

**Human melatonin receptors:
molecular mechanisms of action
and their implication in cell proliferation**

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Abstract

Melatonin entrains the biological clock (the suprachiasmatic nucleus, SCN) by activation of specific melatonin receptors. In mammals, two types (MT₁ and MT₂) of melatonin receptors have been identified by molecular cloning. MT₁ is expressed in the pars tuberalis and in the SCN, i.e., in the presumed sites of the reproductive and circadian actions of melatonin, whereas MT₂ is mainly expressed in the retina. In addition, the distinct peripheral distribution and the numerous physiological processes in which melatonin appears to be involved such as sleep, as well as other circadian, immunological, oncological, cardiovascular, reproductive and anti-oxidant functions, imply that melatonin may signal through a variety of precisely-regulated pathways. The currently available *in vitro* models to study pharmacological and functional properties of human melatonin receptors are based on rodent cells (CHO, NIH3T3), raising the possibility of species-specific effects that could interfere with the interpretation of data obtained. Therefore, in order to study reliably specific properties of the two human melatonin receptors in a homologous context, new cell lines, derived from human cells (SK-UT-1B uterine tumor cells) by stable transfection with either the human MT₁ or MT₂ receptor were developed. Binding studies and the inhibition of forskolin-stimulated adenosine 3'-5'-cyclic monophosphate levels by melatonin confirmed the functional expression of high-affinity melatonin receptors. MT₂-transfected cells modulated cGMP levels in a dose-dependent manner. In contrast, MT₁ receptors had no effect on cellular cGMP levels.

To investigate whether melatonin can alter gene expression and to identify whether these effects are due specifically to either MT₁ or MT₂ receptor activation, microarray analyses from both transfected and untransfected cells following overnight exposure to melatonin were performed. More than twenty independent genes, implicated in different physiological processes such as apoptosis, cell cycle regulation and cell growth, showed a greater than 2-fold change in expression depending on the melatonin receptor subtype involved.

Among the apparently regulated genes by melatonin, two (ERK1/2 and cyclin G2) were examined in more detail. The ERK1/2 pathway is thought to play a role in cell proliferation and differentiation, whereas cyclin G2 is an unconventional cyclin that is highly expressed in cells undergoing apoptosis and has been reported to play a role in cell cycle arrest. Melatonin-induced effects on the expression of these genes were shown in cells expressing the MT₂ melatonin receptor, and 4P-PDOT, a specific antagonist for the MT₂ melatonin receptor, could block these effects. As these actions could be expected to reduce cell proliferation and

in view of the fact that melatonin has already been shown to inhibit the growth of various tumors *in vivo* and *in vitro*, the influence of melatonin on cell proliferation was tested.

The results support the idea that melatonin, especially via the MT₂ receptor, can suppress cell proliferation by regulation of various gene products, including cyclin G2 and MAPK activity.

1 INTRODUCTION

1.1 Melatonin: discovery, chemical structure and synthesis

The indoleamine N-acetyl-5-methoxytryptamine, better known as melatonin, is the principal hormone produced by the pineal gland or epiphysis. The first evidence of biological activity of melatonin can be traced back to 1917 when McCord and Alan discovered that extracts of bovine pineal gland causes blanching of *Rana pipiens* tadpole skin. However, it took more than four decades until Lerner and colleagues (1958) first isolated this agent from the bovine pineal gland. Lerner gave the hormone its name based on its melanin-aggregating properties in amphibian melanophores (“mela”) and its structural similarity to the indoleamine serotonin (“tonin”). In 1963 Wurtman et al. recognized that its rhythmic synthesis is controlled by light and in 1973, Moore et al. proved that the suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the “master oscillator” underlying most of the body’s 24-h rhythmicity, including the synthesis of melatonin.

Melatonin is released from the pineal gland in a circadian manner with melatonin levels peaking at night. Melatonin is a small lipophilic molecule (molecular weight 232), derived from the amino acid tryptophan. Prerequisite for the formation of melatonin is the uptake of the dietary tryptophan from the circulation into the pineal gland. Circulating tryptophan levels do not correlate with pineal tryptophan levels in the rat implying an active transport mechanism (Pardridge et al., 1981). Dietary manipulations have been shown to modify pineal melatonin secretion (Zimmermann et al., 1993). Tryptophan is transformed into 5-hydroxytryptophan (5HTP) by tryptophan-5-hydroxylase (Lovenberg et al., 1967), a mitochondrial enzyme present in high amounts in the pineal, whose activity is two-to-three fold increased at night in the rat (Sitaram et al., 1978). 5-hydroxytryptophan is decarboxylated to 5-hydroxytryptamine (5HT, serotonin) by the cytoplasmatic enzyme aromatic amino acid decarboxylase (Lovenberg et al., 1962), a constitutively active enzyme with little daily variation in the pineal. The cytoplasmatic enzyme Arylalkylamine-*N*-acetyltransferase (AA-NAT) completes the next step by *N*-acetylation of serotonin using acetyl co-enzyme A as a cofactor (Weissbach et al., 1960). The enzyme is present in the pineal cytoplasm and also in the retina and is distinguished from the same enzyme in other tissues by its specificity and the remarkable variation in its activity leading to 70 to 100 fold increases in activity at night in the rat pineal (Klein et al., 1970). Thus, this enzyme appears to be rate limiting in melatonin synthesis. The final step in the pathway is *O*-methylation of *N*-acetylserotonin (NAS) by

hydroxyindole-*O*-methyltransferase (HIOMT) which transfers a methyl group from S-adenosyl methionine (Arendt, 1995). This enzyme does not exhibit diurnal variation but has a high constitutive activity (Sugden et al. 1987). Therefore, NAS levels increase at night through altered activity of the rate-limiting enzyme AA-NAT, and this results in enhanced melatonin synthesis from NAS due to the high constitutive activity of HIOMT (Reiter et al., 1983).

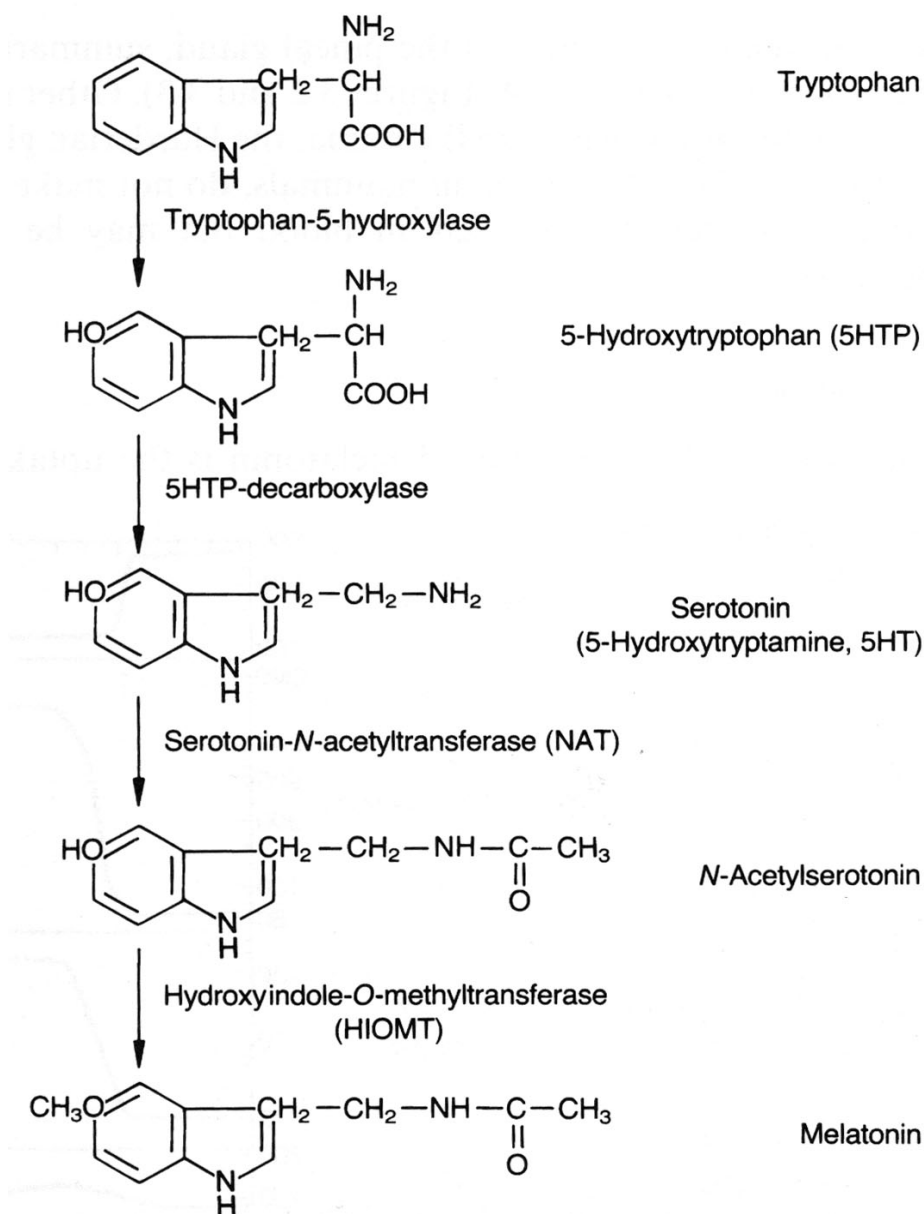


Fig. 1: The chemical structure of melatonin and its pathway of synthesis from tryptophan

1.2 Regulation of melatonin synthesis: sympathetic noradrenergic mechanisms

Photosensory input reaches the pineal gland through a complex multisynaptic neuronal pathway that begins at the retina. In mammals, photoreceptors of the retina convert environment light into electrical impulses, which are sent directly to the suprachiasmatic nucleus (SCN) of the hypothalamus, the site of the mammalian circadian pacemaker, through the retinohypothalamic tract (Sadum et al., 1984). The neurotransmitter involved in this signal pathway appears to be glutamate (Hannibal et al., 2002). From the SCN, neuronal projections make synaptic connections in the paraventricular nuclei (PVN) of the hypothalamus (Swanson et al., 1975), descending through the hindbrain to the spinal cord (Saper et al., 1976), from which preganglionic fibers reach the superior cervical ganglia (SCG). The major control of melatonin synthesis is exerted by sympathetic postganglionic noradrenergic fibers from the SCG, innervating the pineal gland (Moore et al., 1978). Both serotonin and noradrenaline (NA) are present in the nerve endings. Melatonin synthesis is controlled mainly by noradrenaline, released from the sympathetic nerves that innervate the gland (Moore et al., 1996). Noradrenaline release is high at night and low during the day (Brownstein et al., 1974). Noradrenaline binds membrane-bound α_1 and β_1 adrenergic receptors of pinealocytes (Vanecek et al., 1985). Activation of the β_1 receptors activate adenylate cyclase through GTP-binding proteins in the cell membranes, and increases pineal cAMP levels 60-fold (Deguchi et al., 1972). cAMP therefore acts as a second messenger in the pineal by activating protein kinase A (PKA) and subsequently the transcription factor cAMP response element (CRE) binding protein (CREB). The AA-NAT promoter contains several CREs that are also bound by members of the CRE modulator (CREM) family, such as the dominant repressor inducible cAMP early repressor (ICER). The diurnal regulation of AA-NAT depends on the interplay between CREB and ICER and finally results in synthesis and release of melatonin in darkness and its inhibition during daylight (Stehle et al. 1993). In addition, activation of α_1 receptors leads to activation of protein kinase C (PKC) activities which is thought to initiate phosphorylation of cAMP regulatory protein located in the membrane (Sugden et al., 1989).

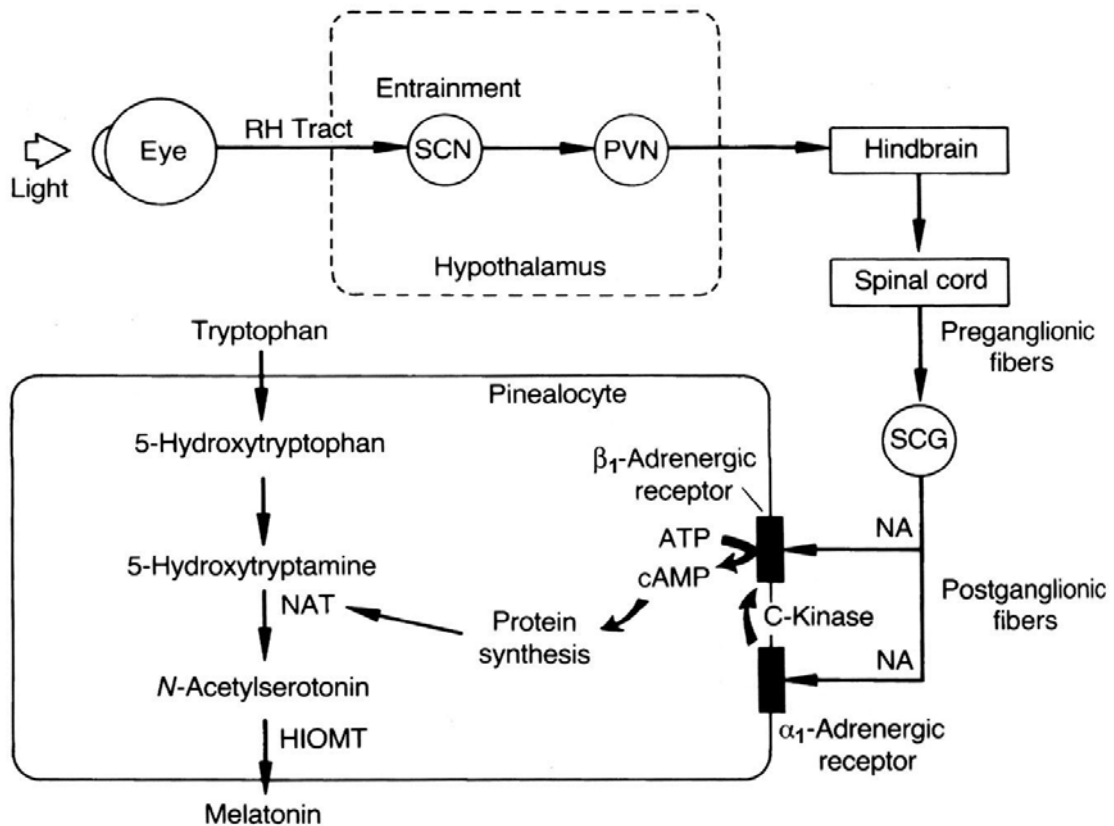


Fig. 2: Noradrenergic control mechanisms in melatonin synthesis

The suprachiasmatic nucleus (SCN) generates rhythmic signals, entrains to 24 h by light entering in the retina, relayed via the paraventricular nucleus (PVN), the hindbrain, spinal cord and superior cervical ganglion (SCG) to β_1 - and α_1 adrenoreceptors within the pineal. RHT: retinohypothalamic tract; AA-NAT: arylalkylamine-*N*-acetyltransferase; HIOMT: hydroxyindole-*O*-methyltransferase; NA: noradrenaline (fig. modified from Arendt, 1995).

1.3 Sites of synthesis and degradation

Apart from the pineal gland, low levels of rhythmic melatonin synthesis may also occur in the vertebrate retina (Iuvone et al., 1990), and in discrete regions of the brain and skin (Slominsky et al., 1996). However, this production does not seem to contribute significantly to the plasma melatonin rhythm (Cogburn et al., 1987), and the biological significance of the synthesis in brain and skin remains uncertain.

As a lipophilic compound, melatonin easily diffuses through biological membranes and readily crosses the hematoencephalic barrier in an albumin-bound form. Therefore, the release of melatonin from the pinealocyte does not seem to require any specialized cellular mechanism (Arendt et al., 1995). Melatonin concentration in blood directly mirror the

changes of pineal melatonin concentrations (Illnerova et al., 1978) where melatonin increases soon after the onset of darkness and peaks in the middle of the night between 2 and 4 a.m. and gradually falls during the second half of the night. During the day, melatonin levels decrease 10-fold and remain low for about 16 hours (Lynch et al. 1975; Reiter et al., 1991). In mammalian species, melatonin is rapidly metabolized, mainly in the liver, by hydroxylation to 6-hydroxymelatonin, and after conjugation with sulphuric or glucuronic acid it is excreted. The urinary excretion of 6-hydroxymelatonin closely parallels serum melatonin concentrations (Lynch et al., 1975). The half-life of melatonin in circulation is circa 10 min (Illnerova et al., 1978). The very dynamic regulation of melatonin levels, established by its rhythmic synthesis and its rapid degradation, suggests that melatonin acts as a precise and efficient mechanism to distribute the message of darkness throughout the body.

1.4 Membrane melatonin receptors

1.4.1 Melatonin receptor subtypes

The physiological effects of melatonin are considered to be mediated via specific melatonin receptors. Hitherto two mammalian melatonin receptors with high affinity for melatonin have been cloned and characterized: MT₁ (Reppert et al., 1994) and MT₂ (Reppert et al., 1995). The classification of melatonin receptors approved by the nomenclature of IUPHAR (Dubocovich et al., 1998) designates now the previously described as Mel1a as MT₁ and the previously described as Mel1b as MT₂. A third mammalian receptor, MT₃ (previously referred to as ML2) is yet to be cloned. The MT₃ melatonin receptor can be activated by both melatonin and its precursor *N*-acetylserotonin and is characterized by a pharmacological profile distinct from any other known mammalian melatonin receptor (Dubocovich et al. 1995; Molinari et al., 1996) and belongs to the family of the quinone reductases. The first known melatonin receptor (Mel1c) was cloned from *Xenopus* dermal melanophores, brain chicken and zebra fish (Ebisawa et al., 1994), but it has not been found in mammals. Additionally, a mammalian melatonin receptor-related receptor (MR-R), structurally related to the melatonin receptors but incapable of binding melatonin, has also been isolated (Reppert et al., 1996). The natural ligand(s) for this receptor have not been identified (Reppert et al. 1996; Drew et al., 1998).

1.4.2 General structure of melatonin receptors

All three cloned melatonin receptors represent classical guanine nucleotide binding protein (G-protein)-coupled receptors (GPCRs) consisting of seven transmembrane domains and three extracellular and intracellular loops each, an extracellular N-terminal domain and an intracellular C-terminal part (Fig. 3). On the basis of structural analyses, melatonin receptors represent a distinct group within the large superfamily of G-protein coupled receptors with the highest similarity to μ -opioid and type 2 somatostatin receptors (Shiu et al., 1997).

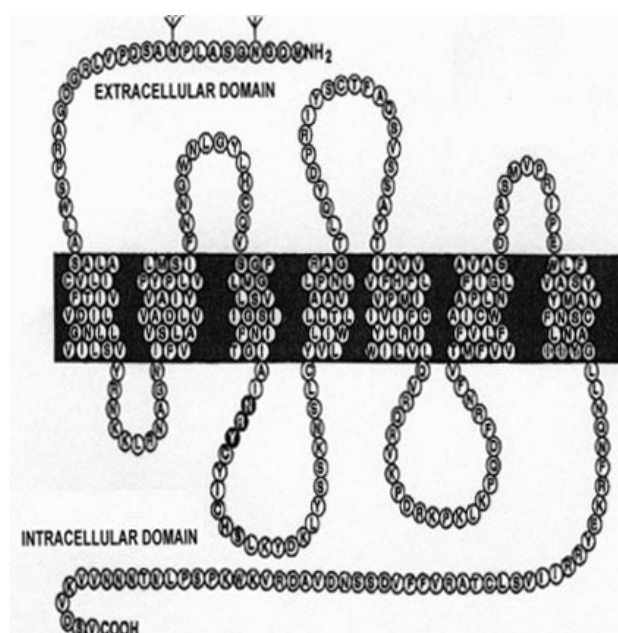


Fig. 3: Scheme of melatonin receptors

The primary structure and predicted topology of the human MT₂ receptor are shown. Shaded amino acids are identical between MT₂ and the human MT₁ receptor (from Reppert et al., 1995).

Melatonin receptors, upon agonist activation, interact with heterotrimeric G proteins, and serve as a guanine-nucleotide exchange factor (GEF) to promote GDP dissociation and GTP binding and activation (Morgan et al., 1989; Laitinen et al., 1990;). This leads to dissociation of the G-protein complexes into an α subunit and a $\beta\gamma$ dimer, which activate several effectors (Barrett et al., 1994). The regulator of G-protein signalling proteins (RGS) are known to be involved in the hydrolysis of GTP to GDP, leading to reassociation of the heterotrimer and termination of the active cycle (Pierce et al., 2002). MT₁ melatonin receptors can couple to a wide variety of G-proteins including G_{iα2}, G_{iα3} and G_{αq} (Brydon et al., 1999), G_{αs}, G_{αz} and

$G_{\alpha 16}$ (Chan et al., 2002; Ho et al., 2001). In case of the MT_2 receptor, only coupling to $G_{\alpha i}$ has been reported (Chan et al., 2002).

1.4.3 Regulation of melatonin receptor subtypes

Because melatonin receptors are normally exposed nightly to melatonin for prolonged periods of time, desensitisation, the process by which melatonin receptors become refractory to their antagonist, is thought to be an essential component underlying the functional effects of melatonin within the body. Prolonged exposure of the MT_1 receptor to melatonin results in desensitisation of both endogenous (Hazlerigg et al., 1993) and recombinant MT_1 melatonin receptors (Witt-Enderby et al., 1998). Additionally, prolonged exposure of recombinant MT_2 receptors to both physiological (up to 1 nM) and pharmacological (>10 nM) melatonin concentrations results in its desensitisation (Jones et al., 2000). G-protein coupling is thought to be involved in this process. In CHO cells expressing the MT_1 melatonin receptor, melatonin exposure induces an increase in the heterotrimeric, undissociated form ($G_{i\alpha\beta\gamma}$) of G_i , resulting in a supersensitive state of the receptor (Witt-Enderby et al., 1998).

In rodent SCN and PT, MT_1 mRNA expression exhibits daily variations, with elevated levels at the day time (Guerrero et al., 1999). In addition, studies involving manipulation of melatonin levels support a role for melatonin in regulating its own receptor (Masson-Pévet et al., 2000). Moreover, a rhythmic regulation of MT_1 mRNA expression that is independent of rhythmic melatonin secretion has been observed (Guerrero et al., 1999; Masson-Pévet et al., 2000). These findings indicate a considerable complexity in the mechanisms regulating melatonin receptor expression.

1.4.4 Gene structure and chromosomal location of melatonin receptor subtypes

The genomic structure of all three cloned melatonin receptors (MT_1 , MT_2 and $Mel1c$) is characterized by the presence of two exons separated by a large intron (>8 kb) in each instance. Exon 1 encodes for the extracellular N-terminal domain, transmembrane domain 1 and the first intracellular loop. The remainder of the receptor is contained within the second exon (Reppert et al. 1994, 1995; Roca et al., 1996). The presence of an intron in the first cytoplasmatic loop of these genes may lead to alternate splicing which could possibly alter receptor structure and function (Reppert et al., 1995), although no reports concerning this issue have yet appeared. In the mouse, a functional promoter (without a TATA box) was

shown to be present in the 1.1-kb 5' flanking region of the MT₁ receptor gene (Roca et al., 1996). The coding regions between the human and sheep MT₁ and MT₂ receptors share a 60% homology at the amino acid level between them and also with that of the *Xenopus* Mel1c (Reppert et al., 1994; 1995). The greatest dissimilarities are found in the N- and C-terminal regions, the latter being 65 amino acid shorter in the mammalian clones (human: 350 aa). The *Xenopus* Mel1c and the mammalian melatonin receptors contain one and two glycosylation sites, respectively. The receptors show several potential protein kinase C phosphorylation sites, which may participate in the regulation of the receptor function. A distinguishing feature of the mammalian receptors is their chromosomal location. The MT₂ melatonin receptor maps to human chromosome 11q21-22 (Reppert et al., 1996). The MT₁ melatonin receptor maps to human chromosome 4q35.1 (Slaugenhaupt et al., 1995). Presently these loci have been not linked to any known genetic diseases. The characteristics of the MT₁, MT₂ and Mel1c melatonin receptors are summarized in Table 1.

1.4.5 Pharmacology of mammalian melatonin receptors

MT₁, MT₂ and Mel1c melatonin receptors show picomolar affinities for the radioligand 2-[¹²⁵I]iodomelatonin, with equilibrium dissociation constants (K_d) of less than 200 pM (Reppert et al., 1994, 1995; Petit et al., 1999). The 5-methoxy group is important for high affinity binding (Sugden et al., 1997). Substitution at the 2 position of the indole ring of melatonin increases affinity and stability considerably. Thus, 2-iodomelatonin (Fig. 4) shows a ~10-fold improvement in affinity at both receptor subtypes (Sugden et al., 1997). When expressed transiently in transfected COS-7 cells (Dubocovich et al., 1997) or stably in NIH3T3 (Nonno et al 1999) and CHO cells (Browning et al, 2000), the recombinant human MT₁ and MT₂ melatonin receptors are characterized by the following rank order of affinities: 2-iodomelatonin ≥ melatonin >> N-acetylserotonin >> serotonin (Table 1). Certain melatonin receptor ligands, however, show distinct selectivity for MT₁ and MT₂ melatonin receptors, allowing the receptors and their functions to be distinguished using these pharmacological tools. For example, the competitive melatonin receptor antagonist 4-phenylacetamidotetraline (4P-PDOT) (Fig. 4) is a selective MT₂ receptor ligand showing higher affinity for the MT₂ than for the MT₁ melatonin receptor. However, the affinity ratios (MT₁:MT₂; 90-22.000) vary depending on the level of the receptor or depending on the signalling pathways present in each recombinant system analyzed (Dubocovich et al., 1997, 1998; Nonno et al., 1999).

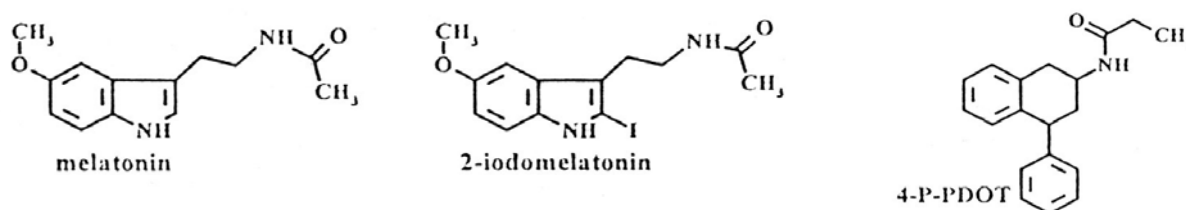


Fig. 4: Structure of melatonin, the melatonin receptor agonist 2-iodomelatonin and the antagonist 4P-PDOT

1.4.6 Distribution and functions of the MT₁ and MT₂ melatonin receptors

MT₁ and MT₂ melatonin receptors differ in their anatomical distribution. MT₁ is expressed primarily in the SCN of the hypothalamus in many species including humans (Reppert et al., 1994) and in the pars tuberalis of the pituitary, whereas MT₂ is mainly expressed in the retina (Reppert et al., 1995) (Table 1). In other regions of the human brain, MT₁ has been detected in the cerebellum, the cortex, the thalamus and the hippocampus (Mazzucelli et al., 1996), whereas MT₂ has been detected in whole brain and hippocampus present in both cases at low expression levels (Reppert et al., 1995). In situ hybridization and RT-PCR experiments suggest that the MT₁ receptor appears to represent more than 99% of all melatonin binding sites in the brain (Liu et al., 1997). MT₁ appears to mediate the inhibitory effects of melatonin in SCN, whereas the MT₂ receptor may be involved in the phase-shifting response of melatonin (Hunt et al., 2001)

In the periphery, MT₁ is expressed in cardiac vessels where it is involved in contraction of smooth muscle cells (Doolen et al., 1998), in rat tail arteries (Ting et al., 1999), the rat uterus endometrium (Zhao et al., 2000), rat ovary (Clemens et al., 2001), guinea pig kidney (Song et al., 1997), epithelial cells (Chan et al., 1997), and in cancer cell lines such as mouse neuroblastoma (Bordt et al., 2001) and breast cancer cells (Dillon et al., 2002).

The MT₂ melatonin receptor is expressed at the peripheral level in human brown adipocytes where MT₂ decreases the expression of the glucose transporter Glut4 and decreases glucose uptake (Brydon et al., 2001) and in human choriocarcinoma cells (Shiu et al., 2000).

Both MT₁ and MT₂ melatonin receptors are expressed in human uterus myometrium (Schlabritz et al., 2003), human granulosa-luteal cells (Woo et al., 2001) and human prostate epithelial cells (Gilad et al., 1996). In the rabbit retina, MT₂ mediates inhibition of dopamine release (Dubocovich et al., 1997).

Experiments using a MT₁ receptor knockout mouse (Liu et al., 1997) indicate that this receptor is essential for melatonin-induced acute inhibition of SCN neuronal firing. Remarkably, Drazen et al. (2001) showed, that MT₂ but not MT₁ melatonin receptors are associated with melatonin-induced enhancement of cell-mediated and humoral immunity. In addition, the fortuitous discovery of a naturally occurring MT₂ knockout in a hamster species, indicate that the MT₂ receptor is not necessary for reproductive and circadian responses to melatonin (Weaver et al., 1996).

	MT₁	MT₂	Mel_{1c}
Source	Mammalian, avian	Mammalian	Amphibian, avian; fish
Kd (pM)	60-170	100-200	200
Exons	2	2	2
Homology (% amino acid)	60	60	60
2nd Messenger	cAMP	cAMP, cGMP	cAMP, cGMP
Human chromosome	4q35,1	11q21-22	?
Central distribution	SNC, PT, brain	Retina, brain	brain
Agonist selectivity	I-Mel>mel>>N-acetyl- serotonin>>serotonin	I-Mel=mel>>N-acetyl- serotonin>>serotonin	I-Mel>mel>>N-acetyl- serotonin>>serotonin
4P-PDOT antagonist selectivity	Affinity ratio MT ₁ :MT ₂ 300-22000	Affinity ratio MT ₁ :MT ₂ 300-22000	—

Table 1: Properties of melatonin receptors

SCN: suprachiasmatic nucleus; PT: pars tuberalis; I-Mel: 2-iodomelatonin; Mel: melatonin; Kd: equilibrium dissociation constant.

1.5 Transduction of melatonin signals

1.5.1 Melatonin effects on cellular cAMP levels

Because melatonin receptors are located on plasma membranes, melatonin is thought to regulate cellular functions through intracellular second messengers. In many tissues, melatonin has been found to decrease intracellular concentrations of cAMP (Carlson et al.,

1989; Morgan et al., 1989). Whenever examined, recombinant human MT₁, MT₂ and Mel1c melatonin receptors have been shown also to inhibit forskolin (a pharmacological activator of adenylyl cyclase)-stimulated cAMP accumulation in various cell models systems (Petit et al., 1999; MacKenzie et al., 2002), confirming the coupling of melatonin receptors to this signal transduction pathway as previously observed in native tissues. This includes decreases in PKA activity and in the phosphorylation of CREB, a transcription factor of cAMP-sensitive genes (McNulty et al., 1994), representing a generalized signalling mechanism proposed for MT₁. This effect of melatonin is pertussis toxin (PTX)-sensitive, indicating coupling of the receptor to a G_{oi} protein (Reppert et al., 1994, 1995)

1.5.2 Melatonin effects on cGMP levels

Cyclic guanosine 3'-5'-monophosphate (cGMP) has also been shown to constitute a second messenger in the transduction of melatonin signals. The Mel1c melatonin receptors from *Xenopus laevis* inhibit intracellular cGMP levels when transfected into cell lines (Jockers et al. 1997). The human MT₂ melatonin receptor has been shown to be coupled to cGMP inhibition in human adipocytes expressing this receptor (Brydon et al., 2001) and in HEK293 transfected cells (Petit et al., 1999), whereas the MT₁ melatonin receptor had no effects on cGMP levels. Synthesis of cGMP is catalyzed by two types of guanylate cyclases: a soluble cytosolic form and a transmembrane form. Soluble guanylate cyclases are activated by nitric oxide, whereas membrane guanylate cyclase are activated by natriuretic peptides. cGMP is an important second messenger involved in many physiological processes such as contractility in smooth muscle (Buhimschi et al., 1995), intercellular communication in the central nervous system (Snyder et al., 1992) and light-triggered rhodopsin signalling in the retina (Yarfitz et al., 1994).

1.5.3 Other effects of melatonin

In addition to a modulation of the nuclear factor CREB, MT₁ can also inhibit the induction of the proto-oncogene c-Fos and Jun B mRNA and c-Fos translation induced by forskolin (Ross et al., 1996). Activation of MT₁ melatonin receptors increase phosphorylation of mitogen-activated protein kinase kinases 1 and 2 (MEK1 and MEK2) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) in CHO cells (Witt-Enderby et al., 2000); however, this effect was not found in NIH3T3 cells expressing the MT₁ receptor (Godson et al., 1997). Activator protein-1 (AP-1), a transcription factor formed by the immediate early gene

products c-Fos and c-Jun, has been shown extensively to be regulated by the mitogen-activated protein kinase (MAPK) pathway (Witt-Enderby et al., 2003). Thus, these findings suggest a mechanism responsible for melatonin's ability to induce differentiation in certain cells (Bordt et al., 2001). Besides the cAMP-dependent cascade, MT₁ melatonin receptors can stimulate phospholipase C (PLC)-dependent signal transduction cascades via G_{βγ} subunits (Godson et al., 1997) and activate protein kinase C (PKC) (Witt-Enderby et al., 2001). PKC activation has been also found with MT₂ melatonin receptors (Hunt et al., 2001). MT₁ melatonin receptor can couple to calcium activated potassium (BKCa²⁺) channels (Geary et al., 1998) and G-protein-activated inward rectifier potassium (GIRK Kir 3) channels (Jiang et al., 1995).

1.6 Antiproliferative effects of melatonin

There is experimental evidence that melatonin influences the growth of spontaneous and induced tumors in animals. E.g., pinealectomy enhances tumor growth, and the administration of melatonin reverses this effect or inhibits tumorigenesis caused by carcinogens (Tamarkin et al., 1981). Low serum melatonin concentrations and low urinary excretion of melatonin metabolites have been reported in women suffering from estrogen-receptor positive breast cancer and in men with prostatic cancer (Tamarkin et al., 1982; Bartsch et al., 1992). Several *in vitro* studies show that melatonin has an antiproliferative effect in numerous cancer cell lines derived from different malignant tissues such as breast cancer (Hill et al., 1992; Cos et al., 1994; Mollis et al., 1994; Blask et al., 1997; Ram et al., 2000), ovarian cancer (Petranka et al., 1999), melanoma (Ying et al., 1993) choriocarcinoma (Shiu et al., 2000), prostate (Zhou et al., 2002) and endometrial cancer (Kanishi et al., 2000).

The mechanism by which melatonin can inhibit tumor growth is not clear, however it is thought that the anti-cancer actions of melatonin are mediated through specific melatonin receptors at physiological concentrations, involving (i) direct anti-proliferative effects of the hormone with respect to inhibition of cell cycle kinetics (Cos et al., 1996; Shiu et al., 1999) or (ii) based on immuno-stimulatory effects (Skwarlo-Sonta et al., 2002). At pharmacological concentrations, the free radical scavenger properties of melatonin are also thought to be involved (Reiter et al., 1995) although recent studies dispute this (Fowler et al., 2003).

1.7 Intracellular actions of melatonin: possibility of nuclear melatonin receptors

The lipophilic properties of melatonin suggest that it may also have intracellular binding sites and actions. In fact, melatonin was shown to act as an intracellular scavenger of hydroxyl and peroxy radicals and to protect against oxidative damage (Reiter et al., 1995). In humans, the antioxidant effect probably occurs only at pharmacological melatonin concentrations, but the decrease of night time serum melatonin concentrations that occurs with aging suggests an antiaging potential of the pineal gland hormone (Reiter et al., 1994). Melatonin was also found to bind cytosolic calmodulin and thus could be involved in modulation of calcium signalling (Romero et al., 1998) (Fig. 5).

Of particular interest, the pineal hormone was shown previously to bind and activate in the low nanomolar range two closely related nuclear receptors (Becker-André 1994), referred to as RZR/ROR α (Becker-André 1993) and RZR β (Carlberg et al., 1994). According to the unified nomenclature of the nuclear receptor superfamily, they are now called NR1F1 and NR1F2, respectively. This nuclear receptor superfamily comprises approximately 100 transcription factors that all contain a highly conserved DNA binding domain with two zinc finger structures (Freedman, 1992). Nuclear receptors regulate gene transcription through binding to specific DNA sequences, called response elements, which are located in the promoter region of their target genes (Carlberg, 1994).

RZR/ROR α and RZR β show different expression patterns, suggesting that both receptor subtypes have different gene control mechanisms in the context of different biological processes. RZR/ROR is rather ubiquitously expressed (Becker-André et al., 1993). Picomolar melatonin concentrations are sufficient for membrane receptor activation, whereas nanomolar melatonin concentrations are required for RZR/ROR α and RZR β activation. However, at a high constitutive activity of RZR/ROR α , a significant ligand activation has not yet been observed (Carlberg, 1994), suggesting effects of nuclear melatonin signalling only under particular conditions.

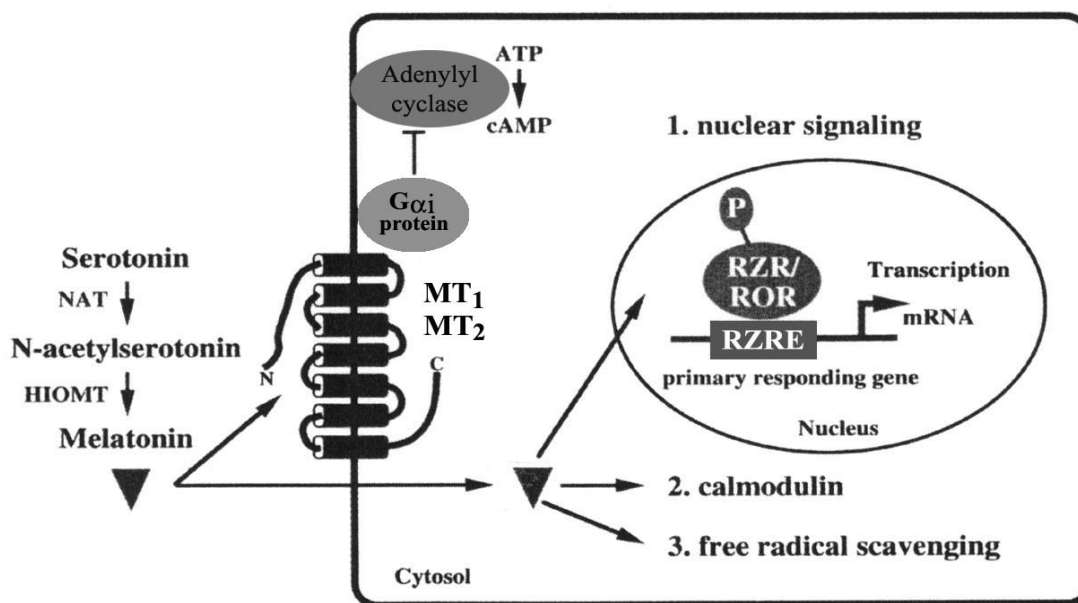


Fig. 5: Melatonin signalling

The hormone binds with high affinity in the picomolar range to its membrane receptors, MT₁ and MT₂, and /or in the nanomolar range to the nuclear receptor RZR/ROR as well as to calmodulin. At pharmacological concentrations, melatonin may have also free radical scavenger functions (fig. modified from Carlberg, 2000)

1.8 Aims of this work

Because little is known on subtype-selective mechanism by which melatonin exert its effects on cells through the human MT₁ and MT₂ melatonin receptors, first aim of the present work was to develop an *in vitro* system in which human cells, stably transfected with either the human MT₁ or the MT₂ melatonin receptor, can serve for comparative analyses between these receptors in a homologous cellular context.

Pharmacological profiles and the inhibition of forskolin-stimulated adenosine 3'-5'-cyclic monophosphate levels by melatonin were examined to confirm a functional expression of high-affinity receptors. In order to define differences in signalling between melatonin receptor subtypes, melatonin-induced modulation of cGMP levels and effects on the proto-oncogene c-Fos were investigated. Generally, potential effects of melatonin on gene expression were screened by microarray analyses. Moreover, investigations were conducted to define the possible role of melatonin receptors for anti-proliferative effects in cancer cell lines. These studies included the assessment of melatonin effects on extracellular kinases ERK1/2 activity and cyclin G2 expression.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines

- SK-UT-1B cell line, which was established in 1972 from a human uterine mixed mesodermal tumor consistent with leiomyosarcoma grade 3 from a 75-yr-old female Caucasian (Fogh et al., 1975): **untransfected cells**.
- SK-UT-1B stably transfected with the human MT₁ melatonin receptor cDNA (Knuffman et al., data not published): **MT₁-transfected cells**.
- SK-UT-1B stably transfected with the human MT₂ melatonin receptor cDNA containing a Flag epitope: **MT₂-transfected cells**.
- SK-UT-1B stably transfected with the human MT₂ receptor cDNA without Flag epitope: **MT₂-transfected cells without Flag**.

2.1.2 Vectors

- pcDNA-3, 5.4 kb, Invitrogen; Insert: human MT₂ receptor cDNA, ca.1100 bp. This vector containing the MT₂ receptor cDNA was a generous gift made to our group from Dr. Steven M. Reppert, Massachusetts General Hospital, Harvard Medical School. Boston, Massachusetts. The MT₂ cDNA sequence (Genbank accession no. U25341) has been published previously (Reppert. et al., 1994).
- pCMV-Tag4 containing a Flag epitope, mammalian expression vector, 4.3 kb; Stratagene.

2.1.3 Other materials

Except where stated, all chemicals were purchased from Sigma,

- LB (Luria-Bertani) medium was prepared by using 10 g of NaCl, 10 g of tryptone, 5 g of yeast extract and doubled distilled H₂O (ddH₂O) to a final volume of 1 liter. The pH was adjusted to 7 by adding 5 N NaOH, and the medium was autoclaved.
- LB-Agar was prepared by using 10 g of NaCl, 10 g of tryptone, 5 g of yeast extract, 5 g of yeast extract and 20 g of agar and ddH₂O to a final volume of 1 liter. The pH was adjusted to 7 by adding 5 N NaOH, and the medium was autoclaved.

- LB-Kanamycin (50 µg/ml) agar: 1 liter of LB Agar was autoclaved, cooled to 55°C, and then mixed with 5 ml of 10 mg/ml-filter-sterilized Kanamycin.
- LB-Kanamycin (50 µg/ml) medium was prepared by adding 5 ml of 10 mg/ml-filter-sterilized Kanamycin to 1 liter of autoclaved LB medium.
- SOC medium was prepared by mixing 0.5 g of NaCl, 20 g of bacto-tryptone, 5 g of bacto-yeast extract, 20 mM glucose and deionized H₂O to a final volume of 1 liter. The pH was adjusted to 7 by adding 5 N NaOH and the medium was autoclaved.
- E-PBS-buffer was prepared by mixing 0.10 M Sodium phosphate, 0.15 M NaCl, 0.005 M EDTA, 0.02% BSA and 0.01% Thimerosal. The pH was adjusted to 7 with NaOH.
- Horseradishperoxidase (HRP)-substrate solution: Equal volumes (500 µl) of 0.2% H₂O₂ in H₂O, substrate buffer (4.80 M sodium acetate, 0.24 citric acid) and TMB (0.5% TMB: 3,3',5,5'-tetramethylbenzidine in dimethyl sulfoxide) were used to prepared HRP-substrate solution.
- 5x TBE: 54 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH 8.0 in 1 l ddH₂O.
- 10x MOPS: 200 mM MOPS pH 7.0, 80 mM sodium acetate, 10 mM EDTA pH 8.0.
- 20x SSC: 175.3 g NaCl, 88.2 g Sodium citrate 2H₂O, in 1 l ddH₂O, pH 7.0.
- 1x PBS: 1.37 M NaCl, 26.8 mM KCl, 43 mM NaH₂PO₄, 14.7 mM KH₂PO₄.
- 1x TGS: 49 mM Tris base, 384 mM Glycine, 0.1% SDS, pH 8.7.
- TE, pH 7.4: 1M Tris base, pH 7.4, 0.5 M EDTA, pH 8.0.
- 1x TBS: 1 g Tris base, 4 g NaCl in 500 ml ddH₂O, pH 7.4.
- 1xTBST: 1x TBS + 0.05% Tween 20.

2.2 METHODS

2.2.1 Cell culture

2.2.1.1 Culture conditions

All cell lines were grown in Dubecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal calf serum (FCS, Greiner), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL) and 2 mM L-glutamine (Gibco BRL), and cultured at 37°C in 5% CO₂. Depending on the purpose, cells were grown on plastic dishes with different sizes (Greiner), or on 24 and 96-well-plates (Costar).

2.2.1.2 Trypsination

Cells were washed twice with sterile PBS and incubated in minimal amount of trypsin-EDTA (0.5 g/l trypsin, 0.2 g/l EDTA, Gibco/BRL) at 37°C until they had detached from the dish. The process was controlled under an inverted microscope. Trypsin was inhibited by addition of growth medium in which the cells were subsequently resuspended. Cell counting was performed, when necessary, using a Neubauer counting chamber, and the cells were plated out or harvested for cryoconservation.

2.2.1.3 Cryoconservation and thawing

Resuspended cells were spun down (1000g for 5 min at 4°C) in 4 ml growth medium. The supernatant was aspirated and the cells resuspended ($1-5 \times 10^7$ cell/ml) in ice-cold freezing medium [DMEM, 20% FCS, 10% dimethylsulfoxide (DMSO) which is cytotoxic at room temperature (RT)]. Cells were kept for 16 h at -80°C and then stored in liquid nitrogen.

For revitalization, frozen cells were quickly thawed, gently transferred to disposable Falcon tubes containing 4 ml cold growth medium and spun down as described above. Supernatant was discarded by aspiration, and cells were plated out after being resuspended in a suitable amount of growth medium.

2.2.2 Preparation of plasmids for transfection

2.2.2.1 Preparation and subsequent subcloning of DNA fragments

The human MT₂ receptor cDNA inserted into the pcDNA vector was a generous gift made to our group from Dr. Steven M. Reppert, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts.

In order to be able to localize the gene product (melatonin receptor) in the MT₂-transfected cells, a pCMV-Tag 4 vector (Stratagene) was used. This vector contains a Flag epitope, coding for an eight amino acid sequence (Asp-Try-Lys-Asp-Asp-Asp-Asp-Lys), that can be recognized by specific anti-Flag antibodies.

Cloning vectors were subjected to restriction endonuclease analyses. Digestions were performed in 50 µl of digestion buffer (Promega). Each probe containing either the pcDNA with the MT₂ cDNA insert or pCMV-Tag 4 vector was first incubated with 20 U of Hind III for 1 h at 37°C. The digestion was completed by following 1 h incubation with 20 U of Xho I at 37°C. DNA fragments were separated by agarose gel electrophoresis. Two bands corresponding to insert (1.1 kb) and vector (5.4 kb) were visualized in probes containing the digested pcDNA vector. Only one band of 4.3 kb, corresponding to the linearized vector, was detectable in probes containing the pCMV-Tag 4 vector. Bands corresponding to the MT₂ melatonin receptor cDNA and to the linearized pCMV-Tag 4 vector were excized from gel with a clean and sharp scalpel and purified with the "Qiaex II agarose gel protocol" (Qiagen) following the protocol of the manufacturer.

2.2.2.2 Ligation-subcloning DNA fragments into plasmid vectors

Restriction endonuclease fragments containing the cDNA expressing the MT₂ melatonin receptor were inserted into a cloning site, generated by the restriction endonuclease Hind III and Xho I (Promega), in the pCMV vector. The ligation was carried out using T4-DNA Ligase (Gibco BRL) at 16°C overnight, in 1x rapid buffer (50 mM Tris-Cl (pH 7.6), 10 mM MgCl₂, 1 mM DDT, 1 mM ATP, 5% w/v polyethylene glycol-8000).

2.2.2.3 Transformation of competent cells

The following transformation step was made by using Epicurian Coli XL1-Blue supercompetent bacteria (Stratagene). After thawing the cells slowly on ice, β-mercaptoethanol (Stratagene) in a final concentration of 25 mM was added to each aliquot of

bacteria. Ligated products were added to the Epicurian Coli XL1-Blue bacteria at a ratio of 1:10 and incubated for 30 min on ice. Cells were heat-shocked at 42°C for 30 sec and cooled on ice. One ml of preheated (42°C) SOC medium was added to each transformation and cells were incubated at 37°C for 1 h.

During this time the cells begin to proliferate, passing from stationary into logarithmic growth phase. After several generations, the bacterial cells possessing the desired recombinant vector will begin to express their antibiotic resistance. Standard cloning vectors generally possess nucleotide sequences for genes that offer specific antibiotic resistance, to kanamycin in this case, via an enzyme that modifies the antibiotic and prevents it from interacting with 70S ribosomes.

Following incubation at 37°C for 1 h, cells were plated on kanamycin agar plates (50 µg/ml) using a sterile spreader. Plates were incubated at 37°C overnight.

2.2.2.4 Plasmid preparations

Twenty-four colonies from plated cells were used to inoculate 3 ml of LB-Kanamycin (50 µg/ml) medium. Cultures were incubated overnight at 37 °C while shaking.

Plasmid DNA was recovered using a QIAprep Spin miniprep kit (Qiagen) for small scale plasmid preparation (mini-prep) and resuspended in 50 µl of TE buffer. This method generally yields 2-3 µg of plasmid DNA. To yield a larger quantity of the desired plasmid DNA (100-500 µg plasmid DNA), the remaining mini-prep cultures were used to inoculate a larger volume of LB-medium (+antibiotic), and were incubated overnight at 37°C while shaking. Cells were harvested by centrifugation at 4°C for 10 min at 400 rpm. Cultures were further processed using a JETSTAR plasmid Midiprep kit (Genomed) for plasmid DNA preparation as described by the manufacturer. Purified DNA was stored at -20°C. DNA concentrations were determined spectrophotometrically.

2.2.3 Spectrophotometric determination of DNA and RNA concentration

For quantification of DNA or RNA of an aqueous solution, the optical density (OD) of the solution is spectrophotometrically measured at wavelengths 260 nm (OD₂₆₀) and 280 nm (OD₂₈₀). the ratio between OD₂₆₀ and OD₂₈₀ (OD₂₆₀/OD₂₈₀) estimates the purity of the nucleic acids. Pure preparations of DNA and RNA have an ratio OD₂₆₀/OD₂₈₀ of 1.8 and 2.0, respectively. An OD₂₆₀ of 1 corresponds to 50 µg/ml doubled-stranded (ds) DNA, 40 µg/ml single stranded (ss) DNA and RNA, and 20 µg/ml for oligonucleotides.

2.2.4 DNA Sequencing

For sequencing plasmid DNA, an ABI PRISM Big DyeTM Terminators v3.0 Cycle Sequence Kit (Applied Biosystems) was used. The kit components were mixed with 250-500 ng DNA template and 10 pmol primer. Twenty-five amplification cycles were run on a Master cycler gradient under the following conditions:

Denaturation	96°C	10 sec
Annealing	50°C	5 sec
Extension	60°C	4 min

The PCR product was added to 2 µl of 3 M sodium acetate pH 5.2 and 50 µl 96% ethanol. The mixture was incubated for 10 min on ice and centrifuged 10 min at 14000 rpm. The resulting DNA pellet was washed twice with 70% ethanol, air-dried and submitted for sequencing. Sequencing was performed by means of an ABI PRISM 377 DNA Sequencer (Applied Biosystems) at the Central Service Laboratory, Institute for Cell Biology and Clinical Neurology, UKE.

2.2.5 Stable transfection of SK-UT-1B cells with the MT₂ receptor cDNA

Transfection is a non-viral method of introducing DNA into eucaryotic cells.

It was performed using the Lipofectin Reagent (Gibco BRL) which is a liposome formulation 1:1 (w/w) of a cationic lipid and dioleoyl phosphatidylethanolamine (DOPE). This reagent interacts spontaneously with DNA to form a lipid-DNA complex (Felgner et al., 1987). The fusion of the complex with cultured cells results in an efficient uptake and expression of the DNA.

Stable transfections were carried out either with the pCMV-Tag4 plasmid containing the MT₂ receptor cDNA (Flag-plasmid) or with pcDNA-3 containing the MT₂ receptor cDNA (without Flag-plasmid). The purpose of this last transfection (without Flag-plasmid) was to investigate whether Flag could alter native binding properties of the receptor to melatonin.

SK-UT-1B cells (2×10^5) were plated out in 75 mm dishes in growth medium supplemented with 10% FCS and incubated for 24 h at 37°C in a CO₂ incubator. Fifty µg of plasmid DNA and 50 µl of Lipofectin Reagent were diluted into 200 µl of DMEM medium and incubated at RT for 10 min. After overlaying the precipitation reaction onto the cells, incubation at 37°C for 24 h was followed. The DNA-containing medium was then replaced with 4 ml of growth

medium supplemented with 10% FCS. After incubation for 48 h, antibiotic selection with kanamycin or ampicillin (for pCMV-Tag 4 and pcDNA transfected cells, respectively), was started at a 1 mg/ml concentration in which selection was carried out up to the moment when single cell colonies were formed.

2.2.6 Cell treatments

Before the RNA isolation (2.2.7), protein extraction (2.2.15), cell proliferation assay (2.2.22) and ELISA (2.2.12, 2.2.13), cells were stimulated with different substances, in order to evaluate their influence on gene expression.

Stimulations were performed in serum-free medium, supplemented with 1% BSA and one of the following substances:

Substance	Final concentration	Stimulation time
Forskolin (FSK), Sigma	10 μ M	15 min
2-iodomelatonin (I-Mel), Sigma	10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} M	7.5, 20, 45 min, 1, 10, 48 h
IBMX (Sigma)	1 mM	15 min
17- β -Estradiol, (E2), Sigma	10 nM	48 h
4-PPDOT, Sigma	10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} M	7.5, 20, 45 min, 1, 48 h

Table 2: Substances used for cell stimulation

2.2.7 Isolation of total RNA from cells

RNA isolation was performed using TRIzol reagent (Gibco BRL).

For this purpose, untransfected as well as MT₁- and MT₂-transfected cells were cultured as described in 2.2.1., trypsinized, centrifugated (1800 rpm, 4 min) and the pellets homogenized with 1 ml of TRIzol Reagent. After 5 min incubation at RT to permit the complete dissociation of nucleoprotein complexes, 200 μ l of chloroform (Merck) were added. Tubes were shaken vigorously by hand for 15 sec and incubated for 3 min at RT. During centrifugation (12000 rpm, 15 min, 4°C), the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase where the RNA

remains exclusively. To precipitate the RNA, 500 µl of isopropyl alcohol (Merck) were added to the aqueous phase and the mixture was kept at -20°C overnight. After incubation at RT for 10 min and centrifugation (12000 rpm, 10 min, 4°C), the RNA was harvested followed by washing with 70% ethanol and centrifuged at 7500 rpm for 5 min at 4°C. RNA pellets were air-dried at 37°C for 10 min and resuspended in RNase-free water. RNA concentration was determined as described in 2.2.5.

2.2.8 Denaturing agarose gel

To check the integrity and size distribution of the total RNA, denaturing agarose gel electrophoresis was performed. For this purpose probes were run on a 1% agarose gel containing 35% formaldehyde (Merck) and 0.5 µg/ml ethidium bromide (Sigma) in 1x MOPS at 40 V. for 5 h, using 1x MOPS as running buffer. The respective ribosomal bands appear as sharp bands at 1.9 kb and 4.7 kb corresponding to the 18S and 28S ribosomal bands.

2.2.9 Reverse transcription

For the reverse transcription of RNA into first-strand cDNA, 1 µg of RNA was first heated at 70°C for 10 min and then incubated at 42°C for 30 min in the presence of 5 mM MgCl₂, 1x transcription buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton-X-100), 1 mM each dNTP, 1 U/µl Recombinant RNAsin ribonuclease inhibitor, 15 U/µl AMV reverse transcriptase and 0.5 µg Oligo(dT) primer. All reagents were supplied by Promega.

To inactivate the reverse transcriptase and to prevent it from binding to the cDNA, samples were heated at 99°C for 5 min followed by 5 min incubation on ice. For PCR analysis, probes were filled up to a final volume of 100 µl with nuclease-free water.

2.2.10 PCR analysis

PCR allows amplification of specific DNA sequences.

The specific primers for amplification of MT₁ and MT₂ cDNA sequences were:

	Primer Sequence	T _m
MT ₁ -specific primer forward	5' tcc tgg tca tcc tgt cgg tgt atc 3'	64.4°C
MT ₁ -specific primer reverse	5' ctg ctg tac agt ttg tcg tac ttg 3'	61.0°C
MT ₂ -specific primer forward	5' tcc tgg tga tcc tct ccg tgc tca 3'	66.1°C
MT ₂ -specific primer reverse	5' agc cag atg agg cag atg tgc aga 3'	64.4°C

Table 3: Sequences and melting temperatures (T_m) of primers used for amplification of MT₁ and MT₂ cDNA by PCR analysis

The specific primers for amplification of the estrogen receptor-alpha (ER α) cDNA sequences were:

Estrogen receptor α forward	5' ctc aac agc gtcg tct ccg ag 3'	61.4°C
Estrogen receptor α reverse	5' cat ttt ccc tgg ttg ctg tcc 3'	59.8°C

Table 4: Sequences and T_m of primers used for amplification of estrogen receptor α cDNA by PCR analysis

Reactions were performed in a PCR thermocycler (GenAmp) with 10 μ l of cDNA (2.2.9) in 30 μ l of Tris-HCl buffer, pH 7.2, containing 50 mM KCl and 1.5 mM MgCl₂ (Genecraft), 10 mM of each dNTP (Genecraft) and 0.5 μ l of Taq polymerase, (5 U/ μ l, Genecraft) in the presence of desired specific primers (25 pmol/ μ l). The reaction mix was denaturated at 95°C for 5 min, followed by the following program: denaturation at 95°C for 20 sec, annealing at 63°C (MT₁), 61°C (MT₂), 57°C (ER α) for 30 sec and extension at 72°C for 1 min. This was followed by a final extension step at 72°C for 10 min. Up to 35 PCR cycles were used.

2.2.11 Separation of DNA on agarose gel

Depending on the size of the DNA fragments, agarose gels with different matrix concentrations (1-1.5%) were cast. Agarose (Seakem EL) was melted in 1x TBE buffer. The solution was cooled down to 55°C, and ethidium bromide was added to a final concentration of 0.5 μ g/ml. Mixed with a respective amount of 6x loading buffer [0.25% bromophenol blue,

0.25% xylene cyanol FF, 40% (w/v) sucrose (Genecraft)], DNA was separated in 1x TBE running buffer in the agarose gel under tension of 80-120 V. The DNA fragments were visualized under UV light by means of a Compact Imaging System, (Imago).

2.2.12 cAMP-ELISA (enzyme-linked immuno sorbent assay)

To study whether melatonin affects the forskolin-stimulated intracellular cAMP accumulation, untransfected as well as MT₁- and MT₂-transfected cells were treated with forskolin alone or in the presence of increasing concentrations of melatonin, and the production of cAMP was determined by ELISA.

Cells were plated onto 24-well-plates and grown until confluence, which took 48 h. After washing the cells with 1x PBS, they were pre-incubated with 1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) for 15 min at 37°C. After aspiration of the medium, cells were then stimulated with forskolin, Sigma, (10 µM) or exposed to both forskolin (10 µM) and increasing (10^{-12} - 10^{-6} M) concentrations of 2-iodomelatonin for 10 min at 37°C. One ml 100% ice-cold ethanol was added to the wells. Plates were placed at -20°C overnight to facilitate the extraction of intracellular cAMP. The mixture was collected and centrifuged for 30 min at 3600 rpm to precipitate proteins and cell fragments. Supernatants were transferred to glass tubes and evaporated to dryness at 50°C for 45 min in a Vortex-evaporator (Vacuubrand). Residues were resuspended in E-PBS buffer. Samples containing forskolin alone or forskolin with melatonin were diluted 1:30. In control samples and those containing melatonin alone, dilutions were not required. A standard curve for cAMP was made using the following cAMP concentrations: 9.72, 3.24, 1.08, 0.36, 0.12, 0.04 and 0 pMol cAMP/ml E-PBS.

Standards and samples were collected for acetylation into small glass tubes. Thereafter, freshly prepared acetylation reagent (triethylamine and acetic anhydride, 2:1, Sigma) was added to all tubes and mixed immediately. Before starting the assay, microtiter strips (immunomodules NUNC coated with goat-anti-rabbit-gamma-globulines) were washed once with E-PBS-buffer. Standards and samples were pipetted first, then cAMP-antiserum 1:100.000 in E-PBS-buffer and finally 80 fmol/ml cAMP-biotin in E-PBS-buffer. Plates were incubated for 20 h at 4°C in a dark and humid chamber. After withdrawal of the solutions, cold HRP-streptavidin (150 ng/ml) was added to the wells, following 40 min incubation at 4°C. Wells were washed 4 times with cold wash-solution (0.02% Tween in H₂O₂, 0,5% NaCl). Plates were warmed up to RT for 5 min and HRP-substrate solution was added to the wells.

Plates were incubated for 45 min at RT in a dark and humid chamber. To stop the reaction 50 μ l of H₂SO₄ (2 M) were added to each well and the absorbance was measured at 450 nm in a ELISA-reader (SLT-Labinstruments).

2.2.13 cGMP-ELISA

To study whether melatonin affects the level of cGMP production, untransfected as well as MT₁- and MT₂-transfected cells were treated with increasing (10^{-12} - 10^{-6} M) concentrations of 2-iodomelatonin in the presence of 1 mM IBMX as described in 2.2.12. The production of cGMP was determined by ELISA. For this purpose assay procedure was performed as described in 2.2.12. A standard curve was made using the following cGMP concentrations: 34.02, 11.34, 3.78, 1.26, 0.42, 0.14 and 0 pMol cGMP/ml E-PBS. Dilutions of samples and acetylation were not required. cGMP-antiserum was diluted 1:80.000 in E-PBS-buffer and the cGMP-biotin concentration used was 170 fmol/ml E-PBS-buffer.

2.2.14 Melatonin bindings assay

2.2.14.1 Protein measurement

(according to Bradford, 1985)

The principle of the assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to proteins occurs. This binding is compared to a standard curve generated by the reaction of known amounts of a standard protein.

Protein concentrations of untransfected as well as MT₁-, MT₂- and MT₂-without Flag transfected cells, were determined prior to binding assays.

For this purpose, all cells were grown until confluence in 75 mm culture dishes. Using a cell scraper, cells were removed from the surface and centrifuged at 1200 rpm for 4 min. Cells pellets were washed twice with 2 ml of 1x PBS, and resuspended in 50 mM Tris-HCl, pH 7.5 and 5 mM MgCl₂. A standard curve was made using the following concentrations of bovine serum albumin (BSA, Sigma): 4, 8, 12 and 16 μ g/ml. The assay was performed using a Bio-Rad protein Assay Kit (Bio-Rad), following the protocol of the manufacturer. The absorbance of the samples was measured at 590 nm.

2.2.14.2 Binding assay

For the binding assay, untransfected as well as MT₁-, MT₂-, and MT₂-without Flag transfected cell samples, corresponding to 50-100 µg of protein were incubated in the presence of increasing concentrations of 2-[¹²⁵I]iodomelatonin (3, 10, 25, 50, 100, 200, 400, 800 and 1600 pM). For competition studies 4-PPDOT at a concentration from 10⁻¹⁰ to 10⁻⁵ M was used. To evaluate non specific binding incubations were performed in the presence of an excess (100 µM) of cold 2-iodomelatonin. After 90 min reactions were stopped by addition of 2 ml of ice-cold Tris buffer. To separate bound and free ligand, the mixture was filtered through GF/B filters, 0.25 mm diameter (Schleicher & Schüll), previously incubated in 50 mM Tris buffer, 5 mM MgCl₂, pH 7.4. Filters were washed twice with 4 ml ice-cold Tris buffer and bound radioactivity was measured in a Gamma-counter 17740 Wizard, Wallac.

2.2.15 Protein extraction by sub-cellular fractionation

For sub-cellular fractionation cells were washed with 1x PBS and harvested by gently scrapping into ice-cold isotonic sucrose buffer containing 10 mM Tris-HCl pH 7.4, 0.25 M sucrose and serine/cysteine protease inhibitors present in the protease inhibitor Cocktail tablets (complete 1 836 170, Roche). Cells were gently homogenized using an all-glass Dounce type homogenizer (Biospec Products). The homogenate was centrifuged at 100xg for 15 min and the supernatant, containing cytosol and a particulate-membrane fraction, was recentrifugated at 100,000xg for 60 min to yield particulate non-nuclear membrane fractions (pellet containing plasma-, microsomal membranes and organelles i.e., mitochondria, lysosomes etc.) and the supernatant (cytosolic fraction). Sub-fractions were filtered through Centricon Ultrafilters with 10k-Cut (Amicon-Millipore) and protein content was determined according to Bradford (2.2.14).

2.2.16 SDS-polyacrilamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE is based on the ability of SDS, an anionic detergent, to denature and charge uniformly negatively protein molecules. Thus, the proteins undergoing an electrophoresis are separated only according to their molecular weight. For a better resolution, a discontinuous SDS-PAGE was performed according to Laemmli (1970).

Discontinuous electrophoresis consisted of a separating gel (10-12% bis:acrylamide solution, 0.375 M Tris/HCl pH 8.8, 0.1% SDS, 0.1% APS, 0.08% TEMED) and stacking gel (5% bis:acrylamide solution, 0.125M Tris/HCl pH 6.8, 0.1% SDS, 0.1% APS, 0.1% TEMED). The

separating gel solution was carefully pipetted down between glass plates, overlaid with ddH₂O and allowed to polymerize at RT for 40 min. After pouring the overlaying ddH₂O, the stacking solution was pipetted down and a comb was inserted. The gel was allowed to polymerize for 60 min. The comb was removed and wells were rinsed with ddH₂O, which was discarded by aspiration. Probes solubilized in a sample buffer, and boiled at 95 °C for 5 min, were loaded on the gel and overlaid with 1x Tris-Glycine (TGS) buffer. Prestained molecular markers (BENCHMARK prestained protein ladder, Gibco BRL) and rainbow coloured protein molecular weight marker (Amersham) were used as standards. Electrophoresis was run at 140 V in a chamber filled with 1x TGS buffer typically for 60 to 90 min.

2.2.17 Protein transfer from SDS-PAGE gels to membranes

Protein electrotransfer from SDS-gels to nitrocellulose or PVDF membranes was carried out by the semidry method according to Bjerrum (1986).

Transfer buffer: 48 mM Tris/HCl, 39 mM Glycin, 0.0375% SDS, 20% methanol (v/v)

Separating gel, nitrocellulose membrane (Millipore) and 7 sheets of 3MM Whatman paper were equilibrated in transfer buffer for 5 min. PVDF membranes (Millipore or Boehringer) were equilibrated in 100% methanol for 5 min, followed by incubation in transfer buffer for 5 min. A transfer sandwich (3 sheets of paper, running gel, membrane, 4 sheets of paper) was assembled in a semidry blotting chamber and run at 150 mA for 60 min.

2.2.18 Detection of proteins in SDS-polyacrylamide gels by Coomassie staining

After transfer, gels were simultaneously fixed and stained in 50% methanol, 10% acetic acid and Coomassie blue R250 (Sigma) at RT for 25 min. Gels were destained in 50% methanol, 10% acetic acid, washed with ddH₂O, and dried between celophane sheets with an air gel dryer (H. Hölzel).

2.2.19 Detection of proteins by Immunoblotting (Western Blot)

Membranes were blocked for 1 to 3 h at RT in I-block solution (Tropix). Blots were incubated for 1h or overnight with primary antibodies (Table 5) diluted in I-block solution. Membranes were rinsed 5 times 10 min with 1x TBST and the respective secondary antibody (Table 6)

was added. After incubation for 1 h at RT, blots were rinsed as before and the antibody binding was visualized with ECL kit (Pierce) by means of X-Omat AR films (Kodak). To reprobe used nitrocellulose and PVDF membranes, bound antibodies were removed by gentle shaking in 0.2 M NaOH for 5 min at RT. After washing membranes twice with ddH₂O for each 5 min, unspecific binding sites were blocked in I-block solution, followed by incubation with primary antibodies.

Primary Antibody	Host	Dilution	Producer
Anti-Flag M2	Mouse	1:1000	Sigma
Anti-human c-Fos	Rabbit	1:1000	Santa Cruz Biotechnology
Anti-human phospho-p44/42 MAPK (Thr202/Tyr204)	Mouse	1:1000	Cell signalling Technology
Anti-β-actin	Mouse	1:15000	Oncogene

Table 5: Primary antibodies used in Immunoblotting

Secondary antibody	Dilution	Producer
Peroxidase-conjugated rabbit anti-mouse IgG	1:3000; 1:5000	Jackson Immuno Research
Peroxidase-conjugated goat anti-rabbit IgG	1:5000, 1:10000	Jackson Immuno Research

Table 6:Secondary antibodies used in Immunoblotting

2.2.20 Competition studies with blocking peptides

Antibody specificities were studied by competing with excess of antigen (peptide). A small volume of antibody was first reacted with peptide to be neutralized. The neutralized antibody can no longer subsequently bind the specific antigen and therefore, the band staining that is competed by the peptide is considered to be specific. If more than one band disappears by peptide competition, those bands have the antigenic determinants and could be considered either fragments of the large antigen or multimers.

Peptides and antibodies were incubated at 4°C overnight in the following molar ratio:

c-Fos blocking peptide (Santa Cruz) + Anti-c-Fos antibody, 1:750

Flag blocking peptide (cell signalling) + Anti-Flag antibody, 1:80

Immunoblots were then performed as described in 2.2.19.

2.2.21 $[Ca^{2+}]_i$ measurements by fluorescence microscopy

For imaging cytoplasmic calcium in individual living cells a ratiometric dye (Fura-2) was used. This fluorophore molecule can be free or bind to Ca^{2+} . This change in the fluorophore molecule will lead to alterations in the fluorescence emission spectrum [from 380nm (Fura-2-free- Ca^{2+}) to 334 nm (Fura-2- Ca^{2+})]. An increase in the intracellular Ca^{2+} concentration will induce an increase in the fluorescence emission at 334 nm.

For the analysis of the intracellular calcium concentration of untransfected, MT₁- and MT₂-transfected cells an Attofluor ratio imaging system (ZEISS) was used. Excitation light was provided by a mercury lamp (Osram HBO 100W/2); excitation wavelengths of 334 and 380 nm were selected by interference filters mounted on a computer-controlled filter changer. Exposure times were controlled by a shutter between the filters and a neutral density filter which was used in conjunction with manual light setting to obtain approximately equal fluorescence intensities at the two different excitation wavelengths. Fluorescent light was collected by an Achrostat 40 x oil-immersion objective (ZEISS), passed through a dichotic mirror (395 nm) and an emission filter (500-530 nm) and finally transmitted to a CCD camera with a photomultiplier. A personal computer with appropriate software (Attofluor Ratio Vision; Atto instruments, Rockville, U.S.A.) was used to control the optical equipment and to record, analyze and store the images and data.

Untransfected as well as MT₁- and MT₂-transfected cells were diluted to 1.5×10^3 cell/ml, immobilized on cover slips 12 mm ø (Roth) previously sterilised in 70% alcohol in 24-well-plates. After cells were attached to the plates (12 h, 37°C), they were loaded with 3 µM Fura-2/AM (Molecular Probes) for 15 min at 37°C in growth medium containing 10% FCS. Subsequently, cover slips were rinsed with saline (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM Hepes, pH 7.4), carefully placed into a superfusion chamber mounted in a heatable stage and superfused with saline at 37°C. Between 19 to 52 cells were analyzed in each experiment. All drugs tested (2-iodomelatonin, Sigma and Uridine 5'-triphosphate UTP used as internal positive control, Pharmacia) were stored frozen as stock

solutions and diluted (100 nM and 10 μ M, respectively) with pre-warmed saline immediately before use. Experiments were performed by stopping the perfusion and adding the drugs with a Pasteur pipette into the perfusion chamber.

2.2.22 Proliferation assays

To determine the number of viable cells, a proliferation assay (CellTiter 96 AQueous assay, Promega) was performed. Two solutions were used for this purpose: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent, phenazine methosulfate (PMS).

MTS is reduced by cells into formazan that is soluble in tissue culture medium. The absorbance of the formazan at 490 nm can be measured directly from 96 well assay plates without additional processing. The conversion of MTS into formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

After cell density was determined Neubauer using a counting chamber, suspended cells were diluted in growth medium to obtain cellular densities of 2×10^4 cells/ml. Cells were plated in 96-well-plates and incubated at 37°C for 5 h. Untransfected as well as MT₁- and MT₂-transfected cells were incubated in the presence of increasing (10^{-12} to 10^{-6} M) 2-iodomelatonin concentrations, 10 nM 17- β -estradiol, 10 nM 4-PPDOT or 0.005% volume of ethanol (vehicle) as described in the results section. After 48 h incubation, 20 μ l of combined MTS:PMS (1:0.2) (w/w) solution were added to each well. Plates were incubated for 4 h at 37°C in a humidified 5% CO₂ atmosphere. The absorbance was recorded at 490 nm using an ELISA plate reader (SLT-Labinstruments).

2.2.23 Microarrays

Microarray experiments rely on the principles of hybridization (base pairing). Complex cDNA probes that have been labelled with cyanine dyes are hybridized to targets (PCR products or oligonucleotides) that have been immobilized on a glass surface. The control cDNA and the experimental cDNA are labelled with two different dyes. The ratio of the dye signal is a measurement of the amount of specific RNA in the samples. The principle of the microarray hybridization is shown in Fig. 6.

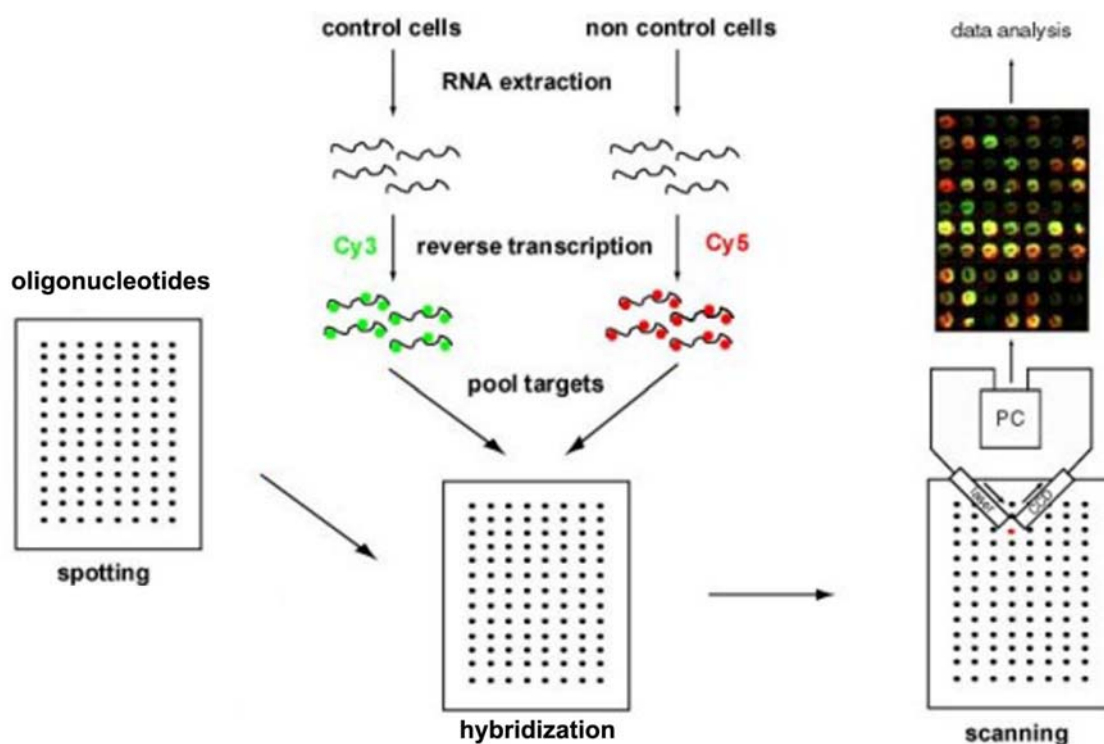


Fig. 6: The principle of microarray hybridization

After hybridization with the experimental probes, the microarray can be used to determine what gene transcripts are expressed by examining the fluorescence present in each probe location.

In order to determine whether melatonin has an effect on gene expression, RNA from untransfected as well as from both MT₁- and MT₂-transfected cells was compared by microarray. All three cell lines were treated with 1 nM 2-iodomelatonin for 10 h and total RNA was extracted as described in 2.2.7. RNA yields and quality were assessed as described in 2.2.3 and 2.2.8, respectively.

To obtain strong hybridization signals, the RNA was amplified with the Message AMP aRNA kit (Ambion). The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter and *in vitro* transcription of the resulting DNA with T7 RNA polymerase to generate hundreds to thousands of antisense RNA (aRNA) copies of each mRNA sample. During this last step Cyanine 3 (Cy3) and Cyanine 5 (Cy5) modified nucleotides are incorporated into the aRNA.

All reagents unless otherwise specified, were provided by Ambion. First-strand cDNA synthesis was performed with 5 µg of total RNA, preheated 10 min at 70°C, in the presence of

T7 Oligo(dT) primer, reverse transcriptase, RNase inhibitor, buffer and dNTPs at 42°C for 2 h. Concentrations were provided by the supplier. Second-strand DNA was synthesized with 20 µl of first-strand cDNA in the presence of buffer, dNTPs, DNA polymerase and RNase H at 16°C for 2 h (concentrations provided by the supplier), followed by treatment with 1.5 µl RNase A (20 mg/µl) and 1 µl of proteinase K (10 mg/µl) at 37°C for 30 min. The cDNA was purified following the protocol of the manufacturer and concentrations were assessed by spectrophotometry as described in 2.2.3. Reverse transcription was performed after concentrating the samples to 3 µl (40 µg) in a speed vacuum centrifuge, in the presence of DNase I, T7 Enzyme Mix, 75 mM T7 ATP, CTP, GTP and UTP solution and 75 mM Cy3/Cy5 UTP (Amersham) for 4 h at 37°C in the dark.

In order to detect which genes are activated in which cell line, RNA from untransfected and MT₁-transfected cells was labelled with Cy3 and RNA from MT₂-transfected cells was labelled with Cy5.

DNase treatment with DNase I at 37°C for 30 min was made prior to aRNA purification (protocol of the manufacturer). Concentration and quality of the labelled aRNA were assessed by a spectrophotometric wavelength scan. In the probes labelled with Cy3, two peaks, one at 260 nm (RNA) and other at 570 nm (Cy3) were detected. In the probes labelled with Cy5, also two peaks were detected, the last one displaced to 670 nm (Cy5). Ten µg of each aRNA from untransfected as well as from MT₁- and MT₂-transfected cells were pooled together and fragmented for 15 min at 94°C in the presence of fragmentation buffer (20 mM Tris/acetate pH 8.1, 50 mM K⁺-acetate, 15 mM Mg²⁺-acetate). Purification of the labelled aRNA prior to slide hybridization was performed with the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. Labelled aRNA was hybridized at 42°C for 16 h to a Pan Human 10k Array (MWG Biotech) consisting of 9.850 genes-specific oligonucleotides (50-mers) spotted onto a glass slide. After gradient stringent washing (2x SSC, 0.05% Triton-X, 10 min; 1x SSC 0.05%, Triton-X, 10 min; 0.2x SSC, 0.05% Triton-X, 10 min), the array was scanned using a microarray laser scanner (Affimetrix 428) at 'gain 60' and 10 micron resolution. For evaluation and calculation of signal intensities, the array image was quantified using the Phoretix Array²-Software (Nonlinear Dynamics, Durham, NC).

2.2.24 Quantitative real time-PCR

Quantitative real-time PCR (RT-PCR) is based on the detection of a fluorescent signal produced proportionally during the amplification of a PCR product. The fluorophore used in this case is SYBR Green, which binds all double-stranded DNA molecules.

In order to confirm the up-regulation of cyclin G2 transcript in MT₂-transfected cells after melatonin stimulation (as shown by microarray analyses), the same RNA extracted from untransfected and from MT₁- and MT₂-transfected cells as used in the microarray experiment was reverse transcribed to cDNA (as described in 2.2.11) and analyzed by RT-PCR.

Amplification was performed with 500 ng of cDNA in 2x Quantitec SYBR green PCR Master MIX (Qiagen), which contains Hotstart Taq DNA polymerase, dNTPs, Quantitec SYBR green PCR buffer and SYBR Green in a concentration provided by the supplier. The primers used are listed in the table 7 and were used in a concentration of 0.5 μ M (for both forward and reverse).

	Primer sequence	T_m
Cyclin G2 forward	5' ggg gtc caa ctt ctt ggg ttg ttg aac g 3'	69.5°C
Cyclin G2 reverse	5' gta cat tta cac tga atc cgg 3'	59.3°C
hGAPDH forward	5' gtc ttc acc acc atg gag 3'	56°C
hGAPDH reverse	5' gtc atg gat gac ctt ggc 3'	56°C

Table 7: Sequences and T_m of primers used by RT-PCR

The reaction conditions for the Light cycler (Roche) were as follows: denaturation step, 95°C, 20 sec; annealing, 57°C (cyclin G2) and 51°C (GAPDH), 20 sec; elongation: 72°C, 25 sec; and acquisition 81°C, 15 sec. Up to 40 PCR cycles were used. Melting curve analysis was performed at the end of each run. Variations in cDNA concentrations were normalized against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a catalytic enzyme involved in glycolysis whose gene is constitutively expressed at high levels in almost all tissues. For each assay internal standard curves were developed.

3 RESULTS

3.1 Development of stable SK-UT-1B cell line expressing the human MT₂ melatonin receptor

The human MT₂ melatonin receptor cDNA, cloned into the expression vector pCMV-Tag 4 containing a Flag epitope was stably transfected into SK-UT-1B cells. The Flag sequence is located in the C-terminus of the protein and was used in order to recognize the gene product (melatonin receptor). Colonies resistant to the antibiotic kanamycin were selected and grown. Eleven out of twenty colonies tested expressed the MT₂ receptor gene as indicated by RT-PCR analyses. All of these bound 2-[¹²⁵I]iodomelatonin (200 pM) in a specific manner. A particularly robust cell line (MT₂-SK-UT-1B), selected through the limited dilution technique, was used for further studies.

To investigate whether Flag could alter the native binding properties of the receptor to melatonin, the human MT₂ melatonin receptor cDNA, cloned into the expression vector pcDNA was also stably transfected into SK-UT-1B cells. Colonies resistant to ampicillin were selected and grown. Three out of 6 colonies tested bound 2-[¹²⁵I]iodomelatonin (200 pM) specifically. One selected cell line (MT₂-SK-UT-1B without Flag), showing a particularly high specific binding to melatonin, was used for further comparative studies.

3.2 Molecular characterization of melatonin receptor subtypes

3.2.1 RT-PCR-Analyses

In order to confirm the expression of MT₂ in the selected MT₂-SK-UT-1B cell line, RT-PCR analyses using MT₂-specific primers were performed.

In addition, to demonstrate the specificity of the reaction and to ensure the absence of an endogenous expression of melatonin receptors in these cells, as well as in untransfected and MT₁-transfected cells, RT-PCR analyses using MT₁- and MT₂- specific primers were performed. These studies included (i) the isolation of cellular RNA (ii) the enzymatic reverse transcription of the RNA into cDNA and (iii) polymerase chain reaction with MT₁- and MT₂-specific primers. To serve as further specificity controls, plasmids containing either MT₂- or MT₁- cDNA were used as templates.

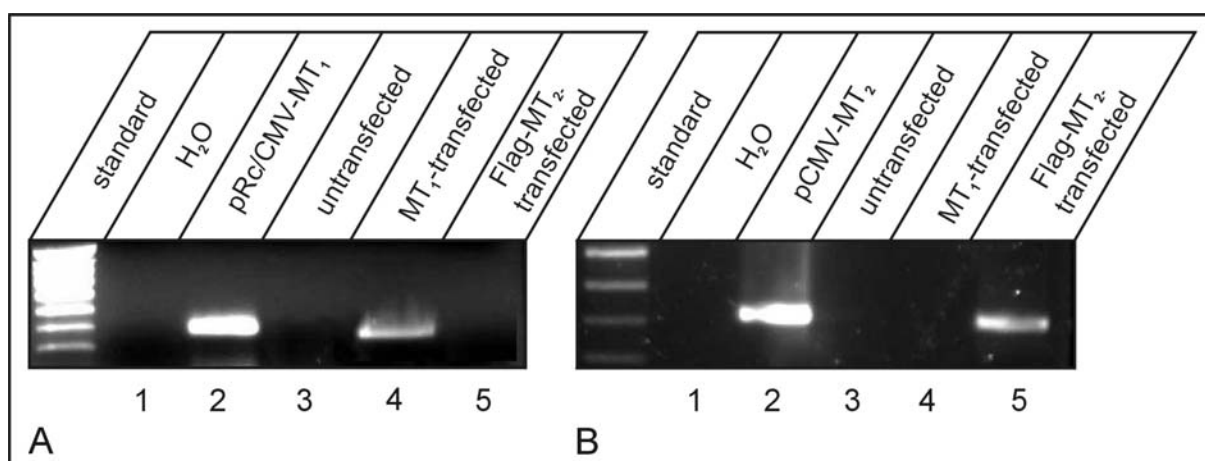


Fig. 7: RT-PCR analyses of melatonin receptor cDNA expression in MT₁- and MT₂-transfected cells using (A) MT₁-specific or (B) MT₂-specific primers

Total RNA was isolated from untransfected, MT₁- and MT₂ transfected cells and analyzed by semiquantitative RT-PCR. Plasmids (pRc/CMV and pCMV) containing the MT₁ or MT₂ receptor cDNAs were used as positive controls. Untransfected cells served as negative controls. PCR products were visualized on agarose gel by ethidium bromide staining. No amplification was observed when the experiments were performed in the absence of DNA (H₂O). The migration of molecular size markers (standard) is indicated.

MT₁ receptor subtype A single PCR product of the expected size (279 bp) was detected after amplification of cDNA from MT₁-transfected cells, Fig. 7A (lane 4), which corresponds to the product found in the positive control (lane 2). No products were found with RNA isolated from untransfected (lane 3) and MT₂-transfected cells (lane 5), as well as when experiments were performed in the absence of DNA (lane 1).

MT₂ receptor subtype A single PCR product of the expected size (320 bp) was detected on amplification of cDNA from MT₂-transfected cells, Fig. 7B (lane 5), which corresponds to the product found in the positive control (lane 2). No products were found with RNA isolated from untransfected (lane 3) and MT₁-transfected cells (lane 4) and when the experiments were performed in the absence of DNA (lane 1).

These studies confirm that the MT₂-SK-UT-1B cells but not the untransfected cells contain MT₂ cDNA. In addition, the experiments demonstrate the specific and transfection-dependent expression of MT₂- or MT₁-cDNA, respectively, in the cells examined.

3.2.2 Western blot analyses demonstrate the presence of MT₂ receptors in MT₂-transfected SK-UT-1B cells

To confirm the presence of the MT₂ melatonin receptor in the cell membrane of the MT₂-transfected cells containing the Flag epitope, immunoblot analyses using a specific anti-Flag antibody were performed. This antibody was used because no specific antibody against the MT₂ receptor subtype is currently available. Fig. 8 shows that the MT₂ melatonin receptor is present in the cell membrane of the MT₂-transfected cells. The apparent molecular mass of the immunoreactive band is approximately 60 kDa, in agreement with the previously reported size of the receptor (Ayoub et al., 2002; Brydon et al., 1999). Controls prepared from untransfected SK-UT-1B cells show no signal. Competition studies with an excess of blocking peptide to neutralize the Flag antibody were performed to confirm antibody specificity. The band representing the MT₂ melatonin receptor disappears after peptide competition.

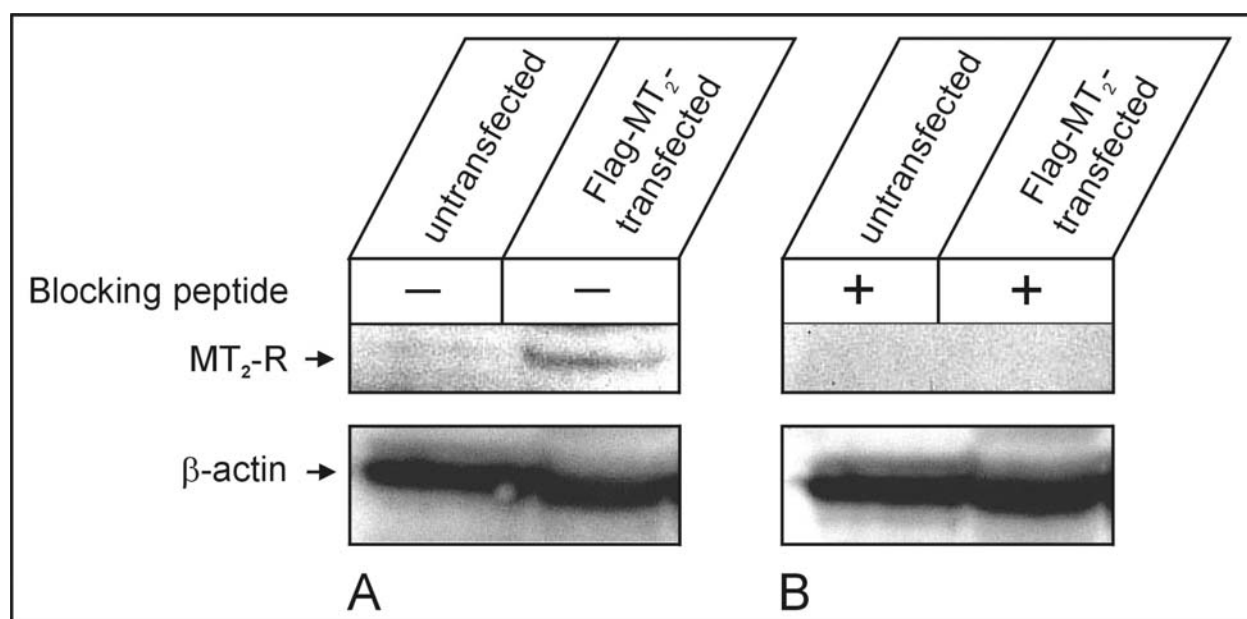


Fig. 8: Detection of the MT₂ receptor by immunoblotting

Crude membranes of untransfected and MT₂-transfected cells were submitted to SDS-PAGE, and immunoreactivity was revealed using anti-Flag antibody (A). Equal amounts of membrane proteins (40 µg) were loaded. (B) Analogous assay performed after pre-incubation of anti-Flag antibody with an excess of Flag-blocking peptide in a 1:80 molar ratio. Comparative assessment of β-actin immunoreactivity served as loading control.

3.3 Biochemical characterization of melatonin receptor subtypes expressed in SK-UT-1B cells

3.3.1 High-affinity binding of 2-[¹²⁵I]iodomelatonin to MT₁- and MT₂-melatonin receptors on SK-UT-1B transfected cells

Saturation binding studies were performed using increasing (25-1600 pM) concentrations of 2-[¹²⁵I]iodomelatonin to verify the functional expression of the melatonin receptor proteins in MT₂-SK-UT-1B cells. A number of colonies obtained were tested for MT₂-receptor expression. The clone expressing the highest 2-[¹²⁵I]iodomelatonin binding (single point assay) was chosen for subsequent studies.

One of the major objectives in this study was to establish an *in vitro* human model system that can be employed to explore the mechanism by which melatonin specifically exerts its effects via the MT₁- or the MT₂-receptor, respectively. Therefore, binding characteristics of MT₁ and MT₂ receptors in MT₁-, MT₂- (with Flag) and MT₂- (without Flag) transfected cells were determined (Fig. 9). Since generally the MT₂-transfected cells with Flag were used in this study, it was important to prove that the Flag epitope does not essentially alter ligand binding properties of the receptor. Based on non-linear regression analyses (PRISM, GraphPad Software, Inc., San Diego, CA) of ligand binding to membrane preparations of MT₁-transfected cells, the binding maximum (B_{max}, receptor density) was found to be 14.21 ± 1.619 fmol/mg protein. The K_d, representing the affinity or equilibrium dissociation constant, based on values for half-maximal melatonin binding was calculated as 125.7 ± 50.15 pM. For MT₂-transfected cells with Flag, these values are 16.6 ± 2.035 fmol/mg protein and 74.79 ± 14.71 pM and for MT₂-transfected cells without Flag 24.45 ± 1.034 fmol/mg protein and 37.84 ± 7.283 pM. No specific binding was observed when experiments were carried out with untransfected cells (data not shown). Data are summarized in Table 8.

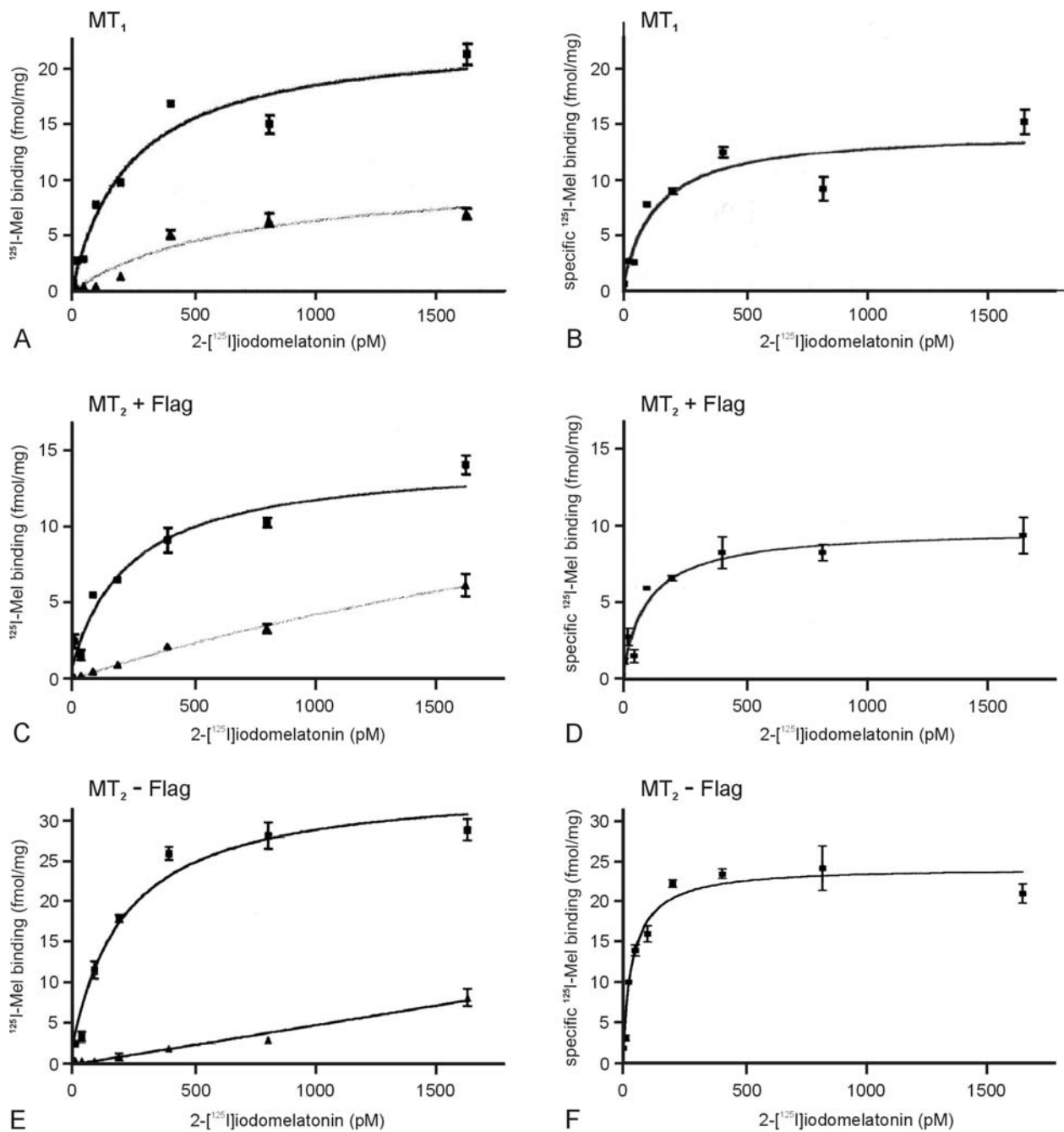


Fig. 9: Representative saturation study of 2-[¹²⁵I]iodomelatonin binding to MT₁-transfected (A,B) MT₂-transfected cells with Flag (C,D) and MT₂-transfected cells without Flag (E,F) Specific melatonin binding (B,D,F) was determined at equilibrium as a function of 2-[¹²⁵I]iodomelatonin concentrations and calculated from the difference between the amount of 2-[¹²⁵I]iodomelatonin bound in the presence (-▲-: non specific binding) or absence (-■-: total binding) of 1 μM unlabelled melatonin (A,C,E). Data are mean ± SE from three independent experiments each.

	K_d (pM)	B_{max} (fmol/mg protein)
MT ₁ -transfected cells	125.7±50.15	14.21±1.619
MT ₂ -transfected cells with the Flag epitope	74.79±14.71	9.617±0.587
MT ₂ -transfected cells without the Flag epitope	37.84±7.283	24.45±7.283

Table 8: K_d and B_{max} values of MT₁-, MT₂- with Flag and MT₂-without Flag transfected cells

These data established the presence of high-affinity melatonin receptors in all three cell lines. Although certain influences of the Flag epitope on MT₂-binding were apparent, the usability of cells transfected with MT₂ plus Flag was proven by specific binding parameters. Moreover, the data obtained were consistent with reported values of receptor binding in native tissues, since the B_{max} values (9.617±0.587 fmol/mg) were similar to those obtained for human PAZ6 adipocytes (7.46±1.58 fmol/mg) natively expressing the MT₂ melatonin receptor (Brydon et al., 2001).

To further prove typical properties of the two receptor subtypes, 2-[¹²⁵I]iodomelatonin binding to the MT₁ and MT₂ melatonin receptors was further examined by competition experiments using the melatonin receptor antagonist 4P-PDOT, which has been reported to have 100 times higher affinity for MT₂ receptors as for MT₁ receptors (Nonno et al., 1999). Comparison of the competition curves obtained between MT₁ or MT₂ membrane preparations revealed a higher potency of 4P-PDOT to block MT₂ receptors. At a concentration of 10 nM, 4P-PDOT was 100 times more efficient in case of MT₂ (-plus Flag-) as compared to MT₁-transfected cells (Fig. 10).

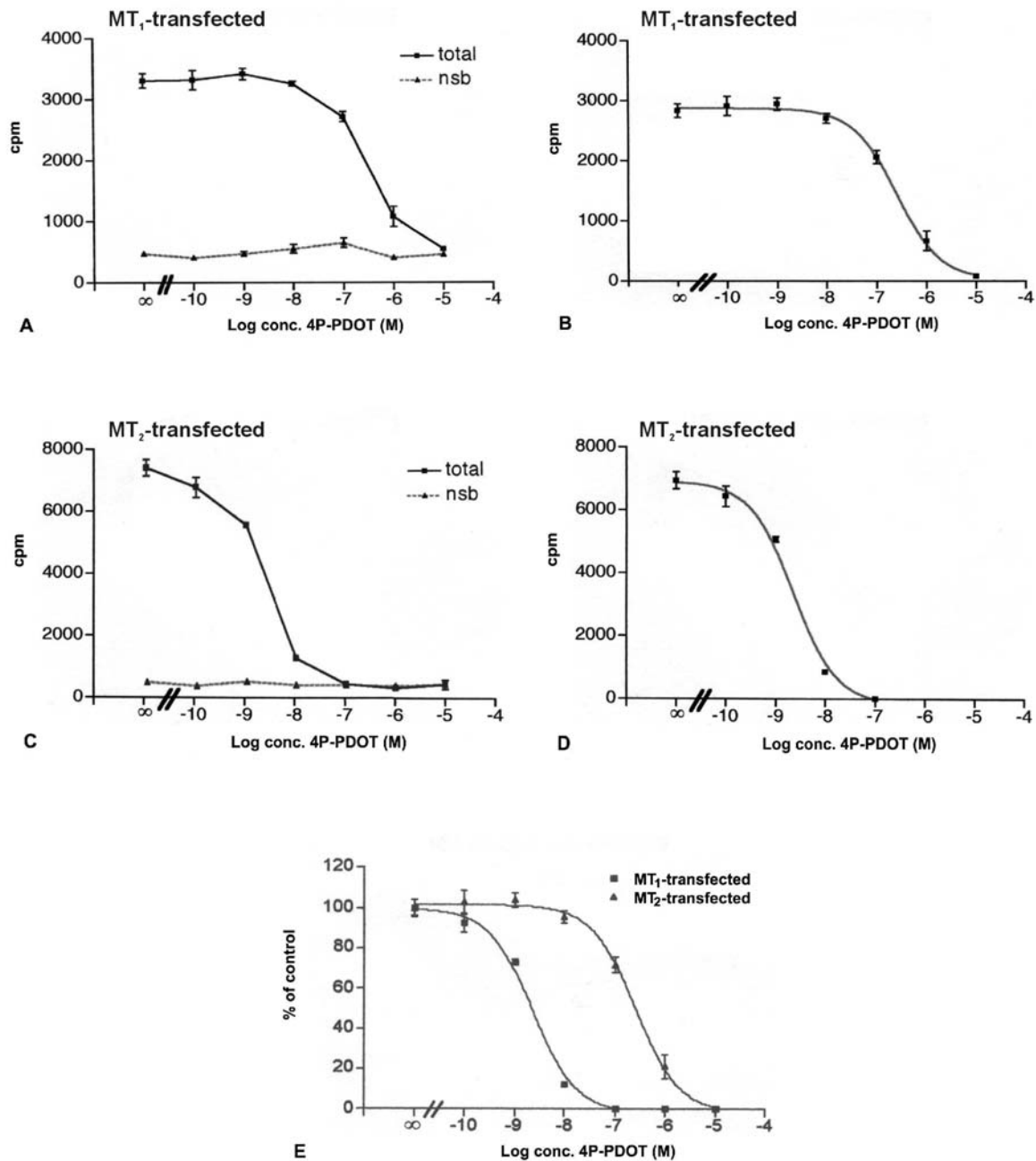


Fig. 10: Competition of 4P-PDOT with 2-[¹²⁵I]iodomelatonin binding to MT₁- and MT₂-transfected cells

Specific melatonin binding was determined at increasing (10^{-10} to 10^{-5} M) concentrations of 4P-PDOT. A: total and non specific binding (nsb) to MT₁-transfected cells. B: specific binding to MT₁-transfected cells. C: total and non specific binding (nsb) to MT₂-transfected cells. D: specific binding to MT₂-transfected cells. E: Comparison between MT₁- and MT₂-transfected cells. Note that the melatonin receptor antagonist shows a higher affinity for the MT₂ as compared to the MT₁ receptor subtype.

3.3.2 Functional assays

3.3.2.1 Assessment of forskolin-stimulated cAMP levels

Melatonin has been shown previously to inhibit forskolin-induced cellular accumulations of cAMP (Reppert et al., 1995; Petit et al., 1999). To prove that MT₁ and MT₂ melatonin receptors in MT₁- and MT₂-transfected cells are functionally coupled to signal transduction pathways, untransfected as well as MT₁- and MT₂-transfected cells were preincubated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and then treated with forskolin (10 μ M) in the presence of different concentrations of 2-iodomelatonin. Incubation of cells with 10 μ M forskolin increased intracellular cAMP concentrations approximately 10-fold. This increase was inhibited by 2-iodomelatonin in a dose-dependent manner, resulting at the highest ligand concentration (1 μ M) in a reduction by 50% of cellular cAMP levels in both cases (Fig. 11). As compared to cAMP levels produced in the absence of melatonin, there is no effect of melatonin in untransfected cells. 2-iodomelatonin alone had no effect on basal cAMP levels.

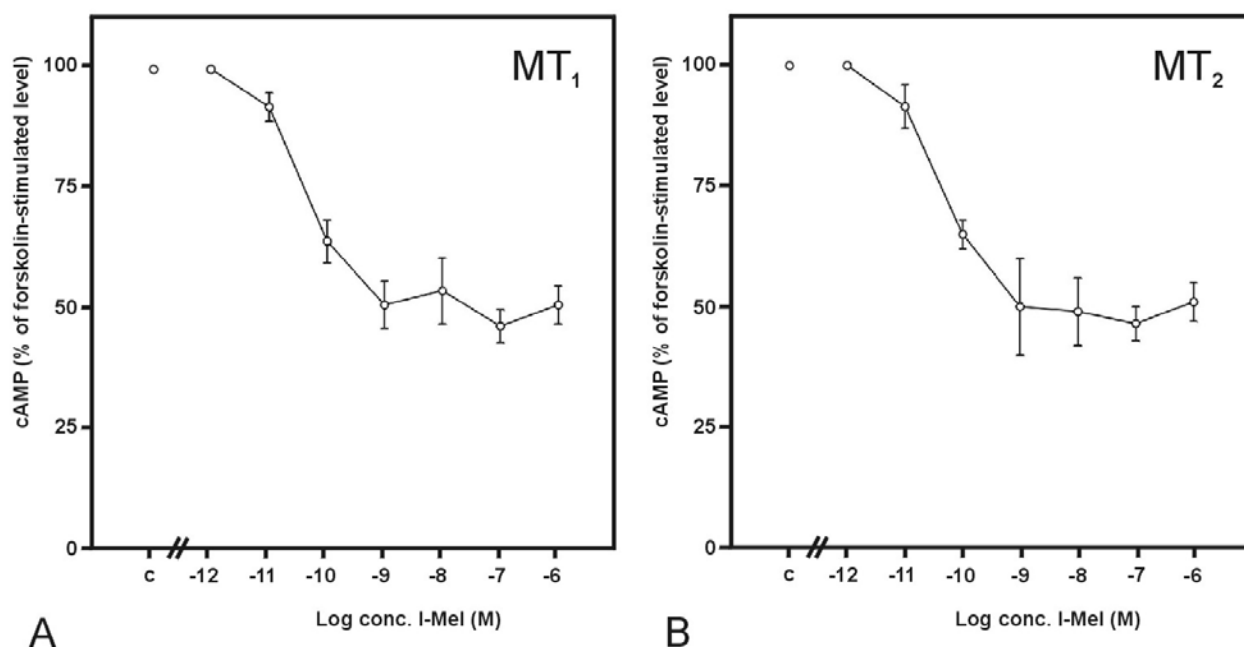


Fig. 11: Modulation of forskolin-stimulated cAMP accumulation by 2-iodomelatonin in MT₁-transfected cells (A) and MT₂-transfected cells (B)

Cells were stimulated for 15 min at 37°C with forskolin (10 μ M) in the presence of the indicated concentrations of 2-iodomelatonin in the presence of 1 mM IBMX. cAMP levels were determined by ELISA. The 100% value corresponds to mean cAMP values in the absence of 2-iodomelatonin.

These findings show that the MT₁- and the MT₂-transfected cell lines both express functionally-active melatonin receptors, confirming that they can serve as appropriate tools to examine specific effects mediated by either MT₁ or MT₂ melatonin receptors.

3.3.2.2 Assessment of cGMP levels

It has been already shown that MT₂ but not MT₁ melatonin receptors modulate cGMP levels in transfected HEK 293 cells (Petit et al., 1999) and in human adipocytes natively expressing the MT₂ melatonin receptor (Brydon et al., 2001). An inhibitory effect of melatonin on cGMP levels was also observed in MT₂-transfected SK-UT-1B cells when cGMP degradation was blocked by IBMX (Fig. 12). Incubation of the cells with IBMX indeed induced a 3-fold increase in basal cGMP levels, which was inhibited by 2-iodomelatonin only in MT₂ transfected cells in a dose-dependent manner with a maximal inhibition level of 50%. These data are in good agreement with those previously reported for the cloned human MT₂ receptor (Petit et al., 1999; Brydon et al., 2001) and support functional expression of this receptor subtype in MT₂-transfected cells.

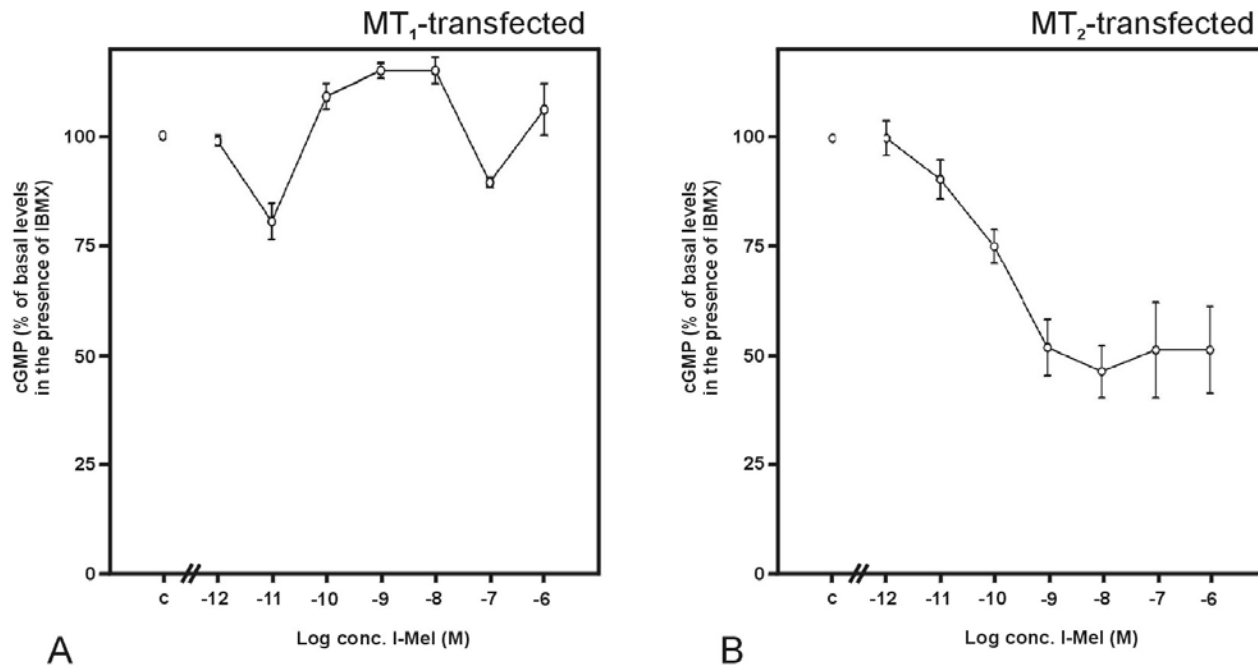


Fig. 12: Melatonin modulates cGMP levels in MT₂- but not in MT₁-transfected cells

Cells were incubated for 15 min at 37°C at the indicated concentrations of 2-iodomelatonin in the presence of 1 mM IBMX and cGMP concentrations were determined by ELISA. The 100% value corresponds to mean cGMP values in the absence of 2-iodomelatonin.

3.4 Melatonin does not induce Ca^{2+} mobilization in either MT_1 - or MT_2 -transfected cells

Melatonin is known to be involved in the transient mobilization of intracellular Ca^{2+} via the inositol-specific phospholipase C pathway in the ovine pars tuberalis (Brydon et al., 1999) and in rat pituitary cells (Vanecek et al., 1992). However, differences in the signalling pathway between the human MT_1 and MT_2 melatonin receptors have not been investigated in a homologous system. To determine whether melatonin has an effect in intracellular Ca^{2+} concentrations and how specific for MT_1 or MT_2 melatonin receptors these effects are, untransfected as well as MT_1 - and MT_2 -transfected cells were loaded with 3 μM of Fura-2/AM, and changes in $[\text{Ca}^{2+}]_i$ were measured by Ca^{2+} -imaging after treatment with 100 nM 2-iodomelatonin or with 10 μM UTP as an internal positive control. UTP was used as a positive control because Ca^{2+} influx is stimulated by UTP in this cell line. 2-iodomelatonin has no effect on $[\text{Ca}^{2+}]_i$ in either untransfected ($n=19$), MT_1 - ($n=49$) or MT_2 -transfected ($n=52$) cells (Fig. 13). In contrast, 10 μM dUTP induced a transient maximum increase in $[\text{Ca}^{2+}]_i$ immediately after the onset of the stimulus in all untransfected, MT_1 - or MT_2 -transfected cells which decreased to basal levels after 2 min.

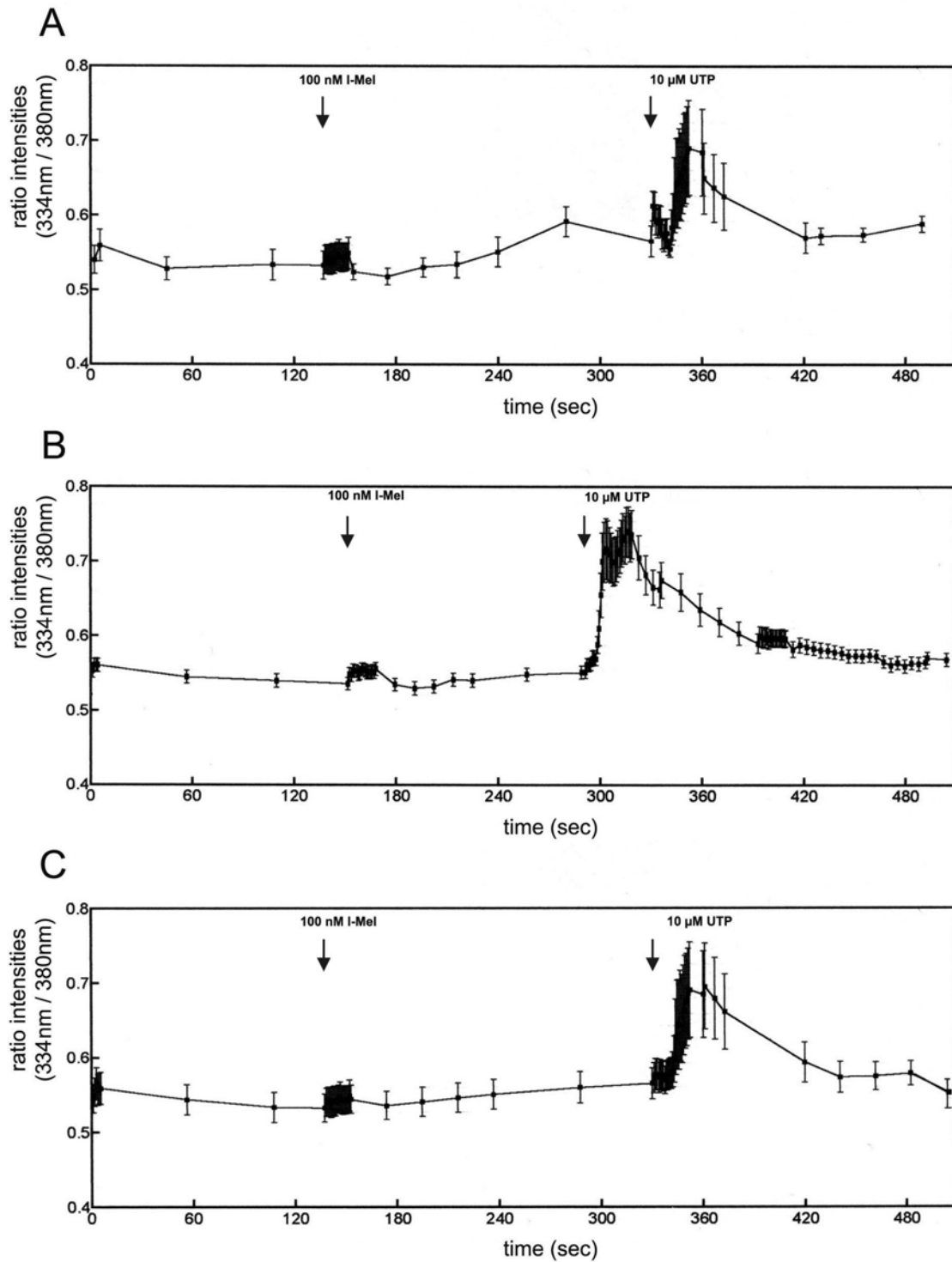


Fig. 13: $[Ca^{2+}]_i$ recordings from untransfected (A) MT₁-transfected (B) and MT₂-transfected (C) cells

Cells were loaded with 3 μ M Fura-2/AM, and changes in $[Ca^{2+}]_i$ were measured as described in materials and methods and expressed as the difference of intensities between 334 and 380 nm. Drugs were applied as indicated by arrows and remained present until the end of tracing.

3.5 Melatonin-induced inhibition of proliferation: possible involvement of the MT₂ receptor

The inhibitory effect of melatonin on cancer growth has already been reported in various human cell lines (Yuan et al., 2002; Shiu et al., 2000). However, depending on the cell line studied, an involvement of either MT₁ or MT₂ melatonin receptors have been purposed. To study the antiproliferative effects of melatonin in SK-UT-1B cells and the specific role of the MT₁ and MT₂ melatonin receptors in melatonin's growth-inhibitory mediation effects, untransfected, as well as MT₁- and MT₂-transfected cells were treated with different concentrations of 2-iodomelatonin in the absence or presence of the melatonin receptor antagonist, 4P-PDOT, and cell proliferation was examined.

After treatment with 2-iodomelatonin at the physiological concentration of 1 nM for 48 h, a maximal significant inhibition by 20% of cell growth in MT₁-transfected cells was detectable (Fig. 14A). The same 2-iodomelatonin concentration elicited a 50% inhibition of cell proliferation in MT₂-transfected cells (Fig. 14B). The antiproliferative effect of 1 nM 2-iodomelatonin is consistent with studies in other cell lines (Kanishi et al., 2000; Yuan et al., 2002). Greater concentration of melatonin (10 nM to 1 μ M) did not significantly suppress the proliferation of MT₁ and MT₂-transfected cells. In addition, the inhibitory effect of melatonin appeared to plateau off when the concentration of the hormone was greater than 100 nM. This decrease in cell proliferation was not due to cell death, since cell viability was greater than 95% after all treatments. Untransfected cells did not respond to melatonin's growth suppressive effect at any concentrations of 2-iodomelatonin administered.

On the other hand, 4P-PDOT at a concentration specific for the MT₂ melatonin receptor (10 nM) blocked the antiproliferative effect of 2-iodomelatonin in MT₂-transfected cells and had no effect in MT₁-transfected cells (Fig. 14). This confirms the involvement of the MT₂ melatonin receptor in inhibiting cell proliferation in these cells.

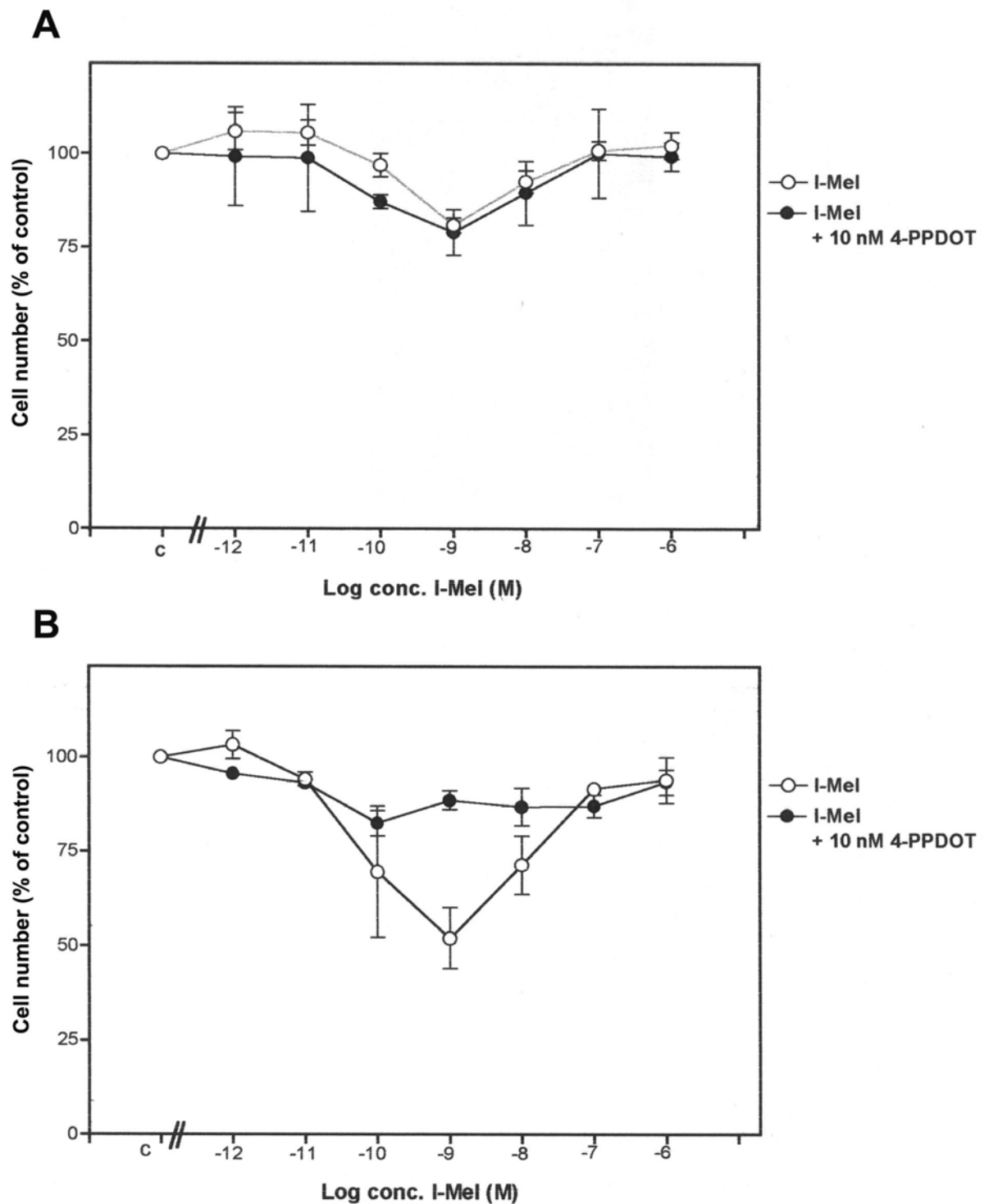


Fig. 14: Antiproliferative effect of 2-iodomelatonin in MT₁- (A) and MT₂-transfected cells (B)
Cells were seeded at a density of 2×10^4 cell/ml. Five hours after seeding 2-iodomelatonin (10^{-12} - 10^{-6} M), 2-iodomelatonin (10^{-12} - 10^{-6} M) plus 10 nM 4P-PDOT, or ethanol (0.01%) were added to the wells. The antiproliferative effect of 2-iodomelatonin is blocked in MT₂-transfected cells by coincubation of 2-iodomelatonin (10^{-12} - 10^{-6} M) with 10 nM of the MT₂ receptor antagonist 4P-PDOT. The data represent cell growth in each treatment group (three wells per group) as a percentage of controls after 2 days of growth.

3.6 Melatonin and the estrogen response system

3.6.1 SK-UT-1B cells are estrogen receptor-alpha (ER α)-positive

ER α expression in breast and endometrium cancer cells correlate with melatonin-sensitivity (Hill et al., 1992; Kanishi et al., 2000). ER α -negative cells are insensitive to melatonin's growth inhibition effect, whereas ER α -positive cells are sensitive. To investigate the ER α status of the SK-UT-1B cells, RT-PCR analyses using ER α -specific primers were performed (Fig. 15). Two products (lane 2) corresponding to the two isoforms of the ER α gene of the expected size (170 and 286 bp) were detected on amplification of cDNA from SK-UT-1B cells. No cDNA amplification was observed when the experiments were performed in the absence of DNA (lane 1). These findings demonstrate that the SK-UT-1B cells are ER α -positive.

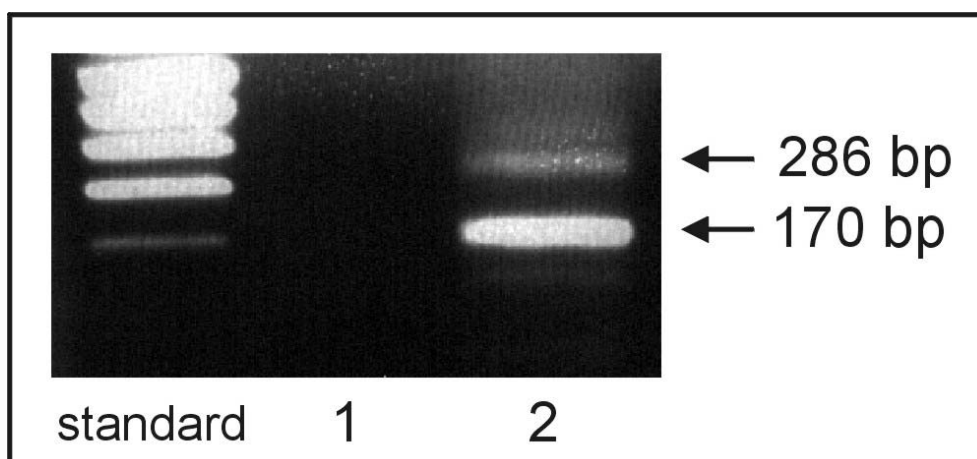


Fig. 15: Expression of the estrogen receptor-alpha gene in SK-UT-1B cells

Total RNA was isolated from SK-UT-1B cells and analyzed by semiquantitative RT-PCR. PCR products were visualized on agarose gel with ethidium bromide staining. No cDNA amplification was observed when experiments were performed in the absence of DNA (H₂O, 1). The migration of molecular size markers (standard) is indicated.

3.6.2 The antiproliferative effect of melatonin is partially prevented by estrogen

After treatment for 48 h with 10 nM 17- β -estradiol (E₂), the cell number was slightly (by 10%) increased in both MT₁- and MT₂-transfected cells compared to untreated controls (Fig. 16). 2-iodomelatonin (1 nM) produced, as expected, an inhibition in both MT₁- and MT₂-transfected cells by 20 or 50% vs control, respectively. The antiproliferative effect of 2-

iodomelatonin was partially prevented in both MT₁- and MT₂-transfected cells by simultaneous treatment of the cells with 10 nM 17- β -estradiol.

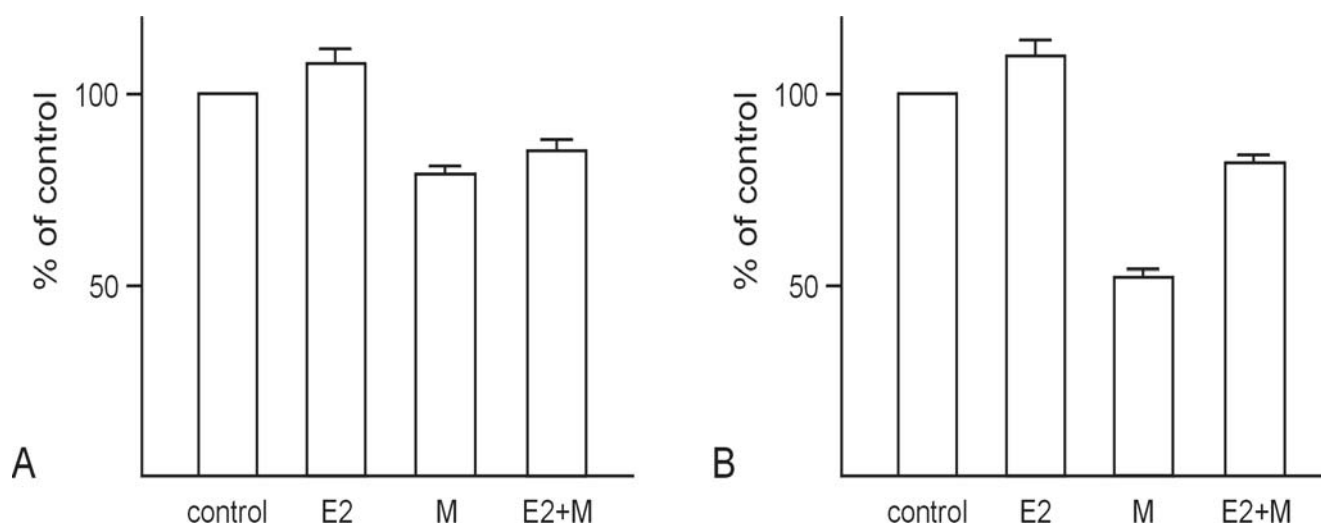


Fig. 16: Effects of 2-iodomelatonin and 17- β -estradiol on MT₁-transfected (A) and MT₂-transfected cells (B)

Cells were seeded at a density of 2×10^4 cells/ml. Five hours after seeding, 10 nM 17- β -estradiol (E2), 1 nM 2-iodomelatonin (M) or 1 nM 2-iodomelatonin plus 10 nM 17- β -estradiol (E2+M) or ethanol (0.01%) was added to the wells. The data represent cells growth in each treatment group (three wells per group) as a percentage of controls after 2 days of growth.

3.7 Melatonin-inhibited protein expression of the proto-oncogene c-Fos

The immediate early gene c-Fos is known to be involved in cell cycle modulation and has been purposed to play an important role in cellular proliferation (Zhou et al., 2002; Dubik et al., 1987). To examine a potential role of melatonin in these processes, experiments were conducted by using c-Fos-specific antibodies. Competition studies served to confirm antibody specificity (Fig. 17). Various bands were blocked by peptide competition, including the one corresponding to c-Fos at 62 kDa. Note however, that only this band is not present in negative controls. Therefore, other bands are unlikely to represent cross-reacting antigens.

To investigate whether melatonin affects c-Fos protein levels, cytosolic protein extracts from untransfected as well as from MT₁- and MT₂-transfected cells were analyzed after 20 or 60 min of 1 nM 2-iodomelatonin treatment by immunoblot analysis using the specific anti-c-Fos antibody (Fig. 18). There is a transient decrease of c-Fos after 2-iodomelatonin exposure in both MT₁- and MT₂-transfected cells with a down-regulation by about 50% when compared to

control levels. However, c-Fos recovered rapidly its elevated basal level within 1 h of 2-iodomelatonin exposure. These values agree well with studies in other cell lines (Mollis et al., 1995; Zhou et al., 2002). Analogous treatments with 2-iodomelatonin did not affect c-Fos levels in untransfected cells. Data were quantified densitometrically (Fig. 18B).

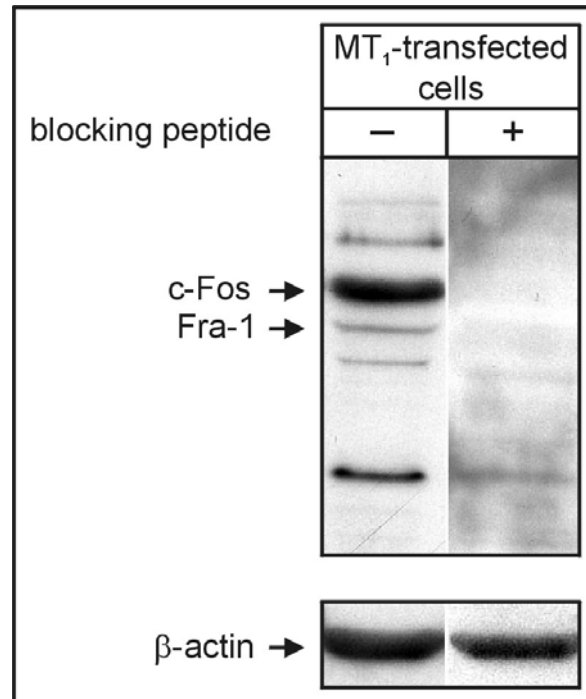


Fig. 17: Competition studies with an excess of c-Fos blocking peptide to neutralize the c-Fos antibody

Cytosolic protein extracts from MT₁-transfected cells were submitted to SDS-PAGE and immunoreactivity was revealed using anti-c-Fos antibody after pre-incubation in the absence (-) or presence (+) of c-Fos-blocking peptide in a 1:180 molar ratio. To demonstrate equal amounts of protein, β-actin levels were visualized.

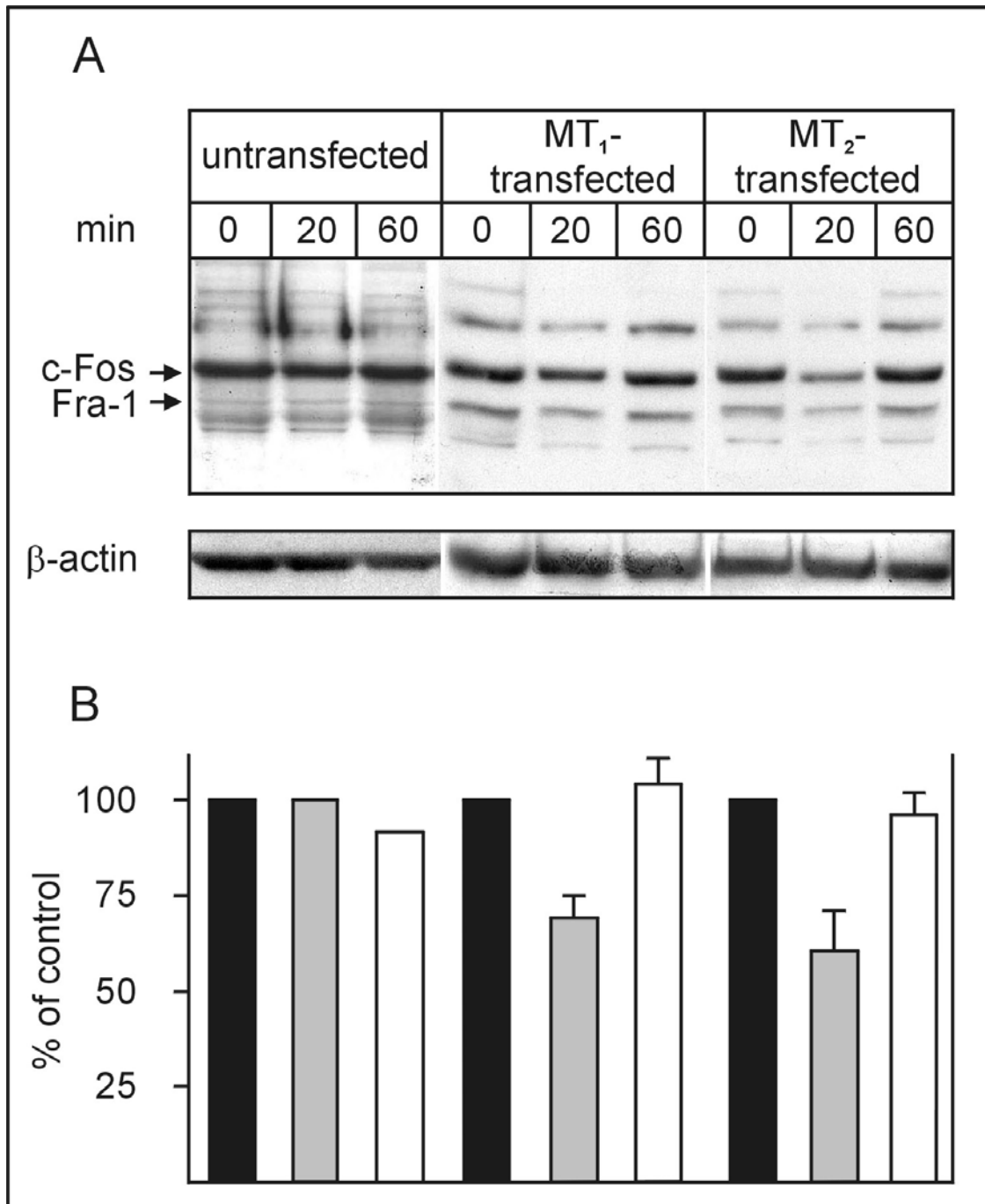


Fig. 18: Time course of the effects of 2-iodomelatonin on c-Fos protein levels in untransfected, MT₁- and MT₂-transfected cells

Cells were incubated with 1 nM 2-iodomelatonin and harvested at the times indicated. c-Fos levels were analyzed by immunoblot. To ensure equal amounts of proteins, a β -actin antibody was used (A), and the intensities of the signals were quantified (B). c-Fos levels are expressed as fold changes as compared to basal levels. Values represent mean $\pm$ SE of three individuals experiments.

3.8 Effects of melatonin on gene expression in MT₁- and MT₂-transfected cells

Altered expression of certain genes in response to melatonin as determined by the microarray technology has already been reported to occur in mouse heart (Anisimov et al., 2001) and in the rat retina (Wiechmann, 2002). In these studies, however, the specific roles of receptor isoforms were not investigated. To identify genes whose expression levels specifically vary in response to melatonin via MT₁- or MT₂ receptor activation, aRNA from untransfected and MT₂-transfected cells (Fig. 19), as well as from MT₁- and MT₂-transfected cells were hybridized to two 10k human chips. All cells were 2-iodomelatonin-treated (1 nM, 10 h). The dosage and timing of the treatment was selected in order to reproduce *in vivo* melatonin exposure conditions.

The primary purpose of this microarray survey was to look for candidate genes which could provide information about differences between both melatonin receptors.

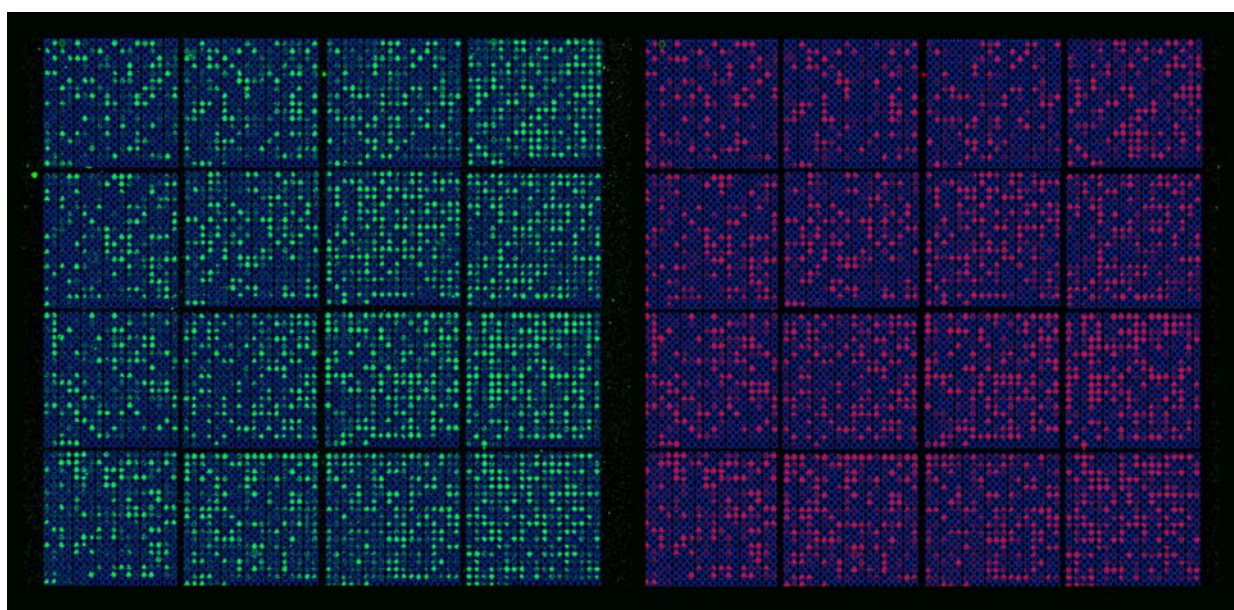


Fig. 19: Cy3-labelled aRNA (MT₁-transfected cells) in green and Cy5-labelled aRNA (MT₂-transfected cells) in red after hybridisation to a 10k human array chip

Both cell lines were treated for 10 h with 1 nM 2-iodomelatonin.

2-iodomelatonin treatment resulted in induction or repression of specific genes in both arrays. According to the ratio intensities of compared samples, 5 genes were up-regulated by melatonin and 5 genes were down-regulated in the array with untransfected and MT₂-transfected cells. In the array comparing MT₁- and MT₂-transfected cells, 9 genes were up-

regulated and 2 genes were down regulated by 2-iodomelatonin (in MT₂- as compared with MT₁-transfected cells).

GenBank accession number	Fold change	Gene name	Putative function
XM_007250	+8	Deiodinase type III (DIO3)	Generation of T3
XM_003628	+5	cyclin G2 (CCNG2)	Involved in cell cycle
XM_011374	+2.8	candidate tumor suppressor protein	tumor growth
XM_003035	+2.7	growth associated protein 43 (GAP43)	tumor growth
XM_004484	+2.5	Tumor protein D52-like 1(TPD52L1)	tumor growth
XM_011739	-4	similar to fibroblast growth factor 20	Cell growth
XM-001542	-3.3	5-hydroxytryptamine (serotonin) receptor 1D(HTR1D)	Binds serotonin
XM_006626	-3.2	C-type lectin, superfamily member 2	Leucocyte recruitment
XM_003760	-2.4	Interleukin 9(IL9)	Role in immune system
XM_002066	-2.3	Regulator of G-protein signalling 16 (RGS16)	G protein regulation

Table 9: Genes differentially expressed in untransfected and MT₂-transfected cells by >2-fold after 10 h 2-iodomelatonin (1 nM) treatment

GenBank accession number	Fold change	Gene name	Putative function
NM_000583	+6	group-specific component (vitamin D binding protein); gc	Role in immune system
NM_002235	+6	potassium voltage-gated channel, shaker-related subfamily, member 6; kcna6	Membrane channel
NM_000336	+5.5	Sodium channel, nonvoltage-gated 1, beta; scnn1b	Membrane channel
NM_004294	+5	mitochondrial translational release factor 1; mtrf1	Mitochondria translocase
NM_001258	+4.5	cyclin-dependent kinase 3; cdk3	involve in cell cycle
NM_012138	+3.7	apoptosis antagonizing transcription factor; ded	Role in apoptosis
NM_002099	+3	glycophorin a precursor; gypa	Blood group antigen
NM_000948	+2.5	prolactin; prl	Pituitary hormone
NM_022112	+2.5	p53-regulated apoptosis-inducing protein 1; p53aip1	Role in apoptosis
NM_000609	-3.5	stromal cell-derived factor 1; sdf1	Involve in B-cell lymphopoiesis
NM_002753	-3	mitogen-activated protein kinase 10; mapk10	cell growth

Table 10: Genes differentially expressed in MT₁- and MT₂-transfected cells by >2-fold after 10 h 2-iodomelatonin (1 nM) treatment

Gene induction or repression was considered significant in case of >two fold average difference intensities (Tables 9 and 10). Several other genes in both cases appeared to show a modest degree of up- or down-regulation (+1.9 to -1.9) but the degree of confidence was not

high enough to warrant further studies. A BLAST search was performed to identify the apparently-regulated genes. The range of gene induction or repression, caused by melatonin treatment, was from +8 to -4 in the untransfected vs MT₂-array (Table 9) and from +6 to -3.5 in the MT₁ vs MT₂-array (Table 10).

Two of the the genes indicated by microarray experiments to respond to melatonin, cyclin G2 (Fig. 20) and MAPK (ERK1/2), were selected for further studies.

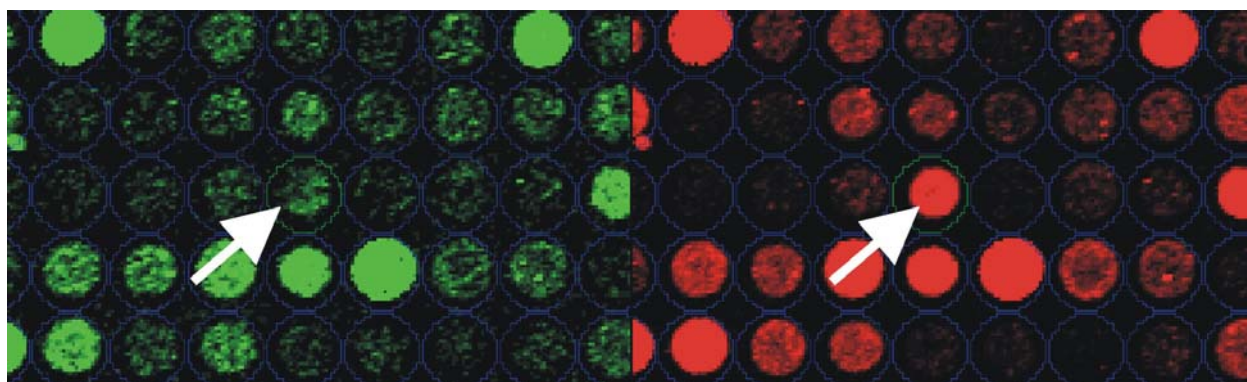


Fig. 20: Cyclin G2 (arrow) is up-regulated in MT₂-transfected cells (Cy5-labelled, red) in relation to untransfected cells (Cy3-labelled, green) after 10 h treatment with 1 nM 2-iodomelatonin

3.9 Activation of the MT₂ receptor in transfected SK-UT-1B cells results in a decrease of phospho-ERK1/2

Because previous investigators had shown that the ERK1/2 pathway may play a role in cell growth and differentiation (Witt-Enderby et al., 2000; Bordt et al., 2001), the effect of melatonin on ERK1/2 phosphorylation in MT₁- and MT₂-transfected cells was examined.

Untransfected, MT₁- and MT₂-transfected cells were analyzed for ERK1/2 phosphorylation by immunoblot analysis using phospho-p44/42 MAPK (Thr202/Tyr204) antibodies to detect the activated form of the kinase (p44 or ERK1 and p42 or ERK2) after 7.5, 45 min or overnight exposure to 2-iodomelatonin. Fig. 21A shows that after 10 h (oN) 2-iodomelatonin (1 nM) treatment, there is no difference in the amounts of phosphorylated protein in untransfected and MT₁-transfected cells at any time, in support of the work of Godson et al. (1996). To ensure that equal amounts of cytosolic protein were used for this experiment, an anti-β-actin antibody was used (Fig. 21A). After quantification of the signal intensities, phospho -ERK1/2

(p-ERK1/2) levels were expressed as relative fold change to basal level in relation to β -actin (Fig. 21B).

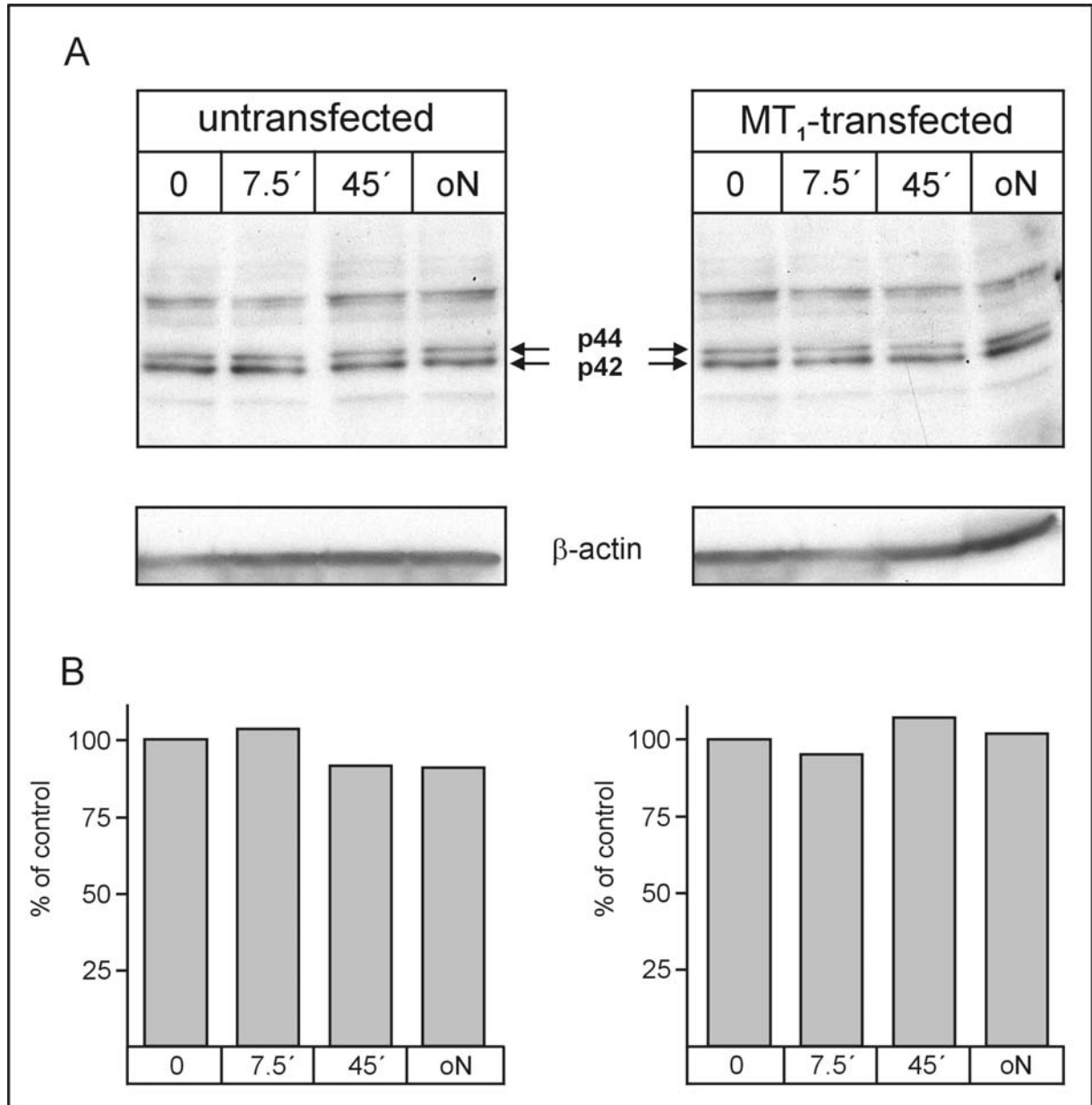


Fig. 21: Time-dependent effects of 2-iodomelatonin on p-ERK1/2 in untransfected and MT₁-transfected cells

Cells were cultured and treated with 1 nM 2-iodomelatonin as described in materials and methods. p-ERK1/2 levels were analyzed by immunoblotting (A) and the intensities of the signals quantified. p-ERK1/2 levels are expressed as fold change relative to basal levels and normalized against β -actin (B). Values represent the mean $\pm$ SE of three individual experiments.

3.10 The decrease of phospho-ERK in MT₂-transfected cells in response to melatonin is mediated through the specific MT₂ receptor

MT₂-transfected cells were also analyzed by immunoblotting using phospho-specific antibodies to detect the activated form of the enzyme. Cells were incubated for 7.5, 45 min and over night in the presence of 1 nM 2-iodomelatonin (Fig. 22A) showing that melatonin produces a rapid decrease in p-ERK1/2 levels in MT₂-transfected cells which was observed within 7.5 min (2-fold under basal levels). This decrease was sustained after over night incubation, consistent with effects observed by Alessi et al. (1995). Remarkably, two additional bands detectable above and below p44/p42 are regulated in the same manner as the known MAPK forms, suggesting that these bands represent MAPK-related proteins.

To demonstrate that the decrease in p-ERK1/2 levels in MT₂-transfected cells in response to melatonin is mediated specifically through the MT₂ receptor, the melatonin receptor antagonist 4P-PDOT was used. Fig. 22B shows that the addition of the MT₂ receptor antagonist 4-PPDOT, totally block the effect of 2-iodomelatonin on p-ERK1/2. To ensure that equal amounts of cytosolic protein were used for this experiment, an anti- β -actin immunoblot was performed (Fig. 22A, B). After quantification of the signal intensities, p-ERK1/2 levels were expressed as relative fold change to basal level in relation to β -actin (Fig. 22C).

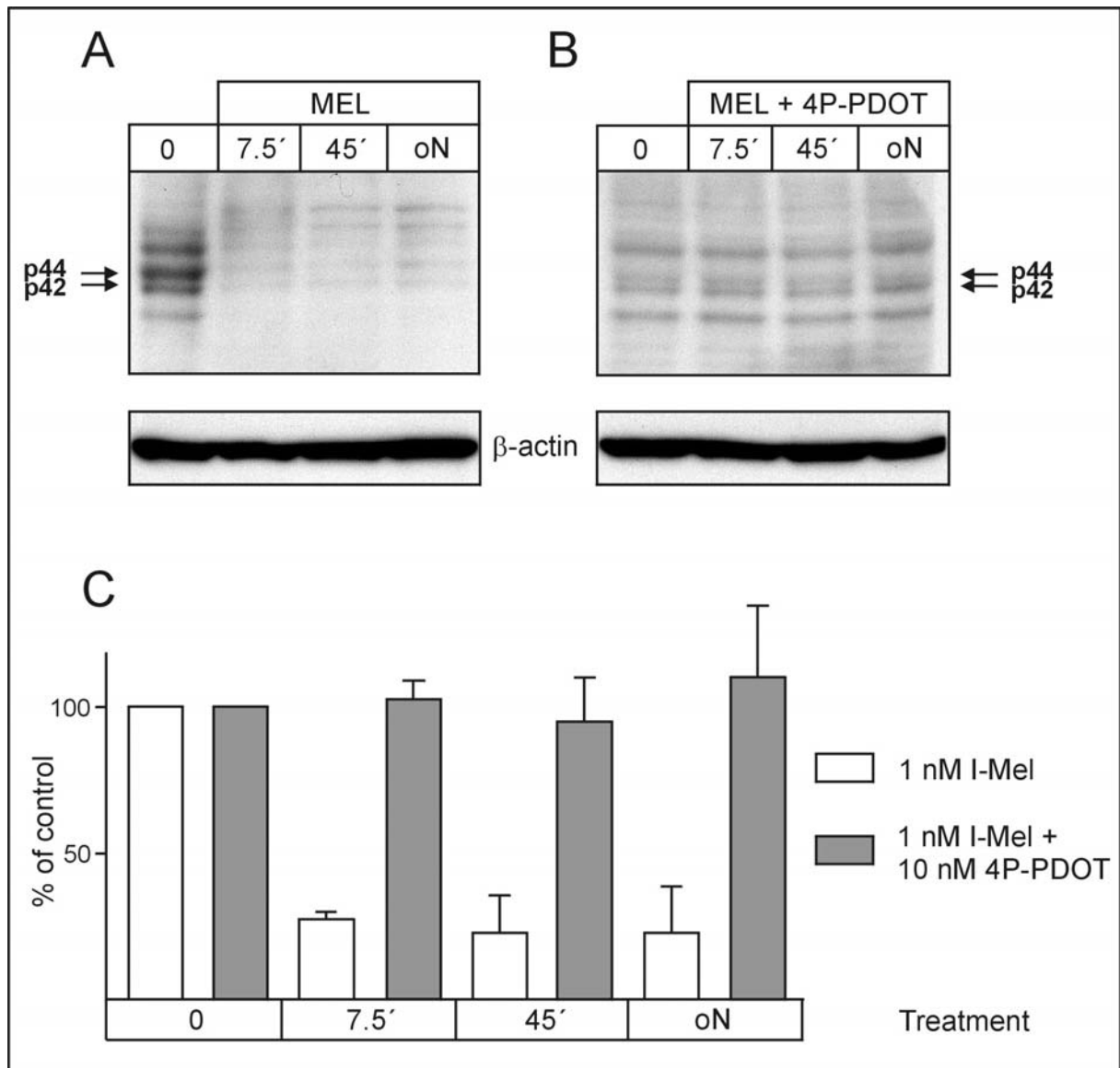


Fig. 22: 4P-PDOT totally blocks the effects of melatonin on p-ERK1/2 in MT₂ transfected cells

Cells were cultured and treated with 1 nM 2-iodomelatonin (A) and 1 nM melatonin plus 10 nM 4P-PDOT (B). p-ERK1/2 levels were analyzed by immunoblot assay and the intensities of the signals quantified (C). p-ERK1/2 levels are expressed as relative fold change to basal levels. Values are mean $\pm$ SE of three individual experiments.

3.11 Cyclin G2 transcript levels increase in response to melatonin in MT₂-transfected cells

Cyclin G2 is an unconventional cyclin highly expressed in cell undergoing apoptosis (Horne et al., 1997) and it can cause cell cycle arrest (Bennin et al., 2002). Using microarray analyses, an up-regulation in cyclin G2 was observed after 10 h 1 nM 2-iodomelatonin treatment only in MT₂-transfected cells. To confirm these findings, transcript levels of cyclin

G2 were assessed by quantitative RT-PCR in untransfected, MT₁- and MT₂-transfected cells (Fig. 23). These assays confirmed the variable expression of this gene. Higher levels of cyclin G2 transcript were found in MT₂-transfected cells after 10 h 1 nM 2-iodomelatonin treatment. Interestingly, in MT₁-transfected cells no statistical relevant change in cyclin G2 transcript levels was observed after 2-iodomelatonin treatment. No differences in cyclin G2 transcript levels were noted also in untransfected cells after 2-iodomelatonin treatment. All values were normalized against human GAPDH. The magnitude of the relative expression difference was greater in microarray assays as compared to quantitative RT-PCR results.

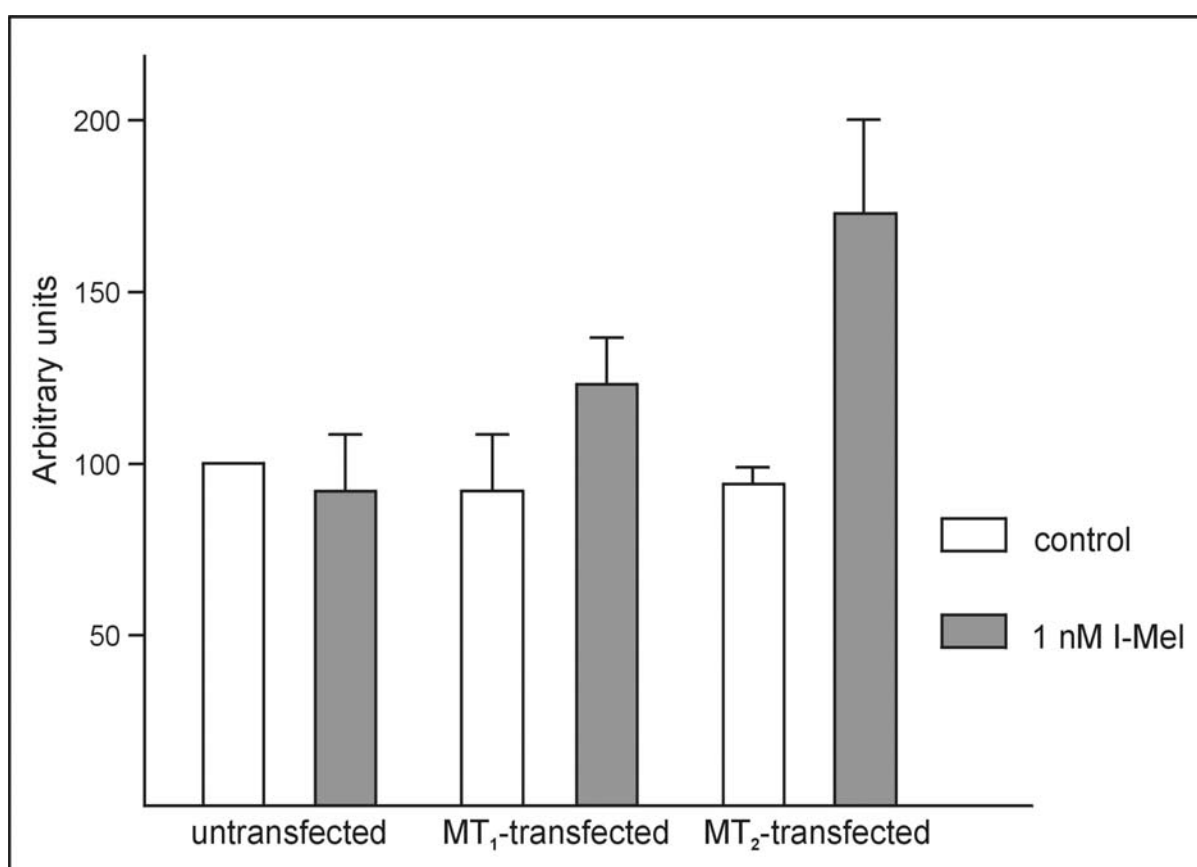


Fig. 23: Quantitative RT-PCR determination of cyclin G2 transcript levels in untransfected, MT₁- and MT₂-transfected cells after 10 h of 1nM 2-iodomelatonin treatment

Variations in cDNA loading were normalized against GAPDH cDNA. Results are expressed as % of control (incubations in the absence of 2-iodomelatonin).

3.12 The effect of melatonin on cyclin G2 is mediated specifically through the MT₂ receptor

To confirm that the increase in cyclin G2 mRNA levels observed in MT₂-transfected cells in response to melatonin is mediated specifically through the MT₂ receptor, the melatonin receptor antagonist 4P-PDOT was used. For this purpose, transcript levels of cyclin G2 were assessed by quantitative RT-PCR in untransfected, MT₁- and MT₂-transfected cells (Fig. 24).

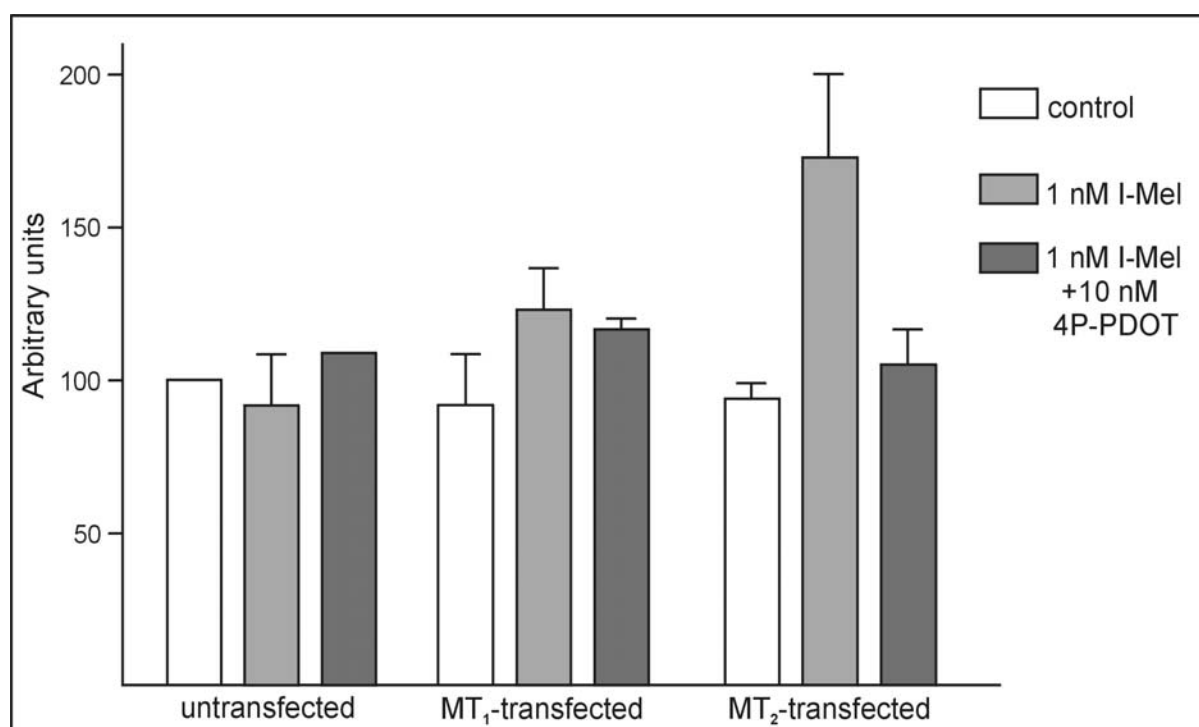


Fig. 24: Effect of 2-iodomelatonin and 10 nM 4P-PDOT in cyclin G2 transcript levels in untransfected, MT₁- and MT₂-transfected cells

Cyclin G2 transcript levels were determined by quantitative real-time PCR. Variations in cDNA loading were normalized against GAPDH cDNA and results are expressed as % of control.

After 10 h coincubation with 1 nM 2-iodomelatonin and the melatonin receptor antagonist 4P-PDOT at a concentration (10 nM) at which the antagonist is specific for MT₂, the effect of 2-iodomelatonin was completely blocked in the MT₂-transfected cells. No effects on cyclin G2 transcript levels were detectable in either untransfected or MT₁-transfected cells by 4P-PDOT treatment. All values were normalized against human GAPDH cDNA.

4 Discussion

4.1 SK-UT-1B: a suitable model system to study melatonin receptor properties

The complexity of melatonin's function within the body is becoming quite apparent. The existence of different receptor subtypes located throughout the body, which theoretically can couple to different signal transduction cascades, may contribute to this diversity. Until recently, it has been difficult to define intracellular signalling pathways activated by melatonin. Expression of recombinant melatonin receptors in mammalian cells provides a useful model to this end. However, most of the comparative studies performed with human melatonin receptors were based on available *in vitro* model systems employing rodent cell lines, which do not provide a human cellular context necessary to understand the actual behaviour of the human melatonin receptors. In the present work, the SK-UT-1B human uterine tumor cell line was used for this purpose. RT-PCR analyses showed that cells transfected with the MT₁ melatonin receptor expressed the MT₁ receptor subtype transcripts, without expressing MT₂ mRNA, whereas only MT₂ transcripts were detectable in SK-UT-1B cells transfected with the MT₂ melatonin receptor. Moreover, MT₂-transfected cells express the MT₂ receptor subtype at the cell surface. A protein band specifically detectable in MT₂-transfected cells (by means of antibodies to the Flag epitope) was found to migrate with an apparent molecular mass of approximately 60 kDa. The cDNA-predicted molecular mass of the MT₂ melatonin receptor is 37 kDa. The Flag epitope increases the expected molecular mass of the receptor protein from 37 to 40 kDa; in addition, the receptor is expected to be present in its glycosylated form, which substantially increases its molecular weight. Glycosylation is characteristic of G-protein coupled receptors (Guillaume et al., 1994) and the presence of two glycosylation sites in the N-terminal domain of the MT₂ melatonin has been reported (Brydon et al., 1999; Ayoub et al., 2002). Furthermore, the MT₁ human melatonin receptor has been reported to migrate at the cDNA-predicted molecular mass of 37 kDa only in the presence of an inhibitor of *N*-glycosylation (Brydon et al., 1999). Recently, Ayoub et al. (2002) suggested the presence of monomeric, dimeric, and higher oligomeric states for the MT₁ and MT₂ melatonin receptors corresponding to three groups of immunoreactive bands with apparent molecular masses of 45-60 kDa, 90-120 kDa and >200 kDa, respectively. However, no evidence for the presence of hetero-oligomers was found by Brydon et al., 1999, although many G-protein coupled receptors have been reported to naturally form dimers and

multimers (Pierce et al., 2002). In the present study, no receptor oligomerization was detectable.

The binding studies revealed that the human MT₁ and MT₂ receptors stably transfected in SK-UT-1B cells specifically bind 2-[¹²⁵I]iodomelatonin with high affinity. The equilibrium dissociation constant values were found to be in the low picomolar range for both receptors, which fits well with the reported K_d values for MT₁ and MT₂ receptors in other transfected cell lines, for example in HEK 293 cells expressing the MT₁ melatonin receptor (Petit et al., 1999) and in Cos-1 cells expressing the MT₂ melatonin receptor (Reppert et al., 1995).

The B_{max} values of 14.21 ± 1.6 and 9.617 ± 0.587 fmol/mg of protein, respectively, found for the MT₁ and MT₂ melatonin receptors in SK-UT-1B cells, correspond to receptor densities reported for high affinity melatonin receptors identified at peripheral sites, such as in adipocytes expressing the MT₂ receptor (Brydon et al., 2001), lymphocytes (Pang et al., 1993) and prostate epithelial cells (Gilad et al., 1996).

In other *in vitro* systems, receptor densities were quite different. For example, in HEK 293 cells expression levels of either the exogenous MT₁ or the exogenous MT₂ melatonin receptor were more than 1200 fmol/mg of protein. Since melatonin receptor overexpression can influence receptor activation (Macewan et al., 1995) and lead to alterations in coupling of the receptor to G-proteins (Burford et al., 1996), it seems advantageous to employ a model system where melatonin receptor expression levels mimic physiological conditions.

The Flag epitope did not alter crucially the expression levels or 2-[¹²⁵I]iodomelatonin binding affinity of MT₂ in transfected cells, as the B_{max} and K_d values for the MT₂-transfected cells without Flag were similar to those found for the MT₂-transfected cells with the Flag epitope. Therefore, the Flag-epitope tagged human MT₂ receptor is thought to provide a suitable model for further investigations.

Importantly, the MT₂ melatonin receptor antagonist 4P-PDOT showed a high affinity for the MT₂ melatonin receptor but only a modest affinity for the MT₁ subtype, thus revealing a very high MT₁/MT₂ selectivity (~1000 fold). 4P-PDOT has already been reported to be a low-affinity MT₁ antagonist and a high-affinity MT₂ antagonist in NIH 3T3 transfected cells (Nonno et al., 1999) and in Cos-7 cells (Dubocovich et al., 1997).

It is well known that melatonin can inhibit cAMP signalling through both the MT₁ and the MT₂ melatonin receptor. This inhibition in forskolin-stimulated cAMP signalling has been observed in numerous transfected cells (Chan et al., 2002; Petit et al., 1999; Reppert et al., 1994, 1995) as well as in cells expressing the endogenous receptors (Schlabritz-Loutsevich et

al., 2003; Brydon et al., 2001). In the present study melatonin inhibited the forskolin-stimulated cAMP levels through both the MT₁ and the MT₂ melatonin receptors with a maximal inhibition (50%) at physiological melatonin concentrations. Incomplete inhibition of cAMP signalling by melatonin has been reported in other transfected cells (Petit et al., 1999; Chan et al., 2002) and cells expressing endogenous receptors (Carlson et al., 1989; Brydon et al., 2001) with considerable variations (10-80%), reflecting the differential sensitivity of this pathway depending on the cellular background. In transfected systems, the receptor density and the coupling-efficiency to available G-proteins can also play an important role in determining how cells respond to physiological levels of melatonin. The most likely intracellular target of melatonin is adenylyl cyclase. The pertussis toxin (PTX)-sensitivity of G_{ai}, the G protein inhibiting the activity of adenylyl cyclase, is well known (Katada et al., 1982). The fact that melatonin effects on cAMP are prevented after incubation with PTX (Reppert et al., 1994, 1995; Godson et al., 1997) is consistent with G_{ai} protein coupling of both melatonin receptors.

Other possibilities, e.g., that melatonin activates phosphodiesterases and decreases cAMP concentrations by increasing its catabolism, is not likely because melatonin acts even in the presence of high concentrations (1 mM) of IBMX. At these concentrations, IBMX strongly inhibits PDE activity, making an effect through this pathway unlikely. Moreover, melatonin has the same inhibitory effect on cAMP accumulation in the presence or absence of IBMX (Morgan et al, 1991).

Another mechanism by which melatonin may influence cellular cAMP relates to intracellular calcium. Nearly all of the nine cloned adenylyl cyclases are regulated by the phospholipase C pathway (Cesnaja et al., 1994). Five of them are calcium-sensitive; two are inhibited by calcium and three are stimulated. Because it has been shown in rat gonadotrophs that melatonin decreases GnRH-induced [Ca²⁺]_i (Zemboka et al., 1997), an inhibition by melatonin of calcium-sensitive adenylyl cyclase activity is conceivable. [Ca²⁺]_i inhibition in cells was observed in the absence of extracellular calcium despite significant decreases in forskolin- or GnRH-induced cAMP-accumulation (Vanecek et al., 1998). However, because in calcium-free medium the forskolin-induced increase in cAMP is much smaller than in normal medium, the decrease in [Ca²⁺]_i is probably not the primary mechanism by which melatonin induces decreases in cAMP. Findings of the present work, that melatonin has no effect on [Ca²⁺]_i during inhibition of the cAMP pathway are consistent with this hypothesis.

There are other possibilities of how melatonin may regulate cAMP. E.g., the products of a phospholipase C- or D- mediated breakdown of various membrane phospholipids have been

shown to inhibit adenylyl cyclase in a number of cells (Diaz-Laviada et al., 1991). Moreover, some of these pathways are pertussis toxin-sensitive. Both MT₁ and MT₂ melatonin receptors have been reported to stimulate the phospholipase C pathway (Godson et al., 1997; Hunt et al., 2001). However, melatonin did not stimulate phospholipases A or D in ovine par tuberalis (McNulty et al., 1994). Therefore, the inhibition of cAMP accumulation by melatonin apparently involves other mechanisms. In conclusion, coupling of melatonin receptors to adenylyl cyclases seems to be the most probable underlying.

4.2 Modulation of cGMP levels by melatonin receptors

Cyclic GMP is an important second messenger in the central nervous system (Snyder et al., 1992), in blood vessels and in the retina, locations where melatonin receptors are expressed (Reppert et al., 1994, 1995; Doolen et al., 1998). Melatonin is secreted by the pineal gland in a circadian manner with maximal levels during the night (Lynch et al., 1975). Cyclic GMP levels have also shown to vary in a circadian manner in the cerebral cortex of the chick, where they are related to blood rhythms of melatonin (Guerrero et al., 1996). Interestingly, cycles of cGMP and melatonin concentrations are inversely correlated. This observation is consistent with an inhibition of cGMP levels by activated melatonin receptors during the night. Furthermore, melatonin has been shown to modulate cGMP levels in cells expressing endogenous receptors such as neonatal rat pituitary cells and in human prostate (Vanecek et al., 1997; Gilad et al., 1996), suggesting that modulation of cGMP levels may be melatonin receptor-mediated. Mel1c receptors from *Xenopus laevis* decrease cGMP levels through the soluble guanylyl cyclase pathway when transfected into different cell lines (Jockers et al., 1997), indicating a direct modulation of cGMP levels by high-affinity melatonin receptors. Mammalian melatonin receptors, namely the human MT₂ subtype, also modulate cGMP levels when expressed in HEK 293 cells (Petit et al., 1999).

The MT₂ receptor subtype is expressed mainly in the retina (Dubocovich et al., 1998) and at low levels also in the SCN (Wan et al., 1999). In these tissues, the second messenger cGMP plays a major role. In the retina of vertebrates, cGMP is involved in light-triggered rhodopsin signalling (Yarfitz et al., 1994) and in the SCN it regulates the circadian rhythm of neuronal activity (Starkey et al., 1996). It is important to note that this modulation in the SCN by cGMP is restricted to night times, and this correlates well with the time-frame of melatonin

receptor activation. Based on these results a role of melatonin in these and other tissues would be to modulate cGMP levels in a highly regulated manner.

The existence of receptor subtypes is a typical feature of G protein coupled receptors (Strosberg et al., 1991) and may confer some of the following advantages to the cell: (i) selectivity for the natural ligand(s); (ii) differential temporal regulation of receptor expression (e.g., during development) and in a tissue specific manner, and (iii) selective intracellular signalling due to coupling to different effectors and selective regulation of signalling by desensitisation or sensitisation processes. In the present work modulation of cGMP levels is specific for the human MT₂ receptor subtype, whereas the human MT₁ receptor is ineffective in modulating intracellular cGMP levels. The maximal inhibition occurs at approximately 1 nM of melatonin, which is in good agreement with values for inhibition of cAMP accumulation. This is in the range of circulating melatonin concentrations and thus of potential physiological relevance. Maximal inhibition (50%) fits well with those observed in HEK 293 cells transfected with the human MT₂ receptor (Petit et al., 1999) and in human PAZ6 adipocytes functional expressing the MT₂ receptor (Brydon et al., 2001). These results indicate for the first time the existence of functional differences between the two human melatonin receptors subtypes in a homologous system. Tissues expressing only MT₁ receptors are expected to be targets for melatonin effects on cAMP levels, whereas tissues expressing the MT₂ receptor are targets for a modulation by melatonin of both cAMP and cGMP pathways. Effects of melatonin receptors on the cAMP pathway thus may represent a general feature of melatonin signalling which, however, according to the subtypes expressed, can be counteracted by modulation of the cGMP pathway.

Cyclic GMP can be generated principally by two types of enzymes, namely soluble or membrane-bound guanylyl cyclases (GCs). Soluble guanylyl cyclases are activated by nitric oxide, whereas membrane-bound GCs are activated by natriuretic peptides. Incubation of HEK 293 cells stably transfected with the human MT₂ melatonin receptor with the nitric oxide donor sodium nitroprusside (SNP) increased basal cGMP levels, indicating a functional guanylyl cyclase in these cells (Petit et al., 1999).

Molecular determinants involved in melatonin signalling are localized to the intracellular receptor domain (Strosberg et al., 1991). Amino acid residues important for the cGMP signalling would be expected to be conserved between the MT₂ and the *Xenopus* Mel1c receptors, since both have been reported to modulate cGMP levels (Petit et al., 1999). Further studies will be necessary to determine which residue(s) of melatonin receptors is/are responsible for the generation of specific effects on cellular cGMP levels.

4.3 Melatonin and cell proliferation

Experimental evidence for a role of melatonin as an antiproliferative or oncostatic molecule has been provided by studies with a variety of human and murine cancer cell lines such as breast (Ram et al., 2000), endometrial (Kanishi et al., 2000), ovarian (Petranka et al., 1999), choriocarcinoma (Shiu et al., 2000), prostate (Zhou et al., 2002), colon, melanoma (Ying et al., 1993) and neuroblastoma cells. These antiproliferative effects are primarily exerted over a concentration range that encompasses levels (1 nM) of melatonin found in the circulating blood during the night (Cos et al., 1994; Kanishi et al., 2000). In the present study, melatonin was most effective in reducing cell proliferation at a concentration of 1 nM, which is in the range of nocturnal melatonin values in mammals. Furthermore, melatonin concentrations greater than 10 nM had no effect on cell proliferation. Interestingly Cos et al. (1994) found that 12-h-pulses with 1 nM melatonin were more effective in inhibiting cancer cell growth when applied at a basal 100 pM concentration than when applied on plates where melatonin was not present in the medium during the intervals between the pulses. This suggests that highest antiproliferative effects are obtained when the pattern of melatonin exposure mimics the physiological changes of serum melatonin concentrations during the light/dark cycle. In mammals, the melatonin rhythm consists of basal levels in the picomolar range which rise to peak levels in the nanomolar range during the period of darkness (Reiter et al., 1991). In this context, it should be mentioned that until recently, the general concept used in toxicology to determine risk assessment is the dose-response relationship, for which two models have traditionally been used, the threshold model and the linear non-threshold model. Calabrese et al. (2003) argue that most significant shape of the dose response curve is neither threshold nor linear, but U-shaped. This U-shape is commonly called hormesis where a modest stimulation of response occurs at low doses and an inhibition of response occurs at high ones. Hormetic responses are thought to have great importance for the biomedical and clinical science. Many antibiotics, antiviral and anti-tumor agents display a hormetic-like biphasic dose response. Some anti-tumor agents (for example, suramin) that inhibit cell proliferation at high doses, where they are clinically effective, act like a partial agonist at lower doses, where they enhance cell proliferation (Calabrese et al., 2003).

Most of what is currently known about the cellular and molecular mechanisms by which melatonin inhibits cancer cell growth is based on extensive *in vitro* studies using estrogen receptor alpha (ER α)-positive MCF-7 human breast cancer cells. Melatonin has no effect on

the proliferation of estrogen receptor-negative human breast cancer cells (Hill et al., 1988), indicating that the estrogen-response pathway is a critical component of the pathway mediating melatonin's oncostatic effects in breast cancer cells. Physiological melatonin concentrations not only inhibit the mitogenic effects of estradiol (E2) on MCF-7 cells (Cos et al., 1991), but they down-regulate the activity of the estrogen receptor via suppressing the expression of the estrogen receptor gene indicated by a time-dependent reduction in estrogen receptor mRNA and protein levels (Mollis et al., 1994). Melatonin itself does not bind directly to the estrogen receptor, indicating that its ability to regulate estrogen receptor expression is exerted via indirect mechanisms rather than by competing with estrogen for the hormone-binding domain of the estrogen receptor (García-Rato et al., 1999). This group argued that the antiestrogenic effects of melatonin could be explained as a consequence of the destabilization by melatonin of estrogen-estrogen receptor (E2-ER) complex binding to DNA. However, they do not exclude that the antiestrogenic effect of melatonin may be also elicited by the down-regulation of estrogen receptors described above (Mollis et al., 1994).

Endometrial cancer, like breast cancer, is known to be an estrogen-dependent neoplasm. Furthermore, melatonin also inhibits the growth of estrogen receptor positive SNG-II endometrial cancer cells, but has no effect in estrogen negative Ishikawa cancer cells (Kanishi et al., 2000). Interestingly, deficient melatonin function has been suggested to be one of the risk factors for the development of endometrial cancer (Sandyk et al., 1992), and its secretion decreases in postmenopausal women, a group showing the highest incidence of endometrial cancer, the most common malignant neoplasm of the female genital tract.

Based on these presently nuclear phenomena, it was decided to investigate the estrogen receptor status in the human uterus cancer cell lines used in the present study. The results of RT-PCR revealed that SK-UT-1B cells are ER α -positive. Moreover, 17- β -estradiol (10^{-8} M) partially blocked the antiproliferative effect of melatonin in both MT₁- and MT₂-transfected cells. However, the fact that estradiol did not completely rescue the cells from melatonin inhibition suggest additional mechanisms through which melatonin exerts its antiproliferative effects in SK-UT-1B cells.

4.4 Possible roles of MT₁ and MT₂ in mediation of antiproliferative effects of melatonin

Several mechanisms have been proposed in the literature to explain the oncostatic action of melatonin, including activation of the immune system (Drazen et al., 2001), free radical scavenging by melatonin (Reiter et al., 1994; 1995) and direct antiproliferative effects of the hormone. Among the above proposed anti-cancer mechanisms, direct antiproliferative effects are thought to involve specific receptor-mediated responses. In the present work, the MT₂ receptor antagonist 4P-PDOT was found to block the antiproliferative effects of melatonin, demonstrating receptor implication.

In addition to the above discussed estrogen and melatonin receptor relationship, there are several other processes by which melatonin receptors appear to mediate antiproliferative effects. In the present study three cellular effects were found to be associated with antiproliferative effects of melatonin: (i) a modulation of MAPK activity via MT₂ melatonin receptor activation (ii) a decrease in the proto-oncogene c-Fos expression by melatonin acting through both MT₁ and MT₂ receptors and (iii) decreases in cyclin G2 expression induced by melatonin via the MT₂ receptor subtype.

Cell surface-associated G protein-coupled receptors play a major role in mediating mitogenic signalling in normal and cancer cells through the modulation of MAPK activity. Mitogen-activated protein kinases, in particular the extracellular signal-regulated kinases ERK1 and ERK2 (MAPK, also called p44 and p42^{mapk}), are activated via proximal kinases, Raf-1 and B-Raf [MAP kinase kinase kinase (MAPKKK)], that phosphorylate and in turn activate MEK1 and MEK2 [MAP kinase kinase (MAPKK)]. These MAP kinases are involved in signal transduction events regulating cell proliferation and differentiation and are thought to mediate pleiotropic responses localized to the membrane, cytoplasm, nucleus or cytoskeleton. Upon activation, MAPKs translocate to the nucleus where they phosphorylate and thereby activate nuclear transcription factors involved in DNA synthesis and mitogenesis (van Biesen et al., 1996).

Activation of MAPK requires phosphorylation of both threonine and tyrosine residues within regulatory site of the enzyme (Anderson et al., 1990). Based on this information, a commercially available antibody that recognizes only the active, dual phosphorylated form of MAPK, was used in the present study. The results show that melatonin treatment causes a decrease in MAPK activation. Furthermore, only the MT₂-transfected but not the MT₁-

transfected cells show this effect, and treatment with a specific MT₂ receptor antagonist blocked totally this effect, confirming MT₂ involvement. A failure of melatonin to modulate MAPK activity through the MT₁ melatonin receptor has also been shown in NIH 3T3 cells transfected with the human MT₁ receptor isoform (Godson et al., 1997). The decrease in MAPK activation by melatonin through the MT₂ receptor subtype was sustained for severe hours. Under these conditions, MAPK is maximally inactivated resulting in decreases in the activation of nuclear transcription factors such as Elk-1 and SRF known to be involved in DNA synthesis and mitogenesis.

The modulation of MAPK phosphorylation is thought to be primarily mediated by reduced cAMP levels (Stork et al., 2002). Since melatonin apparently decreases cAMP levels through the activity of a G_{ai} protein, it is probable that this mechanism is directly linked to the inhibition of MAPK phosphorylation. However, since both MT₁ and MT₂ melatonin receptor are able to mediate cAMP inhibition by melatonin in SK-UT-1B cells and only the MT₂ is able to elicit MAPK inhibition, alternative mechanisms have to be considered. One of these mechanisms could be an activation by melatonin of protein phosphatases that inactivate MAPK and MAPKK. The identity of such phosphatases is still unclear. The catalytic subunits of protein-phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) inactivate MAPKK *in vitro* (Gomez et al., 1991). The threonine residue 183 of p42^{mapk} was found to be dephosphorylated by PP2A and tyrosin 185 by a variety of protein phosphatases (Gomez et al., 1991). A dual specificity protein phosphatase, named CL100 or MAP kinase phosphatase-1, has been also reported to dephosphorylate Thr 183 and Try 185 at similar rates *in vitro* (Sun et al., 1993). However, reliable studies on potential effects of melatonin on the activation of protein phosphatases have not yet been performed.

Another possibility is that the reduction of intracellular cGMP levels is functionally involved in a MAPK-inactivating mechanism. Ho et al. (1999) found in rat pinealocytes that elevation of intracellular cGMP levels leads to activation of the cGMP-dependent protein kinase (PKG) and that this leads to MAPK activation. In SK-UT-1B cells, melatonin inhibits intracellular cGMP levels only in MT₂-transfected cells and selectively these cells show inhibition of MAPK after melatonin treatment. Therefore, the cGMP/PKG signalling pathway in fact could represent a mechanism used by melatonin to inactivate MAPK.

The above interpretation, however, is only valid when the decrease in the phosphorylated form of MAPK is not due to a decrease in the enzyme protein levels. Microarray analyses provided preliminary evidence that overnight melatonin exposure causes a decrease in MAPK gene expression in MT₂-transfected cells when compared to melatonin-exposed MT₁-

transfected cells. Future studies are required to examine the significance of these observations at the protein level.

One of the regulatory pathways responsible for induction of the proto-oncogene, c-Fos, involves increased levels of cAMP acting through a cAMP-response-element (CRE) at the c-Fos promoter (Hoffman et al., 2002). Reduction of cAMP levels by melatonin leads to a decrease in the phosphorylated form of the transcription factor CREB and elicit decrease c-Fos transcription (Ross et al., 1996). Since MT₁- and MT₂-transfected cells show melatonin-induced decreases in causes cAMP and since Fos protein levels are also reduced after melatonin exposure in both cell lines, cAMP-dependent CREB phosphorylation seems to be the most likely pathway regulating c-Fos expression. However, melatonin has been shown to regulate c-Fos gene transcription by the MAPK cascade as well (Chan et al., 2002). This cascade operates via serum response factors (SRFs) which activate a DNA serum response element (SRE) independent of cAMP. In addition, MAPK can also phosphorylate the Jun protein, which together with the c-Fos protein form the transcription factor activator protein-1 (AP-1), (Wit-Enderby et al., 2003). Thus, transient expression of c-Fos in MT₁- and MT₂-transfected cells after melatonin treatment may be explained as follows: after c-Fos is expressed, AP-1 complexes bind to the c-Fos promoter (which has an AP-1 site) and may repress further c-Fos expression (Hoffman et al., 2002).

An up-regulation of cyclin G2 transcript levels was found in MT₂-transfected cells after overnight melatonin treatment. The ability of the MT₂ receptor antagonist 4P-PDOT to block this effect demonstrates a specific MT₂ receptor-mediated mechanism. Cyclin G2, together with cyclin G1 and Cyclin I, defines a novel cyclin family expressed in terminally differentiated tissues including brain and muscle (Horne et al., 1996). Cyclin G2 transcripts show cell cycle periodicity, reaching peak levels at mid-S phase (Horne et al., 1996). Classical cyclins promote cellular proliferation, and they form complexes with specific cyclin-dependent kinases (CDKs), thereby enabling CDK activation. Activated CDKs trigger cell cycle transition through phosphorylation of specific targets. In contrast to conventional cyclins, however, some recently identified cyclins do not promote cell cycle progression *per se* (Gao et al., 1997). Additionally, cyclin G2 expression is independent of the tumor suppressor protein p53 (Horne et al., 1996). Cyclin G2 is up-regulated in cells undergoing apoptosis and in B-cells responding to growth inhibitory stimuli (Horne et al., 1997). In these cells, PKC activation is likely to be important for the regulation of cyclin G2 mRNA. Bennin

et al. (2002) showed that the ability of cyclin G2 to inhibit cell cycle progression correlates with its ability to bind protein phosphatase 2A (PP2A) suggesting that cyclin G2-PP2A complexes inhibit cell cycle progression. Tumor growth ultimately reflects a disruption in the delicate balance between cell proliferation and apoptotic cell loss and/or permanent cell cycle arrest. The ability of melatonin to inhibit cell cycle progression suggest that melatonin's antiproliferative effects on tumor cells may be due in part to its ability to shift the balance from proliferation to differentiation. Supportive evidence for this hypothesis comes from studies showing that melatonin up-regulates the expression of p53 transactivating a group of genes involved in apoptosis and cell cycle arrest (p21/Waf-1 and Bax) resulting in the blockade of cell progression in MCF-7 (Cos et al., 2002) as well as in JAr human choriocarcinoma cells (Shiu et al., 1999).

4.5 Conclusion

In the present study I demonstrate that human MT₁ and MT₂ melatonin receptors, expressed in a human cellular environment, inhibit cAMP levels and c-Fos expression. Modulation of intracellular cGMP levels, as well as of MAPK activity and cyclin G2 expression was shown to be mediated specifically through the MT₂ receptor subtype. The data support the idea that melatonin, particularly via the MT₂ receptor, can suppress cell proliferation. A provisional diagram serves to indicate the proposed signal transduction mechanisms that mediate control of cell growth differentially by MT₁ and MT₂ melatonin receptors (Fig. 25).

The results of this study undercovers that the roles of melatonin in cellular physiology through activation of specific membrane receptors is still insufficiently explained. Research in the next few years should focus on the identification of new melatonin functions and signalling pathways for the "hormone of darkness", which will help to further clarify how melatonin regulates such a diverse number of physiological processes.

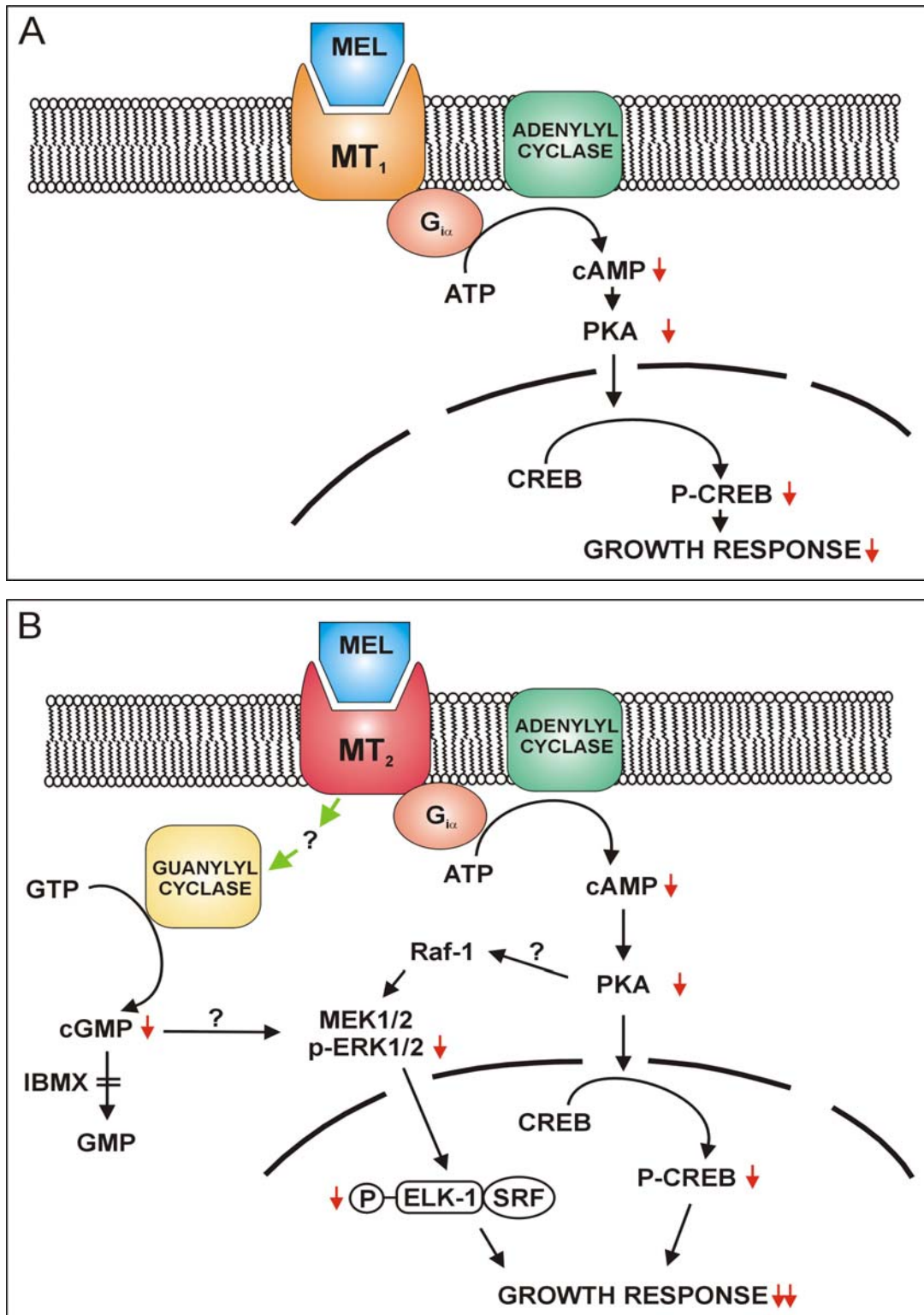


Fig. 25: Schema of the proposed signal transduction mechanisms that mediate control of cell growth through MT₁ (A) and MT₂ (B) melatonin receptors, respectively

Activation of MT₁ receptors by melatonin causes cAMP inhibition and subsequent decrease in PKA and CREB phosphorylation levels, decreasing cell growth. Activation of MT₂ receptors also inhibits cGMP levels and the MEK/MAPK signalling cascade, enhancing cell growth inhibition.

PKA, protein kinase A; CREB, cAMP-responsive element binding protein; IBMX, isobutylmethylxanthine; ATP, adenosine triphosphate; Mel, melatonin; GTP, guanosine triphosphate; GMP, guanosine monophosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; G_{iα}, inhibitory protein α subunit; PKG, protein kinase G; ERK1/2, extracellular signal-regulated kinases 1/2; MEK1/2, MAP kinase kinases 1/2; Raf-1, MAP kinase kinase kinase.

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List of abbreviations

AA-NAT
Arylalkylamine-*N*-Acetyltransferase

aRNA
Antisense RNA

AP-1
Activator protein-1

APS
Amonium per sulphate

ATP
Adenosine triphosphate

BSA
Bovine serum albumin

bp
Base pair

cDNA
Complementary DNA

CTP
Cytosine triphosphate

cAMP
Cyclic adenosine monophosphate

CDK
Cyclin dependent kinase

cGMP
Cyclic guanine monophosphate

CHO
Chinese hamster ovary cell line

COS-7
African green monkey kidney fibroblast cell line

CRE
cAMP response element

CREB
cAMP response element binding protein

cpm
Counts per minute

Cy3/Cy5
Cyanin 3/Cyanin 5

ddH₂O
Double distilled water

DMEM
Dulbecco's modified essential medium

DMSO
Dimethyl sulfoxide

dNTP
Deoxyribonucleoside triphosphate

DNA
Deoxyribonucleic acid

E2
17- β -estradiol

ECL
Enhanced chemiluminescence

EDTA
Ethylenediamine tetraacetic acid

ELISA
Enzyme-linked immuno sorbent assay

EPBS
EDTA-phosphate-buffered saline

ER
Estrogen receptor

ERK
Extracellular-regulated protein kinase

FCS
Fetal Calf Serum

FSK
Forskolin

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanylyl cyclase
Glu	Amino acid; glutamic acid
GnRH	Gonadotropin releasing hormone
GPCR	Guanine nucleotide binding protein coupled receptor
GTP	Guanosine triphosphate
h	Hours
HCl	Hydrochloric acid, hydrochloride
HEK293	Human embryonic kidney cell line
HIOMT	Hydroxyindole- <i>O</i> -methyltransferase
HRP	Horseradishperoxidase
IBMX	3-isobutyl-1-methylxanthine
ICER	Inducible cAMP early repressor
I-Mel	2-iodomelatonin
KCl	Kalium chloride
Kb	Kilobase
K _d	

Equilibrium dissociation constant

kDa

Kilodalton

LB

Laura-Bertani; medium

M

Molarity

min

Minute(s)

MEK

Mitogen-activated kinase kinase

MgCl

Magnesium chloride

MOPS

3-(N-Morpholino)propanesulfonic acid

MTS

(3-(4,5-dimethylthiazol-2-yl)-2-(4-sulfophenyl)-2H-tetrazolium

NA

Noradrenaline

NAS

N-acetylserotonin

NaCl

Sodium chloride

NaOH

Sodium hydroxide

NIH3T3

Mouse embryonic fibroblast cell line

OD

Optical density

PAGE

Polyacrylamide gel electrophoresis

PBS

Phosphate buffered saline

PKA
Protein kinase A

PKC
Protein kinase C

PCR
Polymerase chain reaction

PKG
Protein kinase G

PLC
Phospholipase C

PMS
Phenazine methosulfate

PP2A
Protein phosphatase 2A

4P-PDOT
4-phenyl-2-propionamidotetraline

PVDF
Poly(vinylidene fluoride)

PVN
Paraventricular nuclei

RGS
Regulator of G-protein

RHT
Retinohypothalamic tract

rpm
Rotation per minute

RT
Room temperature

RT-PCR
Real time polymerase chain reaction

SCG
Superior cervical ganglia

SCN
Suprachiasmatic nuclei

SK-UT-1B

Human uterine tumor cell line

SNP

Sodium nitroprusside

SDS

Sodium dodecyl sulphate

SRE

Serum response element

SRF

Serum response factor

ss

Single stranded

SSC

Sodium chloride/sodium citrate buffer

TBE

Tris-Borate-EDTA-buffer

TBS

Tris-buffered saline

TBST

Tris-buffered saline-Tween20

TE

Tris/EDTA buffer

TEMED

N, N, N', N'-tetramethylethylenediamine

TGS

Tris-Glycine buffer

T_m

Melting temperature

TMB

3,3',5,5'-tetramethylbenzidine

Tris

Tris(hydroxymethyl)aminoethane

U

List of Abbreviations

Unit

UTP

Uridine triphosphate

UV

Ultraviolet

V

Volt

v/w

Weight for volume

Curriculum vitae

Name:	Leonor Avila Goñi
Date of birth:	11.11.1972
Origin of birth:	San Sebastian, Gipuzkoa, Spain
Citizenship:	Spanish
Education:	
1978-1986	Virgen del Coro Elementary School in San Sebastian, Spain
1986-1990	J. M ^a Usandizaga High School in San Sebastian, Spain
1990-1996	Bachelor of Science in Biochemistry, University of the Basque Country, Bilbao, Spain
1.10.1999-30.4.2000	Guest scientist in the framework of the European Community Leonardo da Vinci Programme at the Institute for Hormone and Fertility Research, under Dr. A. Mukhopadhyay, in Hamburg, Germany, which focussed on the role of hormones to regulate NO-cGMP-dependent signalling pathways in cultured cells.
1.5.2000-present	Doctoral thesis at the institute for Hormone and Fertility Research, under P.D. Dr. James Olcese, in Hamburg, Germany. The doctoral thesis is titled: "Human melatonin receptors: molecular mechanism of action and their implication in cell proliferation". This was supported by the DFG (Deutsche Forschungs Gesellschaft) in part of the Graduiertenkolleg 336 in Hamburg.

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my grandmother, Leonor Oña Llanos

and my niece, María Avila Montero