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Original article

Polymorphism and signalling of melatonin receptors

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Abstract — Melatonin receptors belong to the superfamily of G protein-coupled receptors. Cloning of Mel1c receptors expressed in Xenopus skin revealed the existence of a polymorphism for these receptors. Heterologous expression of the two allelic isoforms, called Mel1c(α) and Mel1c(β), indicated functional differences in their signalling properties. Both isoforms are coupled to the cAMP and cGMP pathways. However, the α isoform is preferentially coupled to the cAMP pathway, whereas the β isoform couples preferentially to the cGMP pathway. Coupling differences may be explained by the fact that five of the six amino acid substitutions between the two isoforms are localized within intracellular receptor regions potentially involved in G protein coupling. Allelic isoforms were also observed for Mel1a receptors expressed in ovine *pars tuberalis*, suggesting that polymorphism is a general feature of the melatonin receptor family. We also evaluated the potential of the two human melatonin receptor subtypes, Mel1a and Mel1b, to modulate the cGMP pathway. Melatonin inhibited intracellular cGMP levels in a dose-dependent manner in HEK293 cells transfected with the human Mel1b receptor. This was not the case for HEK293 cells transfected with the human Mel1a receptor. In conclusion, our results indicate that the expression of receptor subtypes and isoforms may permit differential signalling between melatonin receptors. © Inra/Elsevier, Paris

melatonin receptor / polymorphism / signalling

Résumé — Polymorphisme et signalisation des récepteurs de la mélatonine. Les récepteurs de la mélatonine appartiennent à la super-famille des récepteurs couplés aux protéines G. Le clonage des récepteurs Mel1c exprimés dans la peau de xénope a révélé l'existence d'un polymorphisme de ces récepteurs. L'expression hétérologue de deux isoformes alléliques, nommées Mel1c(α) et Mel1c(β), a indiqué des différences fonctionnelles dans leur propriétés de signalisation. Les deux isoformes sont couplées aux voies de l'AMPc et du GMPc. Néanmoins, l'isoforme α est couplée préférentiellement à la voie de l'AMPc alors que l'isoforme β l'est à celle du GMPc. Les différences de couplage peuvent être expliquées par le fait que cinq des six substitutions d'acides aminés entre les deux isoformes sont localisées au sein des régions intracellulaires du récepteur potentiellement impliquées dans

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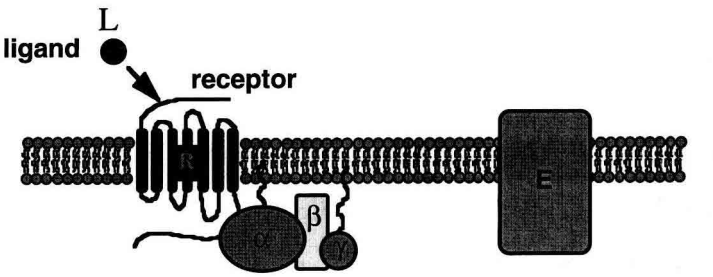
le couplage avec les protéines G. Des isoformes alléliques ont aussi été observées pour les récepteurs Mel1a exprimés dans la pars tuberalis ovine ce qui suggère que le polymorphisme est une propriété générale de la famille des récepteurs de la mélatonine. Nous avons aussi évalué la capacité des deux sous-types de récepteurs de la mélatonine humains, Mel1a et Mel1b, à moduler la voie du GMPc. La mélatonine a inhibé le niveau intracellulaire de GMPc de manière dose-dépendante dans les cellules HEK293 transfectées avec le récepteur Mel1b humain. Ceci n'a pas été observé dans des cellules HEK293 transfectées avec le récepteur Mel1a humain. En conclusion, nos résultats indiquent que l'expression des sous-types et des isoformes de récepteur peut permettre une signalisation différenciée entre les récepteurs de la mélatonine. © Inra/Elsevier, Paris

récepteur à la mélatonine / polymorphisme / signalisation

1. INTRODUCTION

The superfamily of G protein-coupled receptors (GPCRs) comprises more than 200 proteins which allow cells to respond to external stimuli by transmitting information from the extracellular environment across the plasma membrane to intracellular effectors, such as enzymes and ion channels [24]. These receptors typically consist of a single polypeptide chain which traverses the plasma membrane seven times. The N-terminal region is oriented towards the extracellular space, the hydrophobic transmembrane domains are connected via three extracellular loops and three intracellular loops and the C-terminal region is oriented towards the cytoplasmic space (*figure 1*). Ligand binding to a receptor promotes coupling of G proteins (guanine nucleotide-binding proteins) to intracellular receptor domains. Site-directed mutagenesis studies indicate that residues in the second and third intracellular loops and the C-terminus of GPCRs are particularly important for receptor-G protein coupling [1]. On interacting with the receptor, these heterotrimeric G proteins, consisting of α, β and γ subunits, become activated. The α subunit exchanges GDP for GTP and the heterotrimer dissociates into α and $\beta\gamma$ dimers. Both Gα-GTP and G$\beta\gamma$ dimers may interact with effectors and thus transmit a signal [3]. Signal transmission is limited by the hydrolysis of GTP to GDP by an intrinsic GTPase activity of the Gα subunit, leading to re-formation of the G protein heterotrimer. GTP hydrolysis may be accelerated by GTPase-activating proteins (GAPs). The GAP family includes certain G protein-modulated effectors such as cGMP phosphodiesterase and phospholipase C and a more recently identified group of RGS proteins (regulators of G protein-coupled receptor signalling) [18]. At present, 16 different subtypes of G protein α subunits, 5 β and 12 γ have been identified. G proteins can be divided into four subfamilies: the $G_{i/o}$ family whose members are typically pertussis toxin-sensitive and inhibit adenylyl cyclase, the G_s family which stimulate adenylyl cyclase and phosphodiesterases, the $G_{q/11}$ family which stimulate phospholipase C and $G_{12/13}$ proteins which activate small G proteins [13]. Frequently, more than one subtype of receptor exists. This permits an organism to selectively respond to available ligands by coupling to specific G proteins which interact with their corresponding effectors. Subtype division may also contribute to differential regulation of i) receptor signalling, e.g. by desensitization or sensitization; or ii) receptor expression, e.g. in different tissues or during development. It may also lead to variation in post-translational modification of receptors such as phosphorylation or palmitoylation. Polymorphism has been observed for several members of the GPCR family. As receptor subtypes, receptor isoforms frequently show modified signalling and regulation.



GPCR	Gα	Gβ	Gγ	Effector
> 100	16 Gi Gq Gs G12/13	5	12	adenylyl cyclase phospholipase ion channel phosphodiesterase....

Figure 1. Signalling by G protein-coupled receptors. Ligand (L) binding to a receptor (R) at the extracellular surface induces interaction between intracellular receptor domains and heterotrimeric G proteins (α, β, γ subunits). This interaction favours a dissociation of G protein heterotrimers into α subunits and βγ dimers, which transmit a signal through binding to effectors (E).

Melatonin receptors form a distinct group within the GPCR superfamily. Their natural ligand, melatonin, plays a key role in a variety of important physiological responses including circadian rhythmicity, reproduction, sleep and vision [4]. Pharmacological evidence supports the existence of two groups of melatonin receptors, one with a high (10–100 pM) affinity for the radioligand 2-[^{125}I]iodomelatonin (^{125}I-Mel) called Mel1, and another with a low (1–10 nM) affinity for this ligand, called Mel2 or mt_3 [16]. So far, only Mel1 receptors have been successfully cloned and the existence of Mel2 receptors remains a subject of debate. In 1994 the first Mel1 melatonin receptor was cloned from a *Xenopus laevis* dermal melanophore cell line [7]. The originally published sequence of 420 amino acids has not been confirmed in mRNA preparations from *Xenopus laevis* skin where only a shortened receptor with 354 amino acids has been detected [12]. Sequence analysis suggested that this receptor is a member of the GPCR family. The *Xenopus* receptor sequence has since permitted cloning of melatonin receptors from several other species. Sequences were initially separated into three subtypes named Mel1a, Mel1b and Mel1c [21]. Mel1a and Mel1b subtypes have been found in mammals and non-mammals and the Mel1c subtype only in non-mammals. More recently, the International Union of Pharmacology (NC-IUPHAR) proposed to rename mammalian Mel1a receptors mt_1 and mammalian Mel1b receptors MT_2. However, non-mammalian receptors were not considered [5]. Recently, Shiu and Pang presented an update of the phylogenetic tree of all known melatonin receptor protein sequences [23]. The distinction of two major subtypes in mammals was confirmed. However, protein sequences from non-mammalian Mel1b receptors showed more homology with both mammalian and non-mammalian Mel1a receptors than with mammalian Mel1b receptors. The authors propose two subtypes: MT_2 receptors, consisting of mammalian Mel1b receptors, and mt_1 receptors which are subdivided into mt_1a

(mammalian and non-mammalian Mel1a receptors) and mt_1b (non-mammalian Mel1b and Mel1c receptors). All receptors are composed of approximately 350 amino acids and their coding region consists of two exons separated by a large intron. Receptor subtypes have differential, but not exclusive, tissue distributions [16]. All cloned melatonin receptor subtypes have been shown to inhibit forskolin-stimulated cAMP accumulation by pertussis toxin-sensitive $G_{i/o}$ proteins [22]. Cloned Mel1a receptors were also shown to activate Kir3.1/3.2 potassium ion channels, potentiate $PGF2_\alpha$-promoted stimulation of phospholipase C and to modulate PKC and PLA_2 via $G\beta\gamma$ subunits liberated during $G_{i/o}$ protein activation [10, 15, 19]. Information is still lacking about subtype-specific signal pathways which could explain the co-expression of several melatonin receptors. We therefore cloned representative members of all three melatonin receptor subtypes, expressed them in heterologous cell systems and set out to identify additional signal pathways and thus compare signalling between subtypes.

2. RESULTS AND DISCUSSION

2.1. Cloning of Mel1c receptor cDNAs

The Mel1c receptor gene was cloned from *Xenopus laevis* skin mRNA by RT-PCR using primers based on the previously published sequence [7]. The absence of genomic DNA in our mRNA preparation was confirmed in control experiments without reverse transcriptase. The sequence obtained differed from that previously obtained from a melanophore cell line, by 65 amino acids at the C-terminal tail. This led to a shorter protein of 354 amino acids which corresponds to the size of other melatonin receptors. In addition, sequence analyses revealed two populations of Mel1c receptors; one without any substitution other than the shortened C-terminus, called Mel1c(α) and a second one differing from the first by 35 nucleotides and six amino acid substitutions, nominated Mel1c(β) (*figure 2*). Five out of six substituted amino acids are localized in receptor regions predicted to be involved in G protein coupling and receptor regulation [24]. Since the two subtypes are > 98 % homologous at the protein level, they may represent two allelic isoforms of the same gene. Indeed, digestion of PCR-amplified DNA fragments from different *Xenopus* individuals (inbred and foreign) with restriction enzymes specific for Mel1c(α) and/or Mel1c(β) receptors, supported the existence of two allelic receptor isoforms (*figure 3*). A low number of Mel1c(α) homozygotes (2/43) and no Mel1c (β) homozygotes were detected, suggesting that a combination of these two alleles offers some physiological advantage over homozygotes. To test this hypothesis we expressed the two receptor isoforms in heterologous expression systems and characterized their pharmacological and signalling properties.

2.2. Heterologous expression and pharmacological characterization of Mel1c receptors

Mel1c receptor cDNAs were transfected into three different cell lines (human HEK293 and HeLa cells and murine Ltk^- cells). Saturation binding studies with increasing concentrations of ^{125}I-Mel (2–1 500 pM) revealed K_d values of 160 ± 32 and 143 ± 25 pM for Mel1c(α) and Mel1c(β) receptor isoforms, respectively. Accordingly, competition binding studies with ^{125}I-Mel showed that the rank orders of receptor affinities for several ligands were characteristic of high affinity melatonin receptors. Taken together, results show that there are no significant pharmacological differences between Mel1c(α) and Mel1c(β) receptors.

2.3. Signalling of Mel1c receptors via the cAMP pathway

The best-characterized signal pathway mediated by all high affinity melatonin receptor subtypes is the inhibition of

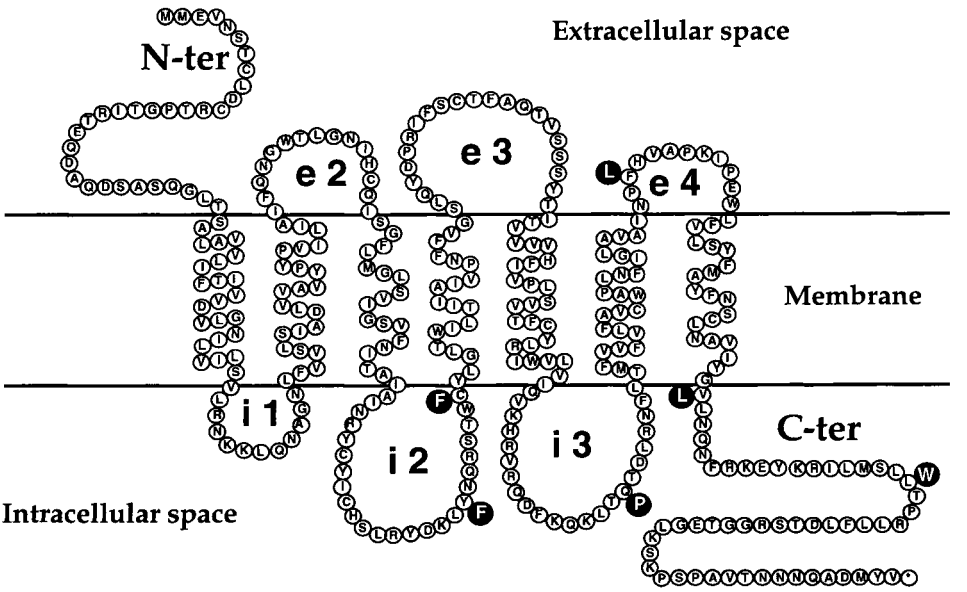


Figure 2. Comparison of Mel1c(α) and Mel1c(β) melatonin receptor sequences. The sequence of the Mel1c(α) melatonin receptor is shown (amino acid sequence in one-letter code in open circles) and amino acid substitutions characterizing the Mel1c(β) receptor (amino acid sequence in one-letter code in closed circles) are indicated.

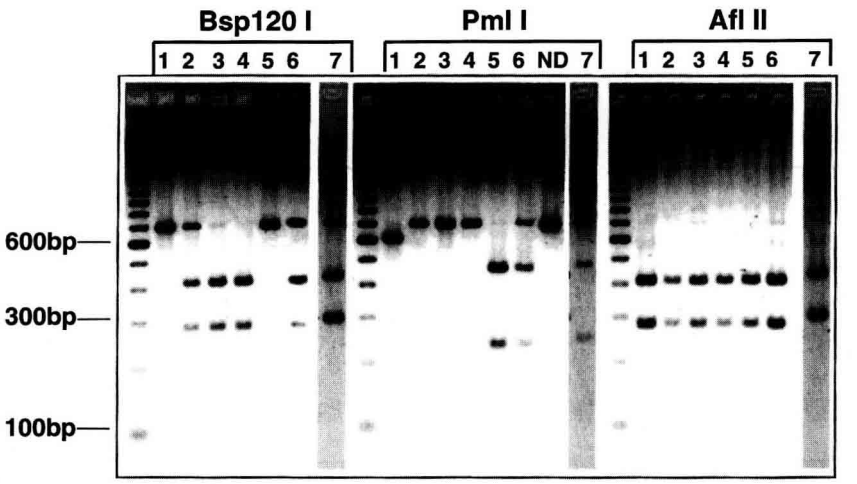


Figure 3. Characterization of the Mel1c(α)/(β) DNAs from various *Xenopus* individuals. The central part of the Mel1c receptor coding region (from position 359–1 029) was amplified from genomic DNA. Restriction enzymes used for digesting PCR products were: Afl II (one site in both Mel1c(α) and Mel1c(β) receptors), Bsp120 I (one site in Mel1c(α), absent in Mel1c(β) receptors), Pml I (one site in Mel1c(β), absent in Mel1c(α) receptors). Restriction analysis of PCR products from representative cDNAs: 1, *X. tropicalis*; 2, *X. ruwenzoirensis*; 3, homozygous *X. laevis* (ff) individual; 4, cloned Mel1c(α) cDNA; 5, cloned Mel1c(β) cDNA; 6, *X. laevis* cDNA amplified from a pool of skin mRNA; 7, heterozygous *X. laevis* (rf) individual; ND, undigested PCR product.

forskolin-stimulated cAMP accumulation. This effect was verified in melatonin receptor-transfected cells. Interestingly, in Ltk cells, inhibition was only observed for the Mel1c(α) subtype ($IC_{50} = 6 \times 10^{-10}$ M) and the Mel1c(β) receptor was without effect (*figure 4*). In HEK293 cells (*figure 4*) and HeLa cells (not shown) transfected with Mel1c(α) or Mel1c(β) receptors, both isoforms promoted the inhibition of forskolin-stimulated cAMP accumulation, with an IC_{50} value of 10^{-10} M. Thus, heterologous expression of both Mel1c receptor isoforms revealed a functional difference in cAMP signalling. Furthermore, this difference appears to be cell type-dependent.

2.4. Mel1c receptors modulate a further second messenger: cyclic GMP

During our search for other potential signal pathways mediated by Mel1c receptors, our interest focused on the observation that melatonin inhibits both cyclic AMP and cyclic GMP accumulation in the rat pituitary, a tissue known to express high affinity melatonin receptors [25]. Intracellular cGMP levels depend on the opposite activities of guanylyl cyclases which catalyse the synthesis of cGMP and phosphodiesterases (PDE) which promote the degradation of cGMP. Ltk$^-$ cells, HEK293 cells and HeLa cells, transfected with either Mel1c(α) or Mel1c(β) receptors were incubated with melatonin and their effect on intracellular cGMP was analysed. In these cell lines, melatonin had no effect on basal cGMP levels. In contrast, in the presence of IBMX, a PDE inhibitor which blocks the degradation of cGMP and thus augments cellular cGMP levels, melatonin inhibited cGMP accumulation in a dose-dependent manner (*figure 5*). Inhibition was strictly receptor-dependent since it was not observed in non-transfected cells. Importantly, the effect of Mel1c(α) receptor activation on cGMP levels was weak compared to Mel1c(β). Combined results indicate that Mel1c receptors from *Xenopus laevis* modulate both cAMP and cGMP levels and that the Mel1c(α) isoform is preferentially coupled to the cAMP pathway, whereas the Mel1c(β) isoform preferentially couples to the cGMP pathway. The physiological relevance of sig-

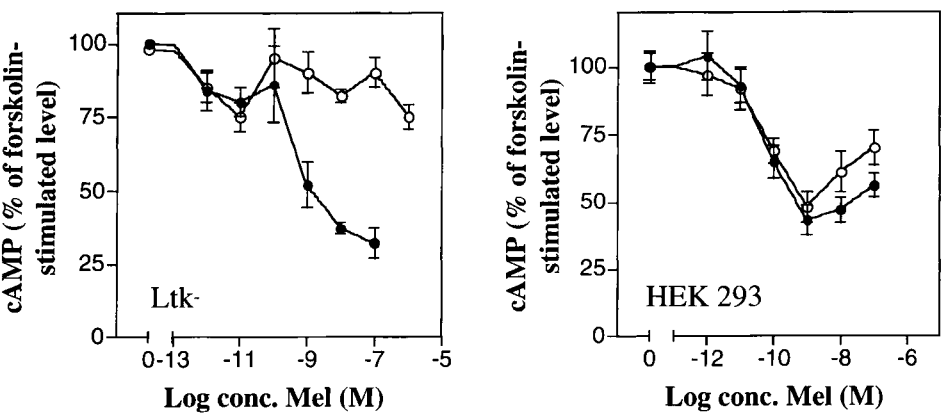


Figure 4. Modulation of forskolin-stimulated cAMP accumulation by Mel1c(α) (●) and Mel1c(β) (○) receptors in Ltk$^-$ and HEK293 cells. Stable clones of Ltk$^-$ and HEK293 cells transfected with either Mel1c(α) or Mel1c(β) melatonin receptor cDNA were stimulated with forskolin (10 μM) in the presence of the indicated concentrations of melatonin and intracellular cAMP levels were determined.

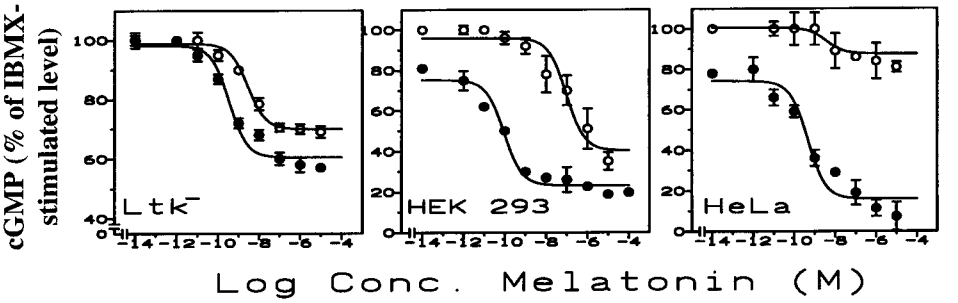


Figure 5. Modulation of cGMP accumulation by Mel1c(α) (●) and Mel1c(β) (○) receptors. Stable clones of Ltk⁻, HEK293 and HeLa cells transfected with Mel1c(α) or Mel1c(β) receptor cDNAs were incubated with the indicated concentrations of melatonin in the presence of 1 mM IBMX and intracellular cGMP concentrations were determined.

nalling differences between Mel1c receptor isoforms should be determined in their natural expression context, the *Xenopus* skin. Taken together, our data demonstrate that both Mel1c receptor isoforms have distinct signalling preferences which may explain the co-expression of both isoforms in *Xenopus* skin as well as the conservation of two allelic isoforms during evolution. The existence of subtype-specific differences in the signalling properties of melatonin receptors was also recently suggested by the phenotype of Mel1a receptor knock-out mice [14]. Whereas the melatonin-induced phase-shift of the circadian rhythm of the SCN was still observed in these mice, the effect of melatonin on the electric activity of the SCN was absent. These results suggest that the effect of melatonin on the electric activity is specifically mediated by the Mel1a receptor and that the phase-shifting effect may be due to the activation of the Mel1b receptor alone or of both Mel1a and Mel1b receptors. The Mel1b receptor has indeed recently been shown to be involved in the phase-shifting properties of melatonin [6].

Interestingly, allelic isoforms of melatonin receptors were also identified in sheep [2]. A novel variant of the Mel1a receptor, named Mel1a(β) was cloned from ovine *pars tuberalis*, which differs from the original sequence, Mel1a(α), by eight nucleotides and three amino acid changes. One of the substituted amino acid residues lies within the third extracellular loop of the receptor, whereas the two other substitutions lie in the carboxy-terminal tail. Despite these variations, the two sheep Mel1a isoforms displayed no apparent difference in ^{125}I-Mel binding or adenylyl cyclase inhibition. Identification of allelic isoforms of different melatonin receptor subtypes in two different species, *Xenopus* and sheep, indicates that polymorphic variation may be a general phenomenon of the melatonin receptor family.

2.5. Modulation of cGMP levels by human Mel1a and human Mel1b receptors reveals functional differences

The above approach was further used to verify whether human Mel1a and Mel1b receptors could also influence the cGMP pathway. In HEK293 cells transfected with either one of these receptors, incubation with IBMX alone increased basal cGMP levels by approximately ten-fold. Stimulation of Mel1b-transfected cells with melatonin inhibited IBMX-elevated cGMP levels with a maximal inhibition of 40 % and an IC_{50} value of approximately 10^{-10} M (*figure 6*). No significant effect was observed

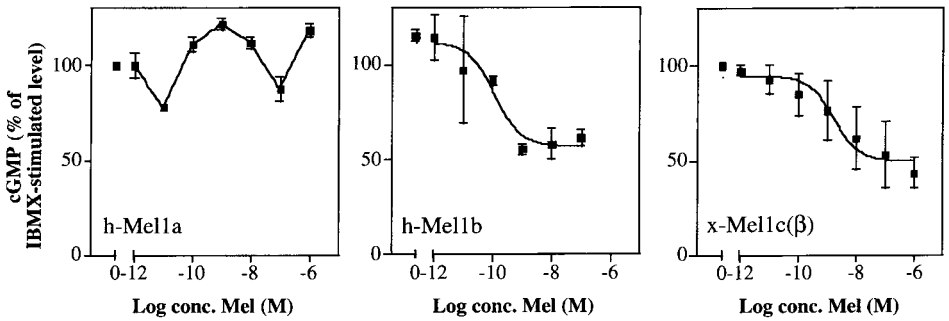


Figure 6. Modulation of cGMP accumulation by Mel1a, Mel1b and Mel1c(β) receptors. HEK293 cells stably transfected with h-Mel1a, h-Mel1b or x-Mel1c(β) receptor cDNAs were incubated with the indicated concentrations of melatonin in the presence of 1 mM IBMX and intracellular cGMP concentrations were determined.

under these conditions in non-transfected or Mel1a-transfected cells. The physiological relevance of these findings is supported by the fact that this effect is observed at normal circulating concentrations of melatonin. This work provides the first evidence of differential signalling between melatonin receptor subtypes.

2.6. Melatonin receptors modulate cGMP levels via the soluble guanylyl cyclase pathway

The cGMP pathway modulated by melatonin receptors was further characterized in a HEK293 cell clone expressing the Mel1c(β) receptor from *Xenopus laevis*. Two major families of guanylyl cyclases exist: soluble guanylyl cyclases which can be stimulated by NO, and membrane-bound guanylyl cyclases which are stimulated by extracellular peptide ligands such as atrial natriuretic peptide (ANP) [8]. Reduction in cGMP levels via melatonin receptors may thus be explained by an inhibition of these cyclases or, alternatively, by activation of cGMP degradation by phosphodiesterases. Since the inhibitory effect of melatonin was observed in the presence of the PDE inhibitor, IBMX, the latter hypothesis is unlikely. The NO donor, nitroprusside (SNP) significantly increased cellular cGMP levels, indicating the presence of a soluble guanylyl cyclase activity (*figure 7*). Furthermore, both IBMX- and SNP-promoted cGMP accumulation could be blocked by the soluble guanylyl cyclase inhibitor, 1H-(1,2,4) oxadiazolo(4,3-α)quinozalin-1-one (ODQ) [9, 17], suggesting that cGMP levels

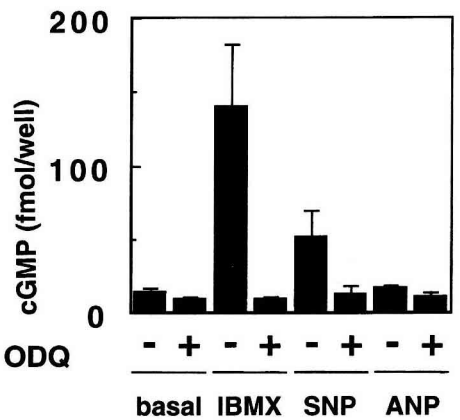


Figure 7. Characterization of the cGMP pathway in HEK293 cells. HEK293 cells stably transfected with the Mel1c(β) receptor were incubated for 15 min with either IBMX (1 mM), sodium nitroprusside (SNP) (300 μM), or atrial natriuretic peptide (ANP) (500 nM) in the presence or absence of ODQ. Cyclic GMP levels were then determined for each condition.

were entirely dependent on soluble guanylyl cyclase activity. Accordingly, no basal or ANP-stimulated membrane-bound guanylyl cyclase activity was detected. Importantly, melatonin had no significant effect on cGMP levels directly stimulated by SNP (not shown). It is thus likely that melatonin receptors modulate the cGMP pathway at a site upstream from soluble guanylyl cyclases. Potential targets for melatonin receptors at this site include NO synthase and NO synthase activators such as calmodulin or divalent Ca^{2+} ions.

3. CONCLUSIONS/PERSPECTIVES

Polymorphism has now been observed for several GPCRs. In some cases allelic isoforms differ in coupling efficiency for an effector system, as has been shown for the Trp64Arg polymorphism of the human β3-adrenergic receptor [20], or differ in receptor regulation, as shown for the Gln27Glu polymorphism of the human β2-adrenergic receptor [11]. Often, these modifications at the molecular level are correlated with modified phenotypes.

Cloning of Mel1c receptors from *Xenopus laevis* skin and Mel1a receptors from ovine *pars tuberalis* has shown that polymorphism also exists for melatonin receptors. In the case of the Mel1c receptor from *Xenopus laevis* the two isoforms showed differences in signalling efficiencies for the cAMP and cGMP pathways. The majority of *Xenopus* individuals tested were heterozygous for Mel1c receptors, suggesting that the conservation of both alleles confers some evolutionary advantage. Isoform-specific signalling preferences might be one of the reasons why expression of both isoforms has been conserved. Studies on endogenously expressed Mel1c receptors in *Xenopus* skin will be necessary to answer this question. A further goal for future studies will be to determine whether polymorphisms also exist for human melatonin receptors and whether these can be associated with any modified phenotypes.

Our results show that the human Mel1b and *Xenopus* Mel1c receptors modulate intracellular cGMP levels. Thus cGMP-regulated effectors such as ion channels, phosphodiesterases and protein kinase G are promising new cellular targets for melatonin signalling. Furthermore, the lack of effect of human Mel1a receptors on intracellular cGMP levels reveals differences in signalling between mammalian melatonin receptor subtypes which may explain the necessity to express different receptor subtypes.

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