes followed by incubation in 0.1 \times SSC for 15 minutes at room temperature. After dehydration in a graded series of ethanol containing 0.3 M ammonium acetate, slides were apposed to Hyperfilm- β

Max (Amersham International, Uppsala, Sweden) or dipped in Ilford K5 or LM-1 photographic emulsion (Amersham International) and exposed for 1–2 weeks, developed, and counterstained with toluidine blue.

Localization of 2-[¹²⁵I]iodomelatonin binding sites

Rainbow trout were killed by anesthesia (2-phenoxyethanol 0.1% (volume/volume). Brains and pituitaries were collected and immediately frozen in isopentane chilled over dry ice. Tissue was stored at -70°C. Cryostat sections 20 μ m in thickness were cut and thaw mounted onto gelatin-coated slides. To localize and characterize 2-[¹²⁵I]iodomelatonin, binding studies were carried out as described previously (Helliwell and Williams 1992). Briefly, slides were incubated with 100 pM 2-[¹²⁵I]iodomelatonin (NEN Dupont Ltd., Stevenage, Herts, United Kingdom) in 25 mM Tris/HCl buffer containing 4 mM CaCl₂ and for 3 hours at room temperature in the presence and absence of either 10^{-7} M melatonin (Sigma Chemical Company, St. Louis, MO) or 10^{-4} M guanosine 5'-O-(3-thiotriphosphate) (GTPS). Slides were then washed in ice-cold buffer, air dried, and apposed to Kodak X-OMAT AR film (Eastman-Kodak, Rochester, NY) along with [¹²⁵I]polymer standards (Amersham International). Films were exposed for between 2 weeks and 6 weeks. Sections were stained with toluidine blue.

Nomenclature

The nomenclature used for brain nuclei is that used recently for the rainbow trout by Meek and Nieuwenhuys (1997).

Computer-generated figures

Films were scanned on a UMAX Power Look II (UMAX Data Systems, Fremont, CA) by using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA). Montages were made by importing images into Freehand 8.0 (Macromedia, San Francisco, CA). No alterations have been made.

RESULTS

PCR amplification

Three different fragments of rainbow trout melatonin receptors were been obtained by PCR of genomic DNA. After subcloning, sequencing, and alignment with the zebrafish sequences (Reppert et al., 1995), the percentages of identity were found to be 80%, 80%, and 74% with the zebrafish melatonin receptors Z1.7, Z1.4, and Z2.6, respectively. Thus, partial sequences of three different rainbow trout melatonin receptors, RT1.7, RT1.4, and RT2.6, were obtained. The amino-acid sequences of these fragments are provided in Figure 1, which also shows that these fragments correspond to the transmembrane domains IV, V, VI, and part of VII of melatonin receptors from all classes of vertebrates. The percentage of nucleotide identity between these three sequences shows that the RT1.4 and RT1.7 are 84% identical and are closely related to the Z1.4 and Z1.7 (Reppert et al., 1995). This fact, together with our phylogenetic analysis (Fig. 2), shows that these two receptor fragments belong to the Mella subtype, whereas the RT2.6 sequence corresponds to the Mellb receptor subtype.

Transmembrane Domain IV	

Zebra Fish 1.7	C---HSLKYDKLYSDKNSVCYVLLIOWALTVLAIVPNLFVGSQYDPRVYSCTFEQSASS
Rainbow Trout 1.7	CYICHSLKYDKLYSDKNSLCYVLLIOWALTIVAIVPNLFVGSQYDPRVYSCTFEQSASS
Chicken 1a	CYICHSLKYDKLYSDKNSLCYVGLIOWALTIVAIVPNLFVGSQYDPRVYSCTFAQSVSS
Zebra Fish 1.4	C---HSLKYDKLFSNKNTVCYVILVWALTVLAIVPNWFVGSQCDPRVFSCTFAQSVSS
Rainbow Trout 1.4	CYICHNLKYDKLFSNQNTVCYVILVWSLTVLAIVPNWFMESLQYDPRVYSCTFAQSVSS
Hamster 1a	CYICHSLKYDRLYSNKNSLCYVFLIOWLTVAIMPNTQTGTQYDPRVYSCTFTQSVSS
Rat 1a	CYICHSLKYDRIYSNKNSLCYVFLIOWLTVAIMPNTQTGTQYDPRVYSCTFTQSVSS
Human 1a	CYICHSLKYDKLYSSKNSLCYVLLIOWLTVAIVPNLFVGSQYDPRVYSCTFAQSVSS
Sheep 1a	CCICHSLRYGKLYSGTNSLCYVFLIOWLTVAIVPNLCVGTQYDPRVYSCTFTQSVSS
Xenopus 1c	CYICHSLRYDKLYNQRTWCYLGTLWLTIIAIVPNFVGSQYDPRVYSCTFAQTVSS
Chicken 1c	CYICHSLRYDKLFNLKNTCCYICLTWLTVAIVPNFVGSQYDPRVYSCTFAQTVST
Zebra Fish 2.3	C---HSLRYDRLYSRNTCLYLLLTWMLTATATVPNLFVGSQYDPRVYSCTFTQTASS
Zebra Fish 2.6	C---HSFAYGRLCSEFRNTLLVALIOWALTVAAILPNFVGSLSYDPRVYSCTFTQTASS
Rainbow Trout 2.6	CYICHNLKYDKLFSNQNTVCYVILVWSLTVLAIVPNWFMESLQYDPRVYSCTFAQSVSS
Xenopus 1b	---HSFVYEKLFSLWNTILYVCLIWLTVAIVPNFVGSLEYDPRVYSCTFVQTASS
Chicken 1b	CYICHSFAYDKVYSWNTMLYVSLIWLTVAIVPNFVGSQYDPRVYSCTFVQTASS
Rat 1b	WCICHSATYHRACSQWHAPLYISLIWLTVAIVPNFVGSLEYDPRVYSCTFIQTAST
Transmembrane Domain V	

Zebra Fish 1.7	AYTIAVVFHFHILPIMIVTYCYLRIWLVLIQVRRRVKNDNRPKITPHDVRNFTMFVVF
Rainbow Trout 1.7	GYTIAVVFHFHILPIMIVTYCYLRIWLVLIQVRRRVKPDNRPKLTPHDERNLPTMFVVF
Chicken 1a	AYTIAVVFHFHILPIAIVTYCYLRIWLVLIQVRRRVKPDNNRPLKPHDFRNFTMFVVF
Zebra Fish 1.4	LYTIMVVVHFIVPIGIVTYCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Rainbow Trout 1.4	LYTITVVVHFIVPIGIVTYCYLRIWLVLIQVRRRVKPDTRPKIKPHDFHIFLTMFVVF
Hamster 1a	AYTIAVVFHFIVPMIIVFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Rat 1a	AYTIALVVFHFIVPMIIVFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Human 1a	AYTIAVVFHFIVPMIIVFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Sheep 1a	AYTIAVVFHFIVPMIIVFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Xenopus 1c	SYTITVVVHFIVPLSVTFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Chicken 1c	SYTITVVVHFIVPLSVTFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Zebra Fish 2.3	SYTVCVLIHFLVPLGVVFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Zebra Fish 2.6	SYTIVVVVHFIVPLSVTFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Rainbow Trout 2.6	SYTITVVVHFIVPLSVTFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Xenopus 1b	SYTITVVVHFIVPLSVTFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Chicken 1b	YYTIAVVFHFIVPIVTFVFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
Rat 1b	QYTMAVVAIHFLPLIAVVSFCYLRIWLVLIQVRRRVKPDNRPKIKPHDFRNFTMFVVF
-membrane Domain VI Transmembrane Domain VII	

Zebra Fish 1.7	V-LFAVCWAPLNFIGLAVAIHP-RLGQSIPEWLFVASYF
Rainbow Trout 1.7	LMLFTVCWAPLNFIGLAVAINPEVVPPIPEWFFVARYF
Chicken 1a	V-LFAVCWAPLNFIGLAVAVDPETIIIPRIPEWLFVSSYY
Zebra Fish 1.4	V-LFAVCWAPLNFIGLAVAIHP-RLGQSIPEWLFVASYF
Rainbow Trout 1.4	V-LFAVCWAPLNFIGLAVAINP-RLGVNIPEWLFVASYF
Hamster 1a	V-LFAICWAPLNFIGLIVASDPATMAPRIPEWLFVASYF
Rat 1a	V-LFALCWAPLNFIGLIVASDPATMAPRIPEWLFVASYF
Human 1a	V-LFAICWAPLNFIGLAVASDPASMPRIPEWLFVASYF
Sheep 1a	V-LFAICWAPLNFIGLVASDPASMAPRIPEWLFVASYF
Xenopus 1c	V-LFAVCWAPLNFIGLAVAINPFHVAPKIPEWLFVLSYF
Chicken 1c	V-LFAVCWAPLNFIGLAVAINPSPKQPHIPEWLFVLSYF
Zebra Fish 2.3	V-LFAVCWAPLNFIGLAVAINPAKVAPNIPEWLFVTSYF
Zebra Fish 2.6	V-LFAICWAPLNFIGLAVAINPEVMAVPRVPEWLFVVSYY
Rainbow Trout 2.6	V-LFAICWAPLNFIGLAVAIIDPETVAPRIPEWLFVVSYY
Xenopus 1b	V-IFAFWCWAPLNFIGLAVAINPTEVAPKIPEWLFVVSYY
Chicken 1b	V-IFAFWCWAPLNFIGLAVAINPSEMAPKVPEWLFVVSYY
Rat 1b	V-VFAICWAPLNCIGLAVAINPEAMALQIPEGLEVTSYF

Fig. 1. Deduced amino acid sequences of the three novel melatonin receptor fragments (1.7, 1.4, and 2.6) cloned from rainbow trout genomic DNA aligned with other known melatonin receptors (GenBank accession numbers: zebrafish 1.7, U31822; zebrafish 1.4, U31823;

chicken 1a, U31820; sheep 1a, U14109; hamster 1a, U14119; rat 1a, U14409; human 1a, U14108; *Xenopus* 1b, U31826; zebrafish 2.6, U31824; chicken 1b, U31821; rat 1b, U28218; *Xenopus* 1c, U05961; zebrafish 2.3, U31825; chicken 1c, U31821).

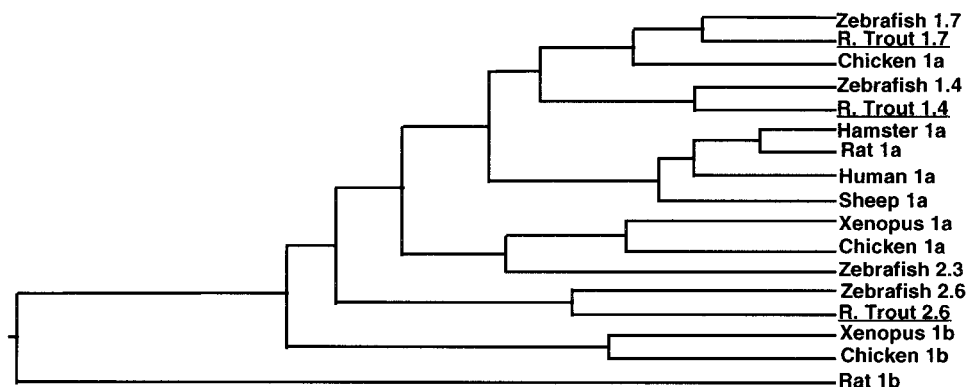


Fig. 2. Phylogenetic analysis of vertebrate melatonin receptors. Rainbow trout segment 1.4 (RT1.4) and RT1.7 correspond to the Mel1a receptor subtype, whereas RT2.6 corresponds to the Mel1b subtype.

2-[¹²⁵I]iodomelatonin binding

The characterization of 2-[¹²⁵I]iodomelatonin binding in the rainbow trout brain has been reported in detail previously (Davies et al., 1994) and corresponds to a single class of high-affinity receptor. All 2-[¹²⁵I]iodomelatonin binding was displaced by an excess (1 μ M) of melatonin and greatly reduced by addition of GTP γ S. The distribution of specific 2-[¹²⁵I]iodomelatonin binding was analyzed in detail in serial sections from three fish (Fig. 3, Table 1). Specific binding was observed consistently in the ventral telencephalon (Fig. 3a), optic tracts (Fig. 3c), pretectal region (Fig. 3e), particularly the posterior pretectal nucleus, hypothalamus (Fig. 3g), optic tectum (Fig. 3c–g), torus semicircularis (Fig. 3g), molecular layer of the cerebellum (Fig. 3i), and medulla oblongata (Fig. 3k). In the preoptic region, no binding was detectable in the nucleus suprachiasmaticus, the nucleus preopticus pars parvocellularis and magnocellularis, or the nucleus parvocellularis pars posterior. Specific binding seemed to be associated with the dorsal optic tracts leading to the optic tectum, pretectal, and thalamic regions (Fig. 3c). In the pretectal region, it was clear that the posterior pretectal nucleus was labeled strongly. It was impossible to ascertain whether or not the closely surrounding nuclei also were positive due to the resolution obtained. In the optic tectum, all layers (periventricular gray, deep white zone, and central white and gray superficial zones) were labeled. In the cerebellum, the binding was restricted to the molecular layer. In the torus semicircularis, 2-[¹²⁵I]iodomelatonin binding appeared to be restricted to the ventral nucleus. No or very low levels of 2-[¹²⁵I]iodomelatonin binding could be detected in the pituitary gland.

In situ hybridization

Riboprobes to all three receptor subtypes were used for in situ hybridization in the brain and pituitary of trout. The results summarized in Table 1 show that the corresponding mRNAs have a widespread distribution but very low expression within the brain. The pattern of expression was similar with the three probes, and that between RT1.4 and RT1.7 was identical, as predicted (Fig. 4). However, in one animal in particular, comparison of adjacent sections clearly showed that, whereas both the RT1.4 and RT2.6 probes labeled the periventricular gray zone of the tectum, the posterior pretectal nucleus was labeled only by the

RT1.4 and RT1.7 probes (Fig. 5). With all probes, the highest signal was detected consistently in the stratum periventriculare of the optic tectum (Fig. 5) and, with the exception mentioned above, in the posterior pretectal nucleus (Fig. 5). With the three probes, a specific hybridization signal was observed consistently at the border between the molecular and the granular layers of both corpus and valvula of the cerebelli (Fig. 6). At the light microscopic level, the hybridization signal could be localized precisely (Figs. 7, 8). In the thalamic region, labeling appeared to be restricted to the dorsomedial nucleus (Fig. 7a–c), whereas, in the optic tectum, the hybridization signal clearly overlapped that of the densely-packed cells of the stratum periventriculare (Fig. 7d–f). The higher resolution obtained with in situ hybridization demonstrated that, in the pretectal region, only the posterior pretectal nucleus exhibited an intense hybridization signal with the RT1.4 and RT1.7 probes (Fig. 8a). The other pretectal nuclei, such as the superficial pretectal nucleus pars parvocellularis and magnocellularis or the central pretectal nucleus, were not labeled (Fig. 8a). In the hypothalamus, the nucleus anterior tuberis, the nucleus lateralis tuberis, and the nucleus recessus lateralis exhibited clear but comparatively weaker labeling than the stratum periventriculare of the optic tectum or the posterior pretectal nucleus (Table 1). In the cerebellum, large cells located at the border between the molecular layer and the granular layer showed gene expression in both the valvula (Fig. 7g–i) and the corpus cerebelli (Fig. 7b,c). The granular layer of the cerebellum exhibited a weaker hybridization signal (Fig. 6). Scattered, weakly labeled cells were detected consistently in the dorsal torus semicircularis and in the nucleus diffusus lobi inferioris. The nucleus anterioris periventricularis also exhibited a weak signal. Although the suprachiasmatic nucleus and the preoptic nucleus pars parvocellularis and magnocellularis were consistently negative, the nucleus periventricularis pars posterior exhibited a significant hybridization signal (Fig. 8a). No significant hybridization signal was detectable in the pituitary gland.

DISCUSSION

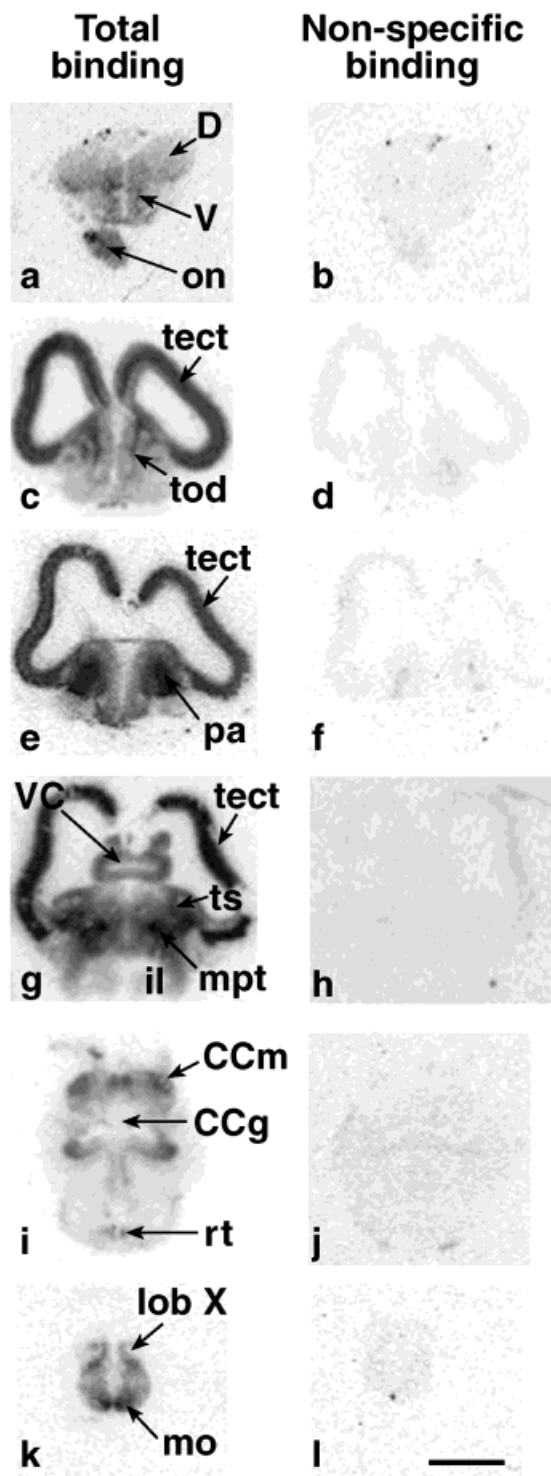
In the present study, we obtained three partial cDNA sequences corresponding to three different melatonin recep-

tor subtypes of the rainbow trout, and we studied their expression sites in the central nervous system compared with the distribution of 2-[¹²⁵I]-iodomelatonin-binding sites. Based on the zebrafish partial sequences obtained by Reppert et al. (1995), we designed degenerate primers to obtain homologous sequences from genomic DNA. Subcloning and sequencing of the PCR products allowed us to obtain three partial sequences, which, once they are

TABLE 1. Summary of the Distribution of Ligand Binding and Gene Expression for Melatonin Receptor in Trout Brain¹

Structure	2-(¹²⁵ I)melatonin	Mel1a	Mel1b
Telencephalon			
Olfactory bulb	nd	nd	nd
Dorsal telencephalon	nd	nd	nd
Ventral telencephalon	+	nd	nd
Preoptic area			
N. parvocellularis pars anterior	nd	++	+
N. preopticus magnocellularis	nd	nd	nd
Chiasma opticum	nd	nd	nd
Optic nerve	+	nd	nd
Tractus opticus dorsalis	++	nd	nd
N. parvocellularis pars posterior	nd	+	+
N. suprachiasmaticus	nd	nd	nd
Thalamus and pretectal region			
Central pretectal nucleus	?	nd	nd
Thalamus dorsalis	+	++	++
Thalamus ventralis	+	+	+
N. pretectalis posterior	+++	+++	+
N. pretectalis superficialis magnocellularis	?	nd	nd
N. pretectalis superficialis parvocellularis	?	nd	nd
Hypothalamus			
N. anterior tuberis	+	+	+
N. recessus lateralis	+	+	+
N. lateralis tuberis	+	+	+
N. diffusus lobi inferioris hypothalami	++	+	+
Pituitary	nd	nd	nd
Posterior tubercular region			
Preglomerular complex	++	+	nd
Migrated posterior tubercular nuclei	++	+	nd
Tegmentum mesencephali			
N. fasciculi longitudinalis medialis	nd	nd	nd
N. oculomotorius	nd	nd	nd
Torus semicircularis	+++	+	+
N. interpeduncularis	nd	nd	nd
Tectum mesencephali			
Stratum periventriculare	++	+++	+++
Stratum album centrale	+++	nd	nd
Stratum griseum centrale	+++	nd	nd
Stratum griseum et album superficiale	+++	nd	nd
Stratum marginale	++	nd	nd
Torus longitudinalis	nd	+	+
Valvula cerebelli			
Granular layer	nd	+	+
Molecular layer	++	nd	nd
Ganglionic layer	?	++	+++
Corpus cerebelli			
Granular layer	nd	+	+
Molecular layer	++	nd	nd
Ganglionic layer	?	++	+++
Crista cerebellaris	nd	nd	nd
Vagal lobe	+	nd	nd
Medulla oblongata	++	nd	nd

¹Nomenclature is from Meek and Nieuwenhuys (1997). Specific binding and hybridization signal levels are +++, high; ++, medium; +, low; nd, not detectable. Mel1a/Mel1b, melatonin receptor subtypes.



aligned with the zebrafish sequences, are likely to correspond to the zebrafish Z1.4, Z1.7, and Z2.6 of Reppert et al. Therefore, according to our phylogenetic analysis and that of Reppert et al., it is believed that two of the sequences (RT1.4 and RT1.7) belong to the Mel1a receptor subtype, whereas the third sequence (RT2.6) corresponds

Fig. 3. Binding of 2-[¹²⁵I]iodomelatonin in the trout brain. X-ray film image of 2-[¹²⁵I]iodomelatonin binding to coronal sections of the brain of the rainbow trout at the levels of the anterior preoptic region (a,b), caudal preoptic region (c,d), pretectal area (e,f), midbrain tegmentum (g,h), cerebellum (i,j), and vagal lobe (k,l) showing total 2-[¹²⁵I]iodomelatonin binding (a,c,e,g,i,k) and nonspecific binding in the presence of 1 μ M melatonin (b,d,f,h,j,l). A high density of specific binding is seen in the tractus opticus dorsalis (tod), the pretectal area (pa), the migrated posterior tubercular nucleus (mpt), and the molecular layer of the cerebellum (CCm). CCg, granular layer of the cerebellum; D, area dorsalis telencephali; il, inferior lobe; lobX, vagal lobe; mo, medulla oblongata; on, optic nerve; rt, rhombencephalic tegmentum; tect, tectum mesencephali; tod, tractus opticus dorsalis; ts, torus semicircularis; V, area ventralis telencephali; VC, valvula cerebelli. Magnification $\times 4$. Scale bar = 2.5 mm.

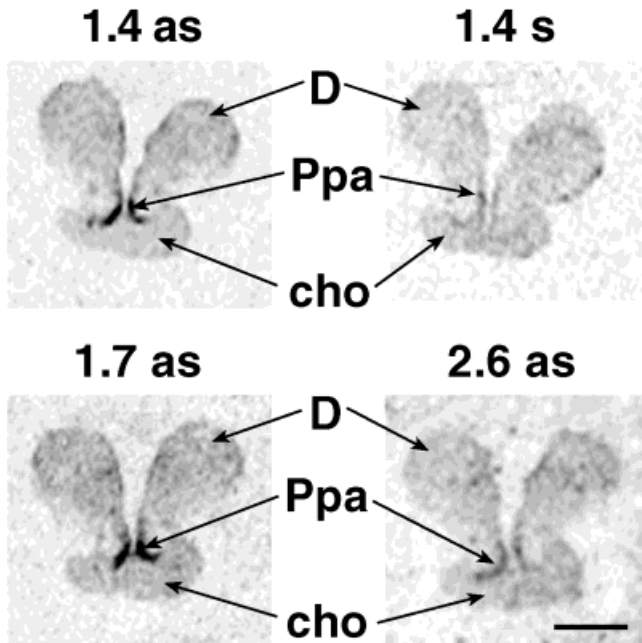


Fig. 4. Melatonin receptor in situ hybridization in the trout brain. X-ray film image of in situ hybridization in the preoptic region using the RT1.4, RT1.7, and RT2.6 antisense (as) probes and the RT1.4 sense (s) probe. A specific signal is detectable only at the level of the nucleus parvocellularis pars anterior (Ppa), whereas the dorsal telencephalon (D) and the optic chiasma (cho) are negative. Magnification $\times 4$. Scale bar = 2.5 mm.

to a Mel1b subtype. To date, the Mel1a receptor is the only subtype found in all groups of vertebrate examined, and it is expressed in the suprachiasmatic nucleus (SCN) in birds and in many, but not all, mammalian species (Reppert et al., 1994, 1995). It is believed that the Mel1a receptor mediates the reproductive and circadian responses to melatonin in mammals. This is reinforced by the fact that, in the Siberian hamster, the Mel1b gene cannot encode a functional receptor, but this species still is capable of showing seasonal and circadian responses to melatonin (Weaver et al., 1996). Different attempts to obtain a Mel1c sequence similar to that of the zebrafish Z2.3 of Reppert et al. (1995, 1996) have failed until now.

The distribution of the mRNA corresponding to the two RT Mel1a subtypes and that of the RT Mel1b subtype was studied by radioactive in situ hybridization, and the results were compared with the distribution of 2-[125 I]iodomelatonin-binding sites in the brain of the rainbow trout. The results confirm the widespread distribution of these binding sites in the brain of a teleost fish and provide for the first time detailed information on the localization of melatonin receptor-expressing sites in a cold-blooded vertebrate. Whereas in situ hybridization provides detailed information on the cells expressing melatonin receptors, binding allows localization of the functional protein; therefore, the two techniques are complementary. Our results show that melatonin receptors are associated tightly with the visual systems: both melatonin binding sites and melatonin receptor-expressing cells were found in majority in the thalamus, the pretectal area, the optic tectum, and the cerebellum. Both Mel-binding sites and Mel-R-expressing cells also were detected to a lesser extent in other brain regions, particularly in the hypothalamus.

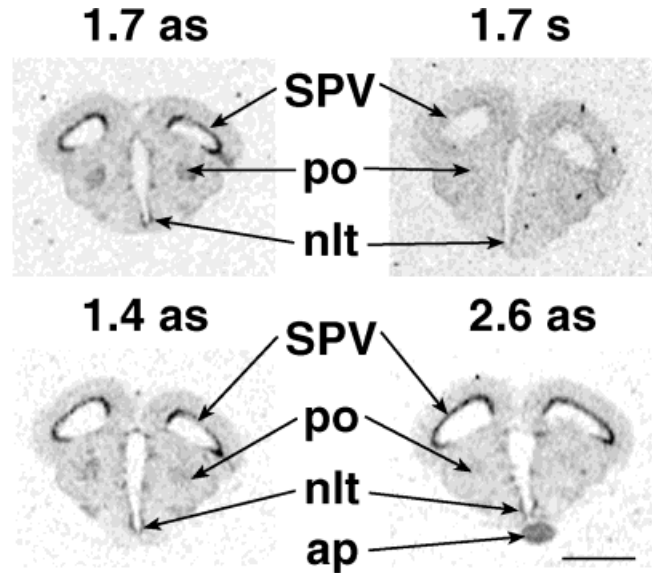


Fig. 5. Melatonin receptor in situ hybridization in the trout brain. X-ray film image of in situ hybridization in the pretectal region using RT1.4, RT1.7, and RT2.6 antisense (as) probes and the RT1.7 sense (s) probe. A specific signal is detectable in the stratum periventriculare of the optic tectum (SPV), the nucleus pretectalis posterior (po), and the nucleus lateralis tuberis (nlt). In the nucleus pretectalis posterior, the signal with the RT2.6 probe appears weaker than that with the RT1.4 or RT1.7 probes. The signal in the optic tectum appears the same for all three probes. ap, Anterior pituitary. Magnification $\times 4$. Scale bar = 2.5 mm.

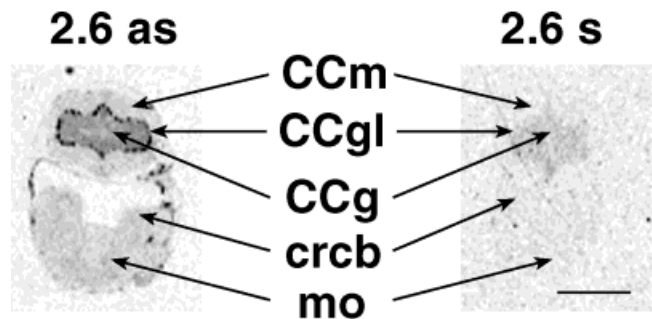


Fig. 6. Melatonin receptor in situ hybridization in the trout brain. X-ray film image of in situ hybridization in the cerebellum using the RT2.6 antisense (as) and sense (s) probes. A strong specific signal is detectable in the ganglionic layer (CCgl), and a less intense signal is observed in the granular layer (CCg). There is no detectable signal over the molecular layer (CCm), the crista cerebellaris (crcb), or the medulla oblongata (mo). Magnification $\times 4$. Scale bar = 2.5 mm.

At present, it is difficult to determine whether our three probes hybridize with the same receptor or to different receptor subtypes. Because of the similarity between the two Mel1a sequences at the nucleotide level, it is very likely that, even under high-stringency washing conditions, the two probes will hybridize with the same mRNAs. Sequence identity is lower (70% at the nucleotide level) between Mel1a and Mel1b, and it is possible that the probes hybridize with different receptor subtypes under our high-stringency conditions. This assumption is reinforced by the fact that, in one animal, the Mel1b probe failed to show any signal over the posterior pretectal

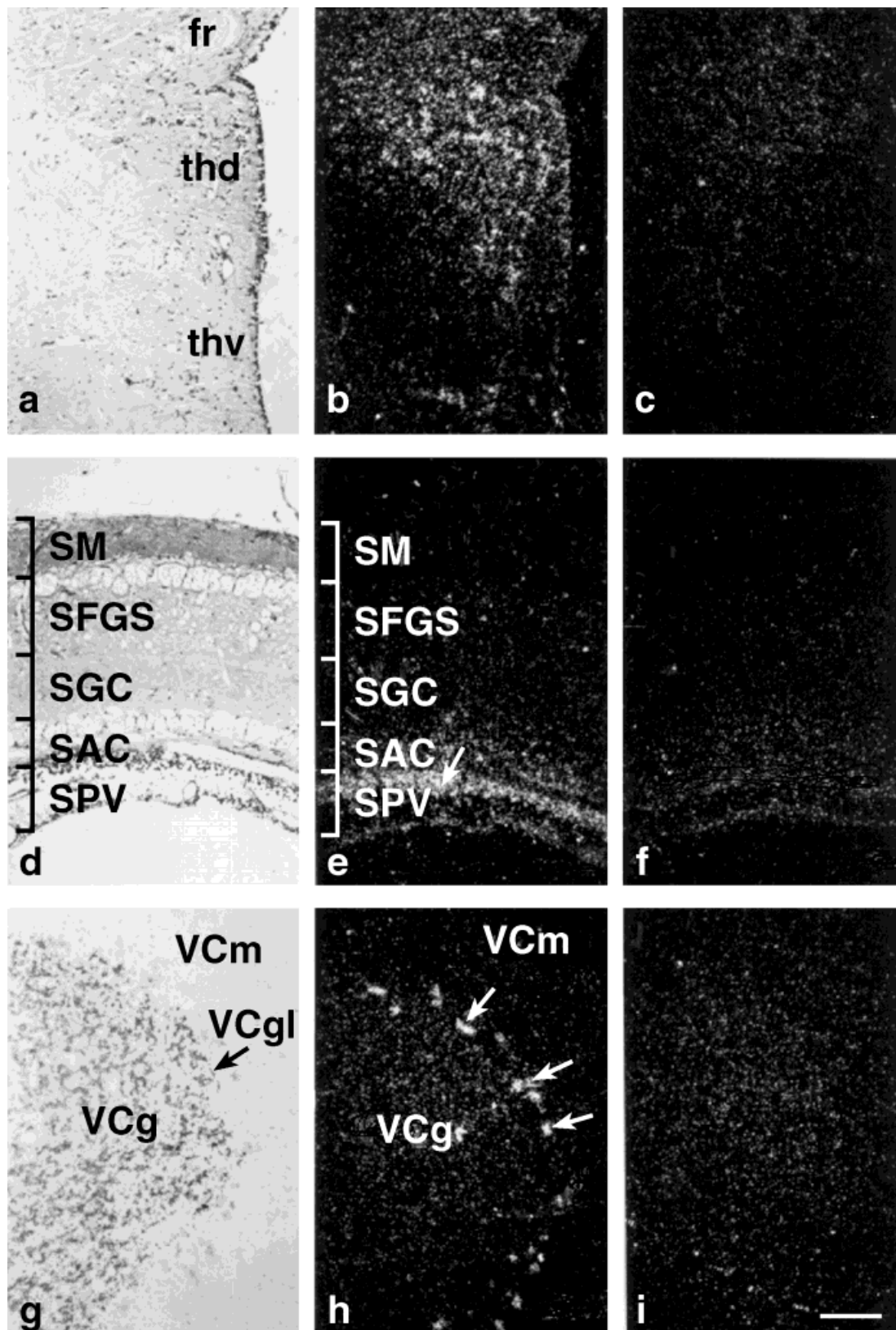


Fig. 7. **a-i:** Melatonin receptor in situ hybridization in the trout brain. Light microscopy of in situ hybridization with the RT1.7 probe on coronal sections of rainbow trout brain. Brightfield (a,d,g) and darkfield (b,e,h) illumination showing the specific signal with the antisense probe in the dorsal portion of the thalamus (thd; a,b), the stratum periventriculare of the optic tectum (SPV; arrow; d,e), and the ganglionic layer of the valvula cerebelli (VCgl; arrows in g,h). Control

serial sections were incubated with the sense probe (c,f,i). fr, Fasciculus retroflexus; SAC, stratum album centrale; SFGS, stratum album and griseum superficiale; SGC, stratum griseum centrale; SM, stratum marginale; thv, ventral thalamus; VCg, granular layer of the valvula cerebelli; VCm, molecular layer of the valvula cerebelli. Magnification $\times 50$. Scale bar = 200 μm .

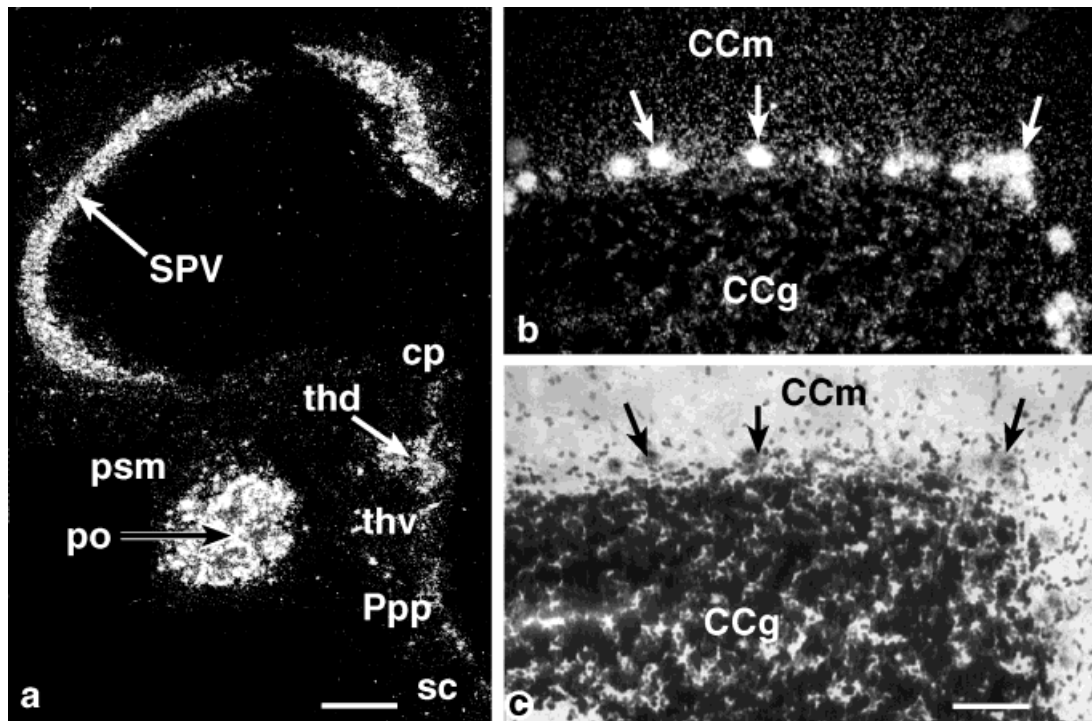


Fig. 8. Melatonin receptor in situ hybridization in the trout brain. Light microscopy of in situ hybridization with the RT1.7 (a) and RT2.6 antisense probes (b,c) on coronal sections of rainbow trout brain. a: A strong hybridization signal is observed in the nucleus pretectalis posterior (po), the stratum periventriculare of the optic tectum (SPV), and the dorsal thalamus (thd). There is no detectable signal in the suprachiasmatic nucleus (sc). Magnification $\times 20$; b,c: Brightfield (b) and darkfield (c) illumination of RT2.6 probe hybridization to large

cells (arrows) in the ganglionic layer of the corpus cerebelli at the border between the molecular layer (CCm) and the granular layer (CCg). The less intense hybridization signal over the granular layer is masked by the counterstaining. Magnification $\times 320$. cp, Commissura posterior; psm, nucleus superficialis parvocellularis; Ppp, nucleus parvocellularis pars posterior; thv, ventral thalamus. Scale bars = 500 μm in a; 30 μm in c (also applies to b).

nucleus, whereas the Mel1a probe on adjacent sections did exhibit a strong hybridization signal. In the other animals, it appeared that the signal obtained with the Mel1a probe in this nucleus was consistently higher than that detected with the Mel1b probe. In the chick, two Mel-R types corresponding to the Mel1a and Mel1c subtypes exhibited similar binding and functional characteristics when expressed in vitro and displayed divergent patterns of expression in the brain, indicating that differential expression may account for specific functions of these two subtypes (Reppert et al., 1995). At this stage, it is difficult to reach a conclusion about the differential expression of the Mel1a and Mel1b subtypes in rainbow trout until more specific probes can be designed; therefore, we will refer to Mel-R-expressing cells in the rest of the text.

The present study confirms and gives a more precise localization of 2-[^{125}I]iodomelatonin binding in salmonids (Ekström and Vanecek, 1992; Pang et al., 1994; Davies et al., 1994). The highest concentrations of 2-[^{125}I]iodomelatonin binding sites and melatonin receptor-expressing cells were associated with visually-related structures, i.e., the pretectal region, the thalamus, and the optic tectum. In rainbow trout, the primary visual structures have been studied in details by using Fink-Heimer degeneration or radioautographic methods, and the results have shown retinal terminal fields in the SCN, the pretectal region, the thalamus, and the optic tectum (Pinganaud and Clairambault, 1979). In mammals, the SCN is a key element of the

retinopineal pathway, regulating circadian release of melatonin, and is considered to be the biological clock (Klein et al., 1991). Melatonin receptors are present in the SCN of many, but not all, mammalian species (Helliwell and Williams, 1992), indicating a feed-back action of melatonin on the brain structure controlling its own secretion. The lack of melatonin receptors in the SCN of the rainbow trout may reflect the lack of an endogenous rhythm in the secretion of melatonin in this species, which would be driven by the SCN (Randall et al., 1991). In the pretectal region, both binding and in situ hybridization studies resulted in a clear signal in the nucleus rotundus of Billard and Peter (1982), actually corresponding to the posterior pretectal nucleus (Butler et al., 1991; Meek and Nieuwenhuys, 1997). A considerable degree of diversity exists in teleosts with respect to the organization of the pretectal nuclei, and this aspect has been dealt with in some studies, which distinguished plesiomorphic characteristics from apomorphic characteristics (Northcutt and Wulliman, 1988; Butler et al., 1991; Butler, 1992). According to these studies, three different patterns (elaborate, intermediate, and simple) of pretectal organization exist in teleosts. In the elaborate pattern, which is found only in the highly evolved percomorphs, visual information from the retina travels to the inferior lobe through different pretectal nuclei, including a well-differentiated nucleus glomerulosus (Wullimann and Meyer, 1990). The simple pattern, which is found only in cyprinids, consists of fewer and less

conspicuously organized pretectal nuclei. The intermediate pattern is found in most other teleostean groups, including salmonids (Wullimann and Meyer, 1990). In this case, it appears that retinofugal fibers contribute to the innervation of the nucleus pretectalis superficialis pars parvicellularis (nucleus geniculatus of Pinganaud and Clairambault, 1979), which does not exhibit Mel-R-expressing cells but projects, in turn, to the posterior pretectal nucleus, which exhibits a high concentration of Mel-R-expressing cells (Wullimann and Meyer, 1990; Butler, 1991). This latter nucleus is believed to be plesiomorphic in teleosts and is present in fish lacking a nucleus glomerulosus (Wullimann and Meyer, 1990; Butler et al., 1991). Although this has not been demonstrated in salmonids, 1,1'-diocetadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) studies in the nonpercomorph *Osteoglossum bicirrhosum* have shown that the posterior pretectal nucleus projects to the inferior lobe of the hypothalamus (Wullimann et al., 1991). Such projections could explain the high density of Mel-binding sites in the dorsal component of the nucleus diffusos lobi inferioris observed in the trout (Davies et al., 1994; current study) and the Atlantic salmon (Ekström and Vanecek, 1992).

In all fish studied until now, another brain region that exhibits a high density of Mel-binding sites is the optic tectum (Martinoli et al., 1991; Ekström and Vanecek, 1992; Davies et al., 1994; Iigo et al., 1994, 1997; Pang et al., 1994). It is noteworthy that, in the deep-sea fish *Coryphaenoides armatus*, living at abyssal depths in the absence of solar light, the optic tectum totally lacks Mel-binding sites, although this fish has well-developed eyes (Smith et al., 1996). The present work indicates that the optic tectum of the rainbow trout also contains a high density of Mel-R-expressing sites; however, there appears to be a differential distribution of the Mel-binding sites and that of Mel-R-expressing cell. The optic tectum of teleosts is a laminated structure consisting of five main layers (Meek, 1983; Northcutt, 1983; Butler, 1992). Of particular interest is our observation that, whereas the Mel-binding sites are observed throughout all layers of the optic tectum, Mel-R-expressing cells were detected only over the periventricular gray zone. The Mel-R-expressing neurons in the periventricular gray zone appear to correspond to densely packed, small piriform neurons that are known to send apical dendrites throughout the stratum album centrale, the stratum griseum centrale, and the stratum griseum et album superficiale, as shown in different teleost species, such as the goldfish or the trout (Pinganaud and Clairambault, 1979; Northcutt, 1983). These dendrites are likely to be the location of the melatonin receptor protein, explaining the high density of 2-[¹²⁵I]iodomelatonin binding sites over the entire ventrodorsal extent of the tectum. Many of these small piriform neurons possess axons branching extensively in the deep white zone and the ventral half of the superficial white and gray zone; therefore, they are likely to be intrinsic to the tectum (Northcutt, 1983). However, the possibility exists that these axons also form part of the efferent pathways. In trout, the main terminal field of retinal fibers within the tectum is the superficial white and gray zone, although the deep white zone also receives retinal input (Pinganaud and Clairambault, 1979). The nature of the cells expressing melatonin receptor genes is unknown; however, in other fish species, such as *Phoxinus phoxinus* (Ekström, 1987), goldfish (Zottoli et al., 1988), and *Porichthys notatus* (Brantley and Bass, 1988),

there is evidence that small numbers of them (less than 10%) are positive for choline acetyltransferase. In European eel, goldfish, and rainbow trout, there also is evidence that a large majority of these piriform neurons in the periventricular gray zone are γ -aminobutyric acidergic (GABAergic) neurons (Martinoli et al., 1990; Médina et al., 1994; I. Anglade, D. Mazurais, and O. Kah, unpublished results). Thus, by implication, GABA neurons of the periventricular gray zone are likely to express melatonin receptors.

High concentration of 2-[¹²⁵I]iodomelatonin binding sites have been reported in both the corpus and the valvula of the cerebellum of fish (Martinoli et al., 1991; Ekström and Vanecek, 1992; Davies et al., 1994; Iigo et al., 1994, 1997; Pang et al., 1994). The valvula of the cerebellum, which is found only in ray-finned fish, is a subdivision of the cerebellum forming a rostral protrusion extending into the mesencephalic ventricle below the optic tectum (Finger, 1983). In teleosts, 2-[¹²⁵I]iodomelatonin binding sites were reported to be located over the molecular layer of the cerebellum (Martinoli et al., 1991; Ekström and Vanecek, 1992; Davies et al., 1994), which is confirmed by the present study. Mel-binding sites also have been reported in the molecular layer of the cerebellum in sheep (Helliwell and Williams, 1992), and Mel1a expression has been found in granule cells in humans (Mazzuchelli et al., 1996). It is noteworthy that large, Mel-R-expressing cells were located in the ganglionic layer at the border between the molecular layer and the granular cell layer, which also contained weak-to-moderate Mel-R-expressing cells. In teleosts, the ganglionic layer contains the Purkinje cells and the cell bodies of cerebellar output, the eurydendroid neurons. The large cells likely correspond to Purkinje cells (Finger, 1983; Meek and Nieuwenhuys, 1997), which send a single apical dendrite branching extensively toward the molecular layer. Therefore, it is possible that the functional protein is located over these dendrites, and this could explain the high density of 2-[¹²⁵I]iodomelatonin binding sites observed in the molecular layer. In teleosts, the chemical nature of the Purkinje cells has been documented only in the European eel by using direct GABA immunohistochemistry (Médina et al., 1994). However, in situ hybridization using a glutamate decarboxylase 65 probe obtained from trout cerebellum has shown that the Purkinje cells of rainbow trout strongly express glutamate decarboxylase (I. Anglade, D. Mazurais, and O. Kah, unpublished results). It is interesting to note that, although it is likely that the cerebellum receives input from virtually every sensory modality (Meek and Nieuwenhuys, 1997), it often has been shown to participate in the processing of visual information in teleosts. In the goldfish, visual stimuli elicit electrophysiological responses in the Purkinje cells of the valvula and the anteromedial part of the corpus cerebelli (Kotchabhakdi, 1976), and, in the trout, Purkinje cell responses were recorded after application of simple visual stimuli, such as flashes or lights off/on (Reid and Westerman, 1975). Recent data in goldfish also suggest that Purkinje cells are involved in the integration of corollary head and eye velocity signals (Pastor et al., 1997).

A high concentration of Mel-binding sites was detected in the torus semicircularis, confirming previous investigations in goldfish (Martinoli et al., 1991), Atlantic salmon (Ekström and Vanecek, 1992), and rainbow trout (Davies et al., 1994). The present work also demonstrates a moderate expression of melatonin receptors in the torus

semicircularis, which is thought to be homologous to the inferior colliculus of mammals (Etcheler, 1984; Wubbels and Schellart, 1997). This structure has been implicated in the processing of auditory stimuli in a number of fish (Etcheler, 1984; Lu and Fay, 1993; Wubbels and Schellart, 1997) but also is implicated in color vision (Schellart, 1983), prey detection, and orientation (Schellart et al., 1987; Coughlin and Hawryshyn, 1994) in rainbow trout.

At the central level, melatonin is believed to mediate the central effects of photoperiod to the neuroendocrine circuits controlling pituitary functions; and, in mammals, there is considerable evidence for pineal involvement in the timing of the preovulatory luteinizing hormone surge (Malpoux et al., 1998). Pinealectomy results in random ovulation in goldfish (Kozuka et al., 1989) and delayed spawning in rainbow trout (Bromage et al., 1995), whereas slow-release melatonin implants cause asynchronous spawning in trout (Bromage et al., 1995). In the Atlantic croaker, melatonin has been shown to influence gonadotropin II (GTH2) secretion directly at the pituitary level and indirectly at the brain level (Khan and Thomas, 1996). In salmonids, ovulation is triggered by a preovulatory surge of GTH2 caused by both gonadotropin-releasing hormone (GnRH) stimulation and removal of an estrogen-dependent dopaminergic inhibition (Linard et al., 1996). GnRH-producing neurons in fish are located in the ventral olfactory bulb, ventral telencephalon, and ventral preoptic region, whereas dopaminergic neurons inhibiting GTH2 secretion are located in the anteroventral preoptic area (Navas et al., 1995; Linard et al., 1996). A weak expression of melatonin receptors was found in the nucleus preopticus pars parvicellularis, and binding sites were detected in the ventral telencephalon and anterior preoptic region; however, it is very unlikely that melatonin receptors are located on either GnRH or dopamine neurons. Therefore, the possibility exists for an indirect influence of melatonin on those cell populations by means of interneurons. Because the pituitary of teleosts receives a direct innervation, the use of DiI retrograde transport has allowed the mapping of neurons projecting to the pituitary in a number of species and has shown that the preoptic region and the mediobasal hypothalamus are the main hypophysiotropic regions (Anglade et al., 1993; Holmqvist and Ekström, 1995). Both Mel-binding sites and Mel-R-expressing cells were detected in the mediobasal hypothalamus, in particular in the nucleus lateralis tuberis pars posterior; however, their functional significance is unknown at the present stage, because the precise role of these structures in the control of pituitary functions still is understood poorly.

In the present study, no Mel-binding sites were detected in the pituitary, in agreement with previous observations in salmonids (Ekström and Vanecek, 1992; Davies et al., 1994) but differing from the goldfish, in which a slight binding was shown in the hypophysis (Iigo et al., 1994). Expression of Mel-R in the pituitary, if any, is likely to be very weak in rainbow trout. In mammals, both 2-[¹²⁵I]iodomelatonin binding and melatonin receptor gene expression are found in high concentrations in the pars tuberalis of the pituitary (Williams and Morgan, 1988; Helliwell and Williams, 1992; Reppert et al., 1994) and mediate the influence of melatonin on seasonal changes in serum prolactin concentration (Morgan and Williams, 1996). However, to our knowledge, there is no functional or morphologic equivalent of the pars tuberalis in the pituitary of teleost fish.

In conclusion, this study provides new information on the expression pattern of melatonin receptors in the brain of a fish model lacking an endogenous rhythm of melatonin secretion. The results show a pattern of expression clearly different from that reported in mammals. Although further studies will be necessary to understand the mechanisms of melatonin action in the brain of teleosts, the present data indicate that one of the functions of melatonin could be to influence neuronal and/or glial cell activities in brain regions primarily involved in processing visual information. How this information is mediated to the hypothalamopituitary complex is still open to speculation.

ACKNOWLEDGMENT

We thank Bob Duthie for assistance with graphics.

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