 ISSN 0172–780X **www.nel.edu**

ORIGINAL ARTICLE

MT1 melatonin receptor mRNA tissular localization by PCR amplification

Vincent-Joseph Poirel, Cathy Cailotto, Dominique Streicher, Paul Pévet, Mireille Masson-Pévet & François Gauer

Laboratoire de Neurobiologie des Rythmes, CNRS-UMR 7518, Université Louis Pasteur, Strasbourg, FRANCE.

Correspondence to: Prof. François Gauer
Laboratoire de Neurobiologie des Rythmes
CNRS-UMR 7518
Université Louis Pasteur
12 rue de l'Université
67000 Strasbourg, FRANCE
PHONE: +33 3 90 24 05 11
FAX: +33 3 90 24 05 28
E-MAIL: gauer@neurochem.u-strasbg.fr

Submitted: *January 9, 2003*
Accepted: *January 15, 2003*

Key words: **Melatonin; pineal gland; circadian system; biological rhythms; suprachiasmatic nuclei; receptors; quantitative PCR**

Neuroendocrinology Letters 2003; ***24****(1/2):33–38 pii: NEL241203A04* *Copyright © Neuroendocrinology Letters* ***www.nel.edu***

Abstract

OBJECTIVES: The pineal gland transduces photoperiodic informations to the neuroendocrine axis through the nocturnally melatonin secretion. This hormonal message plays a major role in the biological rhythm regulation. By autoradiography, more than 130 melatonin putative targets have been reported in the central nervous system (CNS) and in peripheral tissues. However, cross-species consensus concern only a few of them like the suprachiasmatic nuclei (SCN), the master circadian clock, and the pars tuberalis of the pituitary. Recently, MT1 melatonin receptor cDNA have been cloned in several mammals providing us with new tools to investigate its tissular location at the gene level. In the present study, we report a screening for MT1 mRNA by RT-PCR amplification of numerous tissue mRNA.

METHOD: mRNA were extracted from a large variety of rat tissues. To semi-quantify the melatonin receptor mRNA expression level, each cDNA was amplified concomitantly with both β-actin and MT1 specific primers.

RESULTS: In central and peripheral tissues previously reported to bind melatonin, strong PCR signals were logically observed. More surprisingly, a vast majority of studied tissues express MT1 mRNA and then might be responsive to melatonin.

CONCLUSION: Numerous biological functions express diurnal rhythmicity and internal-synchronization. As, most of them apparently do not receive any outcoming neuronal message from the SCN, endocrine communication was proposed to support biological rhythm synchronization. Our present data strengthen the idea that the nocturnally restricted melatonin secretion could be one internal zeitgeber that putatively distributes the endogenous circadian rhythmicity to all tissues expressing melatonin receptors.

Introduction

Rhythmicity is a fundamental endogenous property of living organisms as it provides the living beings with the possibility to anticipate on incoming periodical events. Photoperiodic daily and seasonal variations are the major cues implicated in the synchronization of endogenous rhythmicity with environmental changes. In the pineal gland, the ambient lighting condition changes are transduced into an endocrine message: the secretion of melatonin [1]. Melatonin synthesis is nocturnally restricted, and its duration is proportional to the length of the night, reflecting therefore annual photoperiodic changes [1, 2]. Therefore, the melatonin secretion pattern provides both a daily and seasonal endocrine message, and it is now well established that these messages are directly involved in the regulation of both mammalian circadian and seasonal biological rhythms [1, 2, 3].

As an hormone, melatonin is supposed to deliver its endocrine message to any tissue expressing its own receptors. Over the past 15 years, melatonin receptors, and especially their tissular detection, have been extensively studied. At first, autoradiographic methods to detect melatonin receptors were developed by using 2–^{125}iodo-melatonin as a radioligand [4]. However, this melatonin agonist is up to date the only radioligand available, which considerably limited achievement of exhaustive mappings of the melatonin receptor tissular distribution. Indeed, even if more than 130 melatonin binding site exhibiting areas have been detected in the central nervous system (CNS), the most striking evidence raised by comparing the published mappings is the great variability among species [5, 6, 7, 8, 9]. Two structures only have been reported to be almost consistently labeled in all mammalian species: the pars tuberalis (PT) of the pituitary, which exhibits the highest density of melatonin receptors [7, 8, 10] and is implicated in mediating melatonin photoperiodic effects on the neuroendocrine system [10, 11] and the suprachiasmatic nuclei (SCN), which is the site of the circadian biological clock.

In 1994 and 1995, two mammalian receptor subtypes with high-affinity for melatonin have been cloned and characterized [12, 13] and are now referenced as MT1 and MT2 receptors. When expressed in transfected cells, these subtypes exhibit the pharmacological and functional characteristics of the 2-iodo-melatonin binding sites described so far [14], and MT1 mRNA was clearly reported to be expressed in the PT and the SCN [12, 15]. On the other hand, targeted molecular disruption of the MT1 receptor in mice resulted in the total disappearance of 2-iodo-melatonin binding in brain tissues, in particular in the SCN [16]. Furthermore, 4P-PDOT and 4P-ADOT, that specifically displace 2-iodo-melatonin binding from MT2 binding sites expressed in transfected cells [17], do not displace 2-iodo-melatonin binding in SCN slices [18]. Therefore, to date, MT1 mRNA is the only mRNA that can be correlated to the well known 2-iodo-melatonin binding sites. Several investigations have been conducted to characterize by in situ hybridization the tissues expressing the MT1 mRNA. However, only tissues previously known to express strong 2-iodo-melatonin binding were shown to present detectable MT1 antisense riboprobe hybridization, such as PT, SCN, paraventricular nuclei of the thalamus or cerebellum [12, 19]. In the present study, we decided to reevaluate the inventory of the central and peripheral rat tissues that express MT1 by a highly sensitive RT-PCR method.

Materials And Methods

Animals

Male young adult Wistar rats (200–250 g, Faculté de Médecine, Strasbourg, France) were kept from birth in a 12 h light/12 h dark regime (12L/12D). A dim red light remained on throughout the 24 h period. Food and water were provided *ad libitum*.

cDNA isolation, RT-PCR

All the animals were sacrificed during the second part of the light period (5 hours before lights off). Central and peripheral tissues were immediately dissected and frozen on dry ice. Total RNA was extracted using RNAble (Eurobio, France) according to the protocol of Chomczynski and Sacchi [20]. One µg of total RNA was reverse-transcribed in a reaction volume of 10 µl, containing 2.5 µM of oligo dT, 250 µM of each dNTP (Eurobio, France), 200 units of M-MLV reverse transcriptase (Promega, France), 50 mM Tris-HCL (pH 7.5), 75 mM KCl, 3 mM $MgCl_2$, 10 mM of dithiothreitol and 10 units of RNAsin ribonuclease inhibitor (Promega). The samples were incubated for 60 min at 37°C and then at 95°C for 5 min.

Typically, PCR reactions were carried out in a total volume of 25 µl. The PCR mixtures contained: 1 µl of cDNA, 100 µM of each dNTP, 10 mM Tris-HCL (pH 9.0), 50 mM KCL, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.5 µl S^{35} dATP (12.5 mCi/ml), (NEN, France) and 0.625 units of *Taq* DNA Polymerase (Eurobio, France). In order to control for variations in cDNA synthesis and/or RNA preparations between samples, β-actin cDNA were simultaneously amplified with MT1 cDNA. Therefore, both MT1 and β-actin primers were mixed together in each PCR reaction tubes. The MT1 primers were selected to allow the amplification of a 375 bp specific product. The β-actin primers were selected to frame an intron domain in order to generate either a long PCR product in case on genomic DNA contamination, or a 301 bp long PCR amplification of β-actin cDNA. Several primer concentrations have been tested and finally all PCR reactions contained:

1 µM of both MT1 upstream and downstream oligonucleotide primers: TGCTACATTTGCCACAGTCTCA and CCAGCACAGGGCAAAAAGTA, respectively, and 0.1µM of both upstream and downstream β-actin oligonucleotide primers: TGAACCCTAAGGCCAGT and CGCACGATTTCCCTCTCAGC, respectively. The PCR conditions were: 94°C, 3min and 32 cycles at 94°C, 1 min; 58°C, 1 min; 72°C, 1 min, followed by a 20 min extension at 72°C. Products were run on a 6%

polyacrylamide and 7M-urea gel at 50 °C. The gel was washed with acid acetic 10% and methanol 10%, then rinsed twice in water and dried 2 hours at 80 °C. The amplified radio labeled products were revealed by autoradiography. Amplified bands were semi-quantitative analyzed by using the computerized analysis system Biocom-program RAG 200 [15, 21]. Data normalization of MT1 cDNA abundance was carried out by calculating the product density ratios of MT1/β-actin signals.

The whole procedure was repeated 6 times from 3 independent lots of tissues. An independent series was treated with Rnase free Dnase (Promega, France) prior cDNA synthesis to clearly demonstrate that observed amplifications derived from real tissular MT1 mRNA expression and not from genomic DNA. All the results were expressed as mean ± SEM.

Results and discussion

The typical amplification pattern that we observed on autoradiograms clearly showed that only two bands were observed after the 32 amplification cycles, the 301 bp and the 375 bp products resulting from β-actin and MT1 cDNA amplification, respectively. The absence of smears, background, or even surnumerous amplification products attest that the primer specificity, the annealing temperature, the number of cycles and salt concentrations (especially Mg^{2+}) were suitably selected (Fig. 1).

Experiments have been carried out to check that the selected primer concentrations and annealing temperature would guarantee that PCR experiments were conducted and stopped in the exponential phase of the amplification.

The β-actin amplification pattern appeared to be highly reproducible between samples and independent experiments, attesting that cDNA MT1 amplification level variations that we observed between the different tested tissues were not due to cDNA synthesis variations. Therefore, the absence of MT1 amplification product at the end of the PCR amplification clearly signifies that MT1 mRNA level in the starting tissue samples is either absent or below the detection limit. On the other hand, PCR positive results could signify genomic DNA contamination in the extracted total RNA. β-actin primers were selected to span an intron boundary in order to allow detection of genomic DNA contamination [22]. The band that would witness DNA contamination, was never observed in any tissue sample. However, a complete set of total RNA was treated with RNAse free DNAse prior cDNA synthesis. The obtained amplification pattern was similar for DNAse treated and untreated RNAs. Direct PCR product sequencing reactions were carried out to check the identity of the predicted 375 bp MT1 amplification product. Therefore, positive or negative results, we observed on PAGE gels, really attest the presence or the absence of MT1 mRNA expression in the starting tissue samples, respectively.

Quantitative estimation of MT1 PCR products was normalized by calculating the ratio between MT1/β-actin PCR amplification level (Fig. 2). There is, however, no precise evidence attesting that an housekeeping gene like β-actin has the same mRNA expression level in all the tissues. Actually, our repetitive experiments showed that slight β-actin mRNA expression tissular variations do exist, but that they are however strongly weaker than MT1 mRNA expression inter-tissular variations. Therefore, even if normalizing quantitative estimation of MT1 PCR by calculating the MT1/β-actin ratios does not provide an absolute quantification of inter-tissular MT1 mRNA expression level, we nevertheless used this tool to classify the different tissues in 4 different categories: tissues with strong, mid-

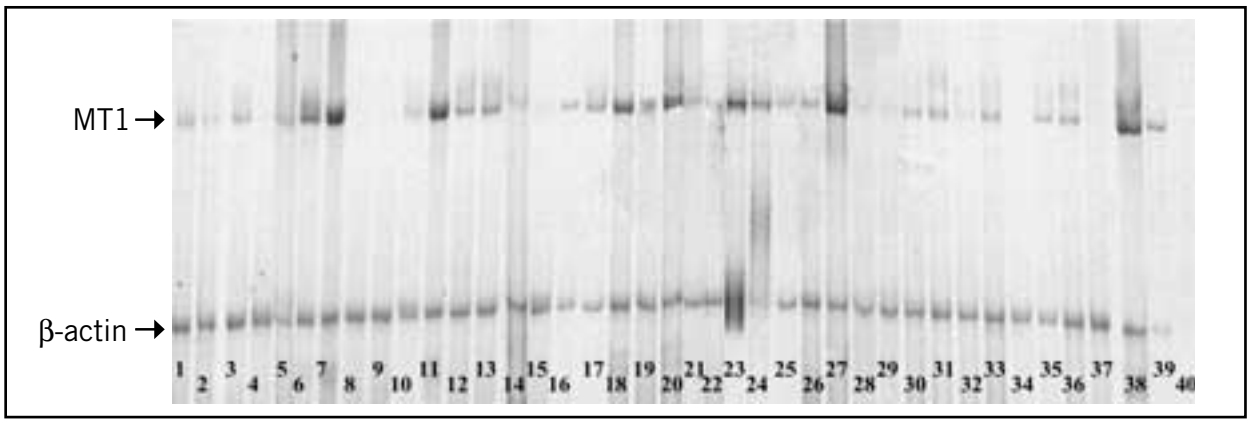

Fig 1. Electrophoretic mobility of MT1 and β-actin S^{35}-dATP radiolabeled RT-PCR fragments amplified from cDNA transcribed from total RNA extracted from different rat tissues. The cDNA were synthesized with reverse transcriptase using oligo dT. PCR parameters and gel electrophoresis conditions are described in the text.

issues by alphabetical order and their corresponding electrophoresis pattern number:
Adenohypophysis: 1; Adrenal glands: 35; Area postrema: 2; Blood: 32; Bone Marrow: 39; Brown fat: 17; Caudal artery: 3; Cerebellum: 5; Colon: 7; Duodenum: 10; Entorhinal cortex: 8; Epididymis: 11; Harderian gland: 18; Heart: 6; Ileon: 19; Kidney: 30; Liver: 13; Lung: 27; Muscle: 21; Neurohypophysis: 22; Occipital cortex: 9; Olfactory bulb: 4; Pancreas: 23; Pars tuberalis: 24; Pineal gland: 26; Prostate: 27; Retina: 31; SCN: 33; Seminal glands: 34; Skin: 25; Spinal cord: 20; Spleen: 29; Stomach: 12; Superior cervical ganglia: 14; Salivary glands: 15; Testis: 36; Thymus: 37; Thyroid: 38; White fat: 16.

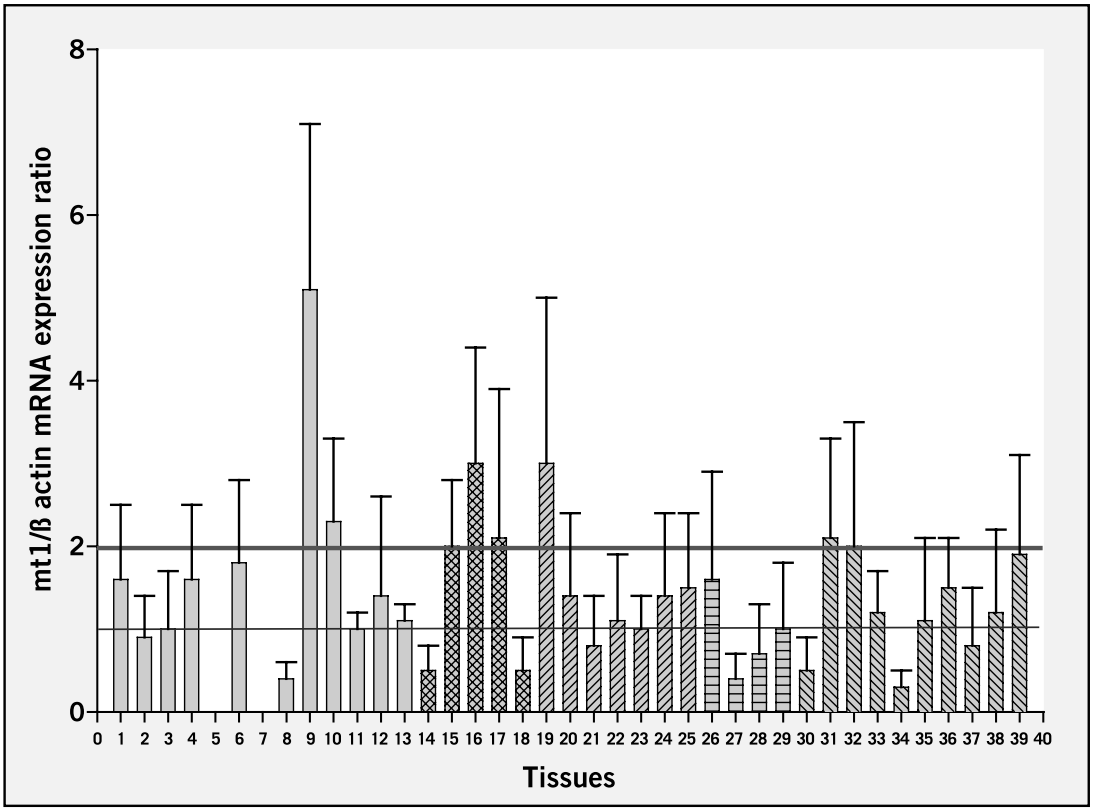

Fig. 2. Expression of the MT1 melatonin receptor subtype mRNA in rat various tissues. The values are expressed as a ratio between the optical density of the MT1 and β-actin PCR amplification signal. Values are the mean ±SEM of 6 independent PCR amplifications. The number below the axis bar refers to tissue names listed below. Tissues with a high, middle or low amplification level were arbitrary define with a ratio value above 2, between 1 and 2, or below 1 respectively.
1: Adenohypophysis; 2: Area postrema; 3: Cerebellum; 4: Superior cervical ganglia; 5: Entorhinal cortex; 6: Neurohypophysis; 7: Occipital cortex; 8: Olfactory bulb; 9: Pars tuberalis; 10: Pineal gland; 11: Retina; 12: Spinal cord; 13: Suprachiasmatic nuclei; 14: Blood; 15: Caudal artery; 16: Heart; 17: Lung; 18: Spleen; 19: Colon; 20: Duodenum; 21: Ileon; 22: Liver; 23: Pancreas; 24: Salivary glands; 25: Stomach; 26: Prostate; 27: Seminal glands; 28: Epididymis; 29: Testis; 30: Bone Marrow; 31: Brown fat; 32: Harderian gland; 33: Kidney; 34: Skin; 35: Squeletic muscle; 36: Adrenal glands; 37: Thymus; 38: Thyroid; 39: White fat.

dle, low or no MT1 mRNA expression (Fig. 2). Surprisingly, only very few tissues have no detectable MT1 mRNA expression. Actually, among all the tested tissues, only enthorhynal and occipital cortex never exhibited any MT1 cDNA amplification signal.

Most of the tissues presenting a very high amplification signal, (listed in Fig. 2), were previously reported to bind specifically the 2-iodo-melatonin ligand. Particularly, the PT of the adenohypophysis, which is the tissue with the highest MT1 mRNA expression is also known to be the tissue with the highest melatonin receptor density. Data accumulated so far brought evidences that these receptors are implicated in mediating some of the photoperiodic effects on the neuroendocrine system like the photoperiodic control of prolactin secretion [10]. The caudal artery is also known to be a clear melatonin signal target as melatonin is known to potentiate adrenergic vasoconstrictor effect on the tail artery [23, 24, 25]. In colonic tissues, the MT1 mRNA expression correlates with the high melatonin binding site density reported in both the mucosa, and the blood vessels of rodent and human colon sections [26]. At the present time, it is difficult to correlate these melatonin binding sites to any particular function of the melatonin signal. Indeed, data have suggested that melatonin could inhibit the spontaneous motility of rat intestine tissue through an indirect inhibition of the contractile response to serotonin [27, 28]. However, the large melatonin concentrations requested to get these effects suggest that they do not result from MT1 picomolar melatonin receptor stimulation [26]. More surprinsingly, lung and heart expressed also very high MT1 mRNA. Indeed, until now, only bird lungs and hearts had been reported to putatively bind melatonin [29, 30]. The shared characteristics between colon, heart and lung is their high content in blood vessels, suggesting that MT1 mRNA expression positive signal in these tissues could be interpreted as vascular melatonin receptors. The pineal gland presents also a quite strong PCR signal. This endocrine organ is known to present a high melatonin receptor density at birth [31], but the receptor density decreases rapidly afterwards, and in adult any signal is detected by classical autoradiographic methodology [31]. This situation is quite similar with melatonin receptor expression in the SCN of the Syrian hamster, where a severe loss of receptor is observed in adulthood, even if the MT1 mRNA is still largely expressed [32].

Among all the other tissues exhibiting either middle or low MT1 cDNA amplification signal are previously described binding melatonin tissues such as pituitary, area postrema, cerebellum, olfactive bulbs, retina, spinal cord, SCN, spleen, prostate, epididymis, testis, thymus, harderian and adrenal glands [7, 9]. However, some new putative targets of the melatonin signal which are detected in this study have to be added to this non exhaustive list such as liver, pancreas, salivary and seminal glands, bone narrow, fatty tissues, kidney, squeletic muscle and thyroid. Actually, at a first glance, the most intriguing results is that the SCN, one of the most studied melatonin target, does not belong to the tissues with the highest MT1 cDNA amplification. However, among all the tested tissues, the SCN belongs to the few whose dissection into a pure preparation is rendered very difficult due to the minute size of these cerebral nuclei. Therefore, the SCN specific cDNA concentration submitted to the MT1 primers is decreased by non SCN mRNA in the total RNA extracted from explants, which could explain the obtained results.

The main question raised by our data is the significance of the expression of MT1 mRNA in almost all the rat tissues tested. Melatonin is known to play a major role in regulating several circadian and seasonal rhythms. In the SCN and PT, well known melatonin responsive tissues, precise actions of melatonin have been established, even if the underlying mechanisms are not yet elucidated. The same holds true for the well characterized action of melatonin in potentiating the norepinephrine induced blood vessel vasoconstriction. In all the other tissues, many role have been proposed, but the real physiological function of the melatonin hormone remains speculative. In some way, until now, each new discovered melatonin target had to be presented with its particular role in decoding the melatonin message. Actually, new discoveries in biological rhythmicity should invite us to investigate the melatonin message function on different basis. Our data clearly suggest that the vast majority of the mammalian tissues might be responsive to melatonin. The recently discovered clock genes, which are the molecular basis of the SCN circadian oscillation [33], are not actually restricted to the circadian biological clock. They are also expressed in a vast variety of tissues such as heart, liver, retina, PT, fibroblast, etc [34]. However, their specificity in the SCN is to generate circadian oscillations which persists for long times in vitro [33] while in other tissues, they generally oscillate on a circadian basis for a only few days [35]. It thus seems that intra-tissular oscillation loops in these tissues requires an external zeitgeber to both maintain the oscillation, and synchronize it with the whole body time, since most biological parameters appear to be synchronized together. However, most of them apparently do not receive any SCN out-coming neuronal message suggesting a endocrine synchronizing message. The melatonin hormone could be one internal zeitgeber that distributes the endogenous circadian rhythmicity originating in the SCN. As its secretion is nocturnally restricted melatonin could distribute the day/night information to all tissues that putatively express its receptors. Our present data support the idea that the number of both central and peripheric structures which receive this temporal message is extremely large.

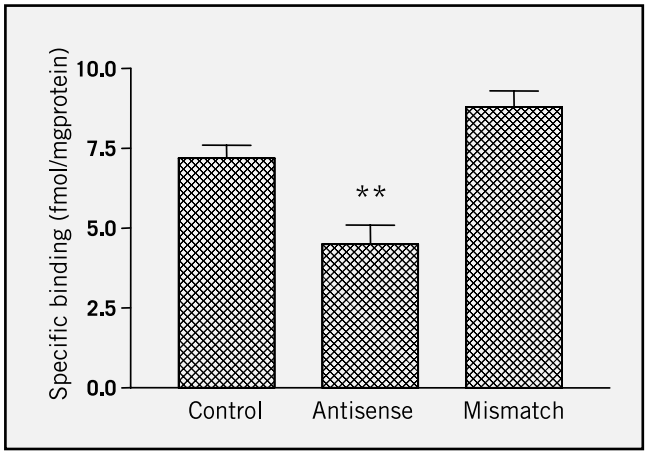

Fig. 3. In antisense experiments, injection cannula (final external and internal diameters were 150 and 53 µm respectively) were implanted using standard stereotaxic procedures close by the SCN. Distribution of the antisense oligonucleotides was estimated after injecting fluorescein labeled oligonucleotides (Molecular research Laboratories, LCC, USA).
Melatonin receptor density was measured in the SCN of Siberian hamster daily injected for 6 days with 1.5 µl of either a saline solution (control), a saline solutions containing 0.75 nmol of a MT1 antisense (TGCTGCGAGGCATTGAGCAG) or a mismatch (TGGTGCGACGCTATGAGCAG) oligonucleotide, respectively (Molecular research Laboratories, LCC, USA). Animals were sacrificed on day 7 at 2.00 pm. Autoradiographic procedures were conducted as previously described [20, 29]. The results are expressed in fmol/mg of protein. Values are the mean ± SEM of 7 animals.

In a large majority of the tissues studied by PCR, the attempt to detect MT1 mRNA by in situ hybridization failed. From the general opinion, the MT1 mRNA expression level is very low, making difficult to differentiate specific signal from background noise. The apparent discrepancy between the high melatonin receptor density measured for example in the rodent PT and the apparently low MT1 mRNA expression level [15, 36] could be explained by a long half-life of the melatonin receptor. Pilot experiments using antisense oligonucleotide invalidation of MT1 melatonin receptor were carried out in the SCN of the Siberian hamster which lacks the MT2 melatonin receptor subtype [37]. The SCN melatonin binding sites expressed in the Siberian hamster are then only due to MT1 mRNA translation. We observed that daily injections of oligonucleotides for 7 days decreased the melatonin receptor density in all the injected animals, but only by 30%, strongly suggesting that the melatonin receptor is a very stable protein (Fig. 3). Continuous renewal of the protein might therefore only require a quite low MT1 mRNA expression level which would explain the encountered difficulties to detect MT1 mRNA by in situ hybridization. More than ever, new specific pharmacological tools are required to investigate widely the physiological function of the pineal hormone melatonin, and especially to

demonstrate its possible implication as a master endocrine internal synchronizer of biological rhythmic functions.

Acknoledgements

The authors wish to thank D. Bonn for his excellent technical assistance. This study was partly supported by IRIS (Courbevoie, France), the *Simone and Cino Del Duca* Foundation (France), "la Région Alsace" (France) and by a grant from *La Fondation pour la Recherche Médicale* (France).

REFERENCES

1 Reiter RJ. The melatonin rhythm: both a clock and a calendar. Experientia 1993; **49**:654–664.
2 Reiter RJ. Melatonin synthesis: multiplicity of regulation. Adv Exp Med Biol 1991; **294**:149–158.
3 Cassone VM. Effects of melatonin on vertebrate circadian systems. Trends Neurosci 1990; **13**:457–464.
4 Vanecek J, Pavlik A, Illnerova H. Hypothalamic melatonin receptor sites revealed by autoradiography. Brain Res 1987; **435**:359–362.
5 Weaver DR, Provencio I, Carlson LL, Reppert SM. Melatonin receptors and signal transduction in photorefractory Siberian hamsters (Phodopus sungorus). Endocrinology 1991; **128**:1086–1092.
6 Stankov B, Cozzi B, Lucini V, Fumagalli P, Scaglione F, Fraschini F. Characterization and mapping of melatonin receptors in the brain of three mammalian species: rabbit, horse and sheep. A comparative in vitro binding study. Neuroendocrinology 1991; **53**:214–221.
7 Masson-Pevet M, George D, Kalsbeek A, Saboureau M, Lakhdar-Ghazal N, Pevet P. An attempt to correlate brain areas containing melatonin-binding sites with rhythmic functions: a study in five hibernator species. Cell Tissue Res 1994; **278**:97–106.
8 Morgan PJ, Barrett P , Howell HE, Helliwell R. Melatonin receptors: localization, molecular pharmacology and physiological significance. Neurochem Int 1994; **24**:101–146.
9 Pang SF, Li L, Ayre EA, Pang CS, Lee PPN, Xu RK, Chow PH, Yu ZH, Shiu SYW. Neuroendocrinology of melatonin in reproduction: recent developments. J Chem Neuroanat 1998; **14:** 157–166.
10 Morgan PJ. The pars tuberalis: the missing link in the photoperiodic regulation of prolactin secretion? J Neuroendocrinol 2000; **12**:287–295.
11 Lincoln GA, Clarke IJ. Evidence that melatonin acts in the pituitary gland through a dopamine-independent mechanism to mediate effects of daylength on the secretion of prolactin in the ram. J Neuroendocrinol 1995; **7**:637–643.
12 Reppert SM, Weaver DR, Ebisawa T. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron 1994; **13**:1177–1185.
13 Reppert SM, Godson C , Mahle CD, Weaver DR, Slaugenhaupt SA, Gusella JF. Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. Proc Natl Acad Sci USA 1995; **92**:8734–8738.
14 Reppert SM, Weaver DR, Godson C. Melatonin receptors step into the light: cloning and classification of subtypes. Trends Pharmacol Sci 1996; **17**:100–102.
15 Schuster C, Gauer F, Guerrero H, Lakhdar-Ghazal N, Pevet P, Masson-Pevet M. Photic regulation of mt1 melatonin receptors in the Siberian hamster pars tuberalis and suprachiasmatic nuclei: involvement of the circadian clock and intergeniculate leaflet. J Neuroendocrinol 2000; **12**:207–216.
16 Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VKet al. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. Neuron 1997; **19**:91–102.
17 Dubocovich ML, Masana MI, Iacob S, Sauri DM. Melatonin receptor antagonists that differentiate between the human Mel1a and Mel1b recombinant subtypes are used to assess the pharmacological profile of the rabbit retina ML1 presynaptic heteroreceptor. Naunyn Schmiedebergs Arch Pharmacol 1997; **355**:365–375.
18 Dubocovich ML, Yun K, Al Ghoul WM, Benloucif S, Masana MI. Selective MT2 melatonin receptor antagonists block melatonin-mediated phase advances of circadian rhythms. FASEB J 1998; **12**:1211–1220.
19 Mazzucchelli C, Pannacci M, Nonno R, Lucini V, Fraschini F, Stankov BM. The melatonin receptor in the human brain: cloning experiments and distribution studies. Brain Res Mol Brain Res 1996; **39**:117–126.
20 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; **162**:156–159.
21 Gauer F, Masson-Pevet M, Skene DJ, Vivien-Roels B, Pevet P. Daily rhythms of melatonin binding sites in the rat pars tuberalis and suprachiasmatic nuclei; evidence for a regulation of melatonin receptors by melatonin itself. Neuroendocrinology 1993; **57**:120–126.
22 el Husseini AE, Paterson JA, Myal Y, Shiu RP. PCR detection of the rat brain basic fibroblast growth factor (bFGF) mRNA containing a unique 3' untranslated region. Biochim Biophys Acta 1992; **1131**:314–316.
23 Viswanathan M, Laitinen JT, Saavedra JM. Expression of melatonin receptors in arteries involved in thermoregulation. Proc Natl Acad Sci USA 1990; **87**:6200–6203.
24 Krause DN, Barrios VE, Duckles SP. Melatonin receptors mediate potentiation of contractile responses to adrenergic nerve stimulation in rat caudal artery. Eur J Pharmacol 1995; **276**:207–213.
25 Bucher B, Gauer F, Pevet P, Masson-Pevet M. Vasoconstrictor effects of various melatonin analogs on the rat tail artery in the presence of phenylephrine. J Cardiovasc Pharmacol 1999; **33**:316–322.
26 Poon AM, Chow PH, Mak AS, Pang SF. Autoradiographic localization of 2[125I]iodomelatonin binding sites in the gastrointestinal tract of mammals including humans and birds. J Pineal Res 1997; **23**:5–14.
27 Harlow HJ, Weekley BL. Effect of melatonin on the force of spontaneous contractions of in vitro rat small and large intestine. J Pineal Res 1986; **3**:277–284.
28 Bubenik GA. The effect of serotonin, N-acetylserotonin, and melatonin on spontaneous contractions of isolated rat intestine. J Pineal Res 1986; **3**:41–54.
29 Pang SF, Dubocovich ML, Brown GM. Melatonin receptors in peripheral tissues: a new area of melatonin research. Biol Signals 1993; **2**:177–180.
30 Pang SF, Cheng KM, Pang CS, Wang ZP, Yuan H, Brown GM. Differential effects of short photoperiod on 2-[125I]iodomelatonin binding in the testis and brain of quail. Biol Signals 1993; **2**:146–154.
31 Zitouni M, Masson-Pevet M, Gauer F, Pevet P. Influence of maternal melatonin on melatonin receptors in rat offspring. J Neural Transm Gen Sect 1995; **100**:111–122.
32 Gauer F, Schuster C, Poirel VJ, Pevet P, Masson-Pevet M. Cloning experiments and developmental expression of both melatonin receptor Mel1A mRNA and melatonin binding sites in the Syrian hamster suprachiasmatic nuclei. Brain Res Mol Brain Res 1998; **60**:193–202.
33 Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng Bet al. Interacting molecular loops in the mammalian circadian clock. Science 2000; **288**:1013–1019.
34 Balsalobre A. Clock genes in mammalian peripheral tissues. Cell Tissue Res 2002; **309**:193–199.
35 Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 1998; **93**:929–937.
36 Poirel VJ, Masson-Pevet M, Pevet P, Gauer F. MT1 melatonin receptor mRNA expression exhibits a circadian variation in the rat suprachiasmatic nuclei. Brain Res 2002; **946**:64–71.
37 Weaver DR, Liu C, Reppert SM. Nature's knockout: the Mel1b receptor is not necessary for reproductive and circadian responses to melatonin in Siberian hamsters. Mol Endocrinol 1996; **10**:1478–1487.