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Original article

Homology modeling of MT_1 and MT_2 receptors

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Abstract

Melatonin is a neurohormone synthesized and secreted mainly during the dark period of the circadian cycle by the pineal gland. It has already been proved to be involved in a number of chronobiological processes, most of them being mediated by its membranar receptors MT_1 and MT_2. Both are members of the GPCR class and, despite the interest they elicit, their 3D structure is still to be described. Models for both human MT_1 and MT_2 receptors have been constructed by homology modeling, using the X-ray structure of bovine rhodopsin as template. These models have been evaluated in terms of hydrophobic properties of the helices and refined to take into account the rearrangement of GPCRs necessary for their activation, thus leading to a putative activated model for each subtype.

Keywords: Melatonin; GPCRs; Activation; Docking

1. Introduction

Melatonin, *N*-acetyl-5-methoxytryptamine (Fig. 1) was isolated and its structure described in 1959 [1]. It is principally synthesized by the pineal gland and secreted during the dark period of the circadian cycle [2], due to the suppressive effect of light on the stimulatory retinohypothalamic pathway [3,4]. This light-regulated cycle gives to melatonin a major role in seasonal and chronobiological processes. It is already employed for veterinary use, such as stimulation of fur growth for the mink or regulation of the ovine reproduction [5,6]. Among the possibilities under investigation for its use in human clinics, its chronobiotic properties are widely involved. Numerous works have been devoted to the resynchronization of disturbed biological rhythms such as sleep shift caused by jet lag or night work, insomnia and seasonal affective disorders [7–11]. Overall, two kinds of melatonin binding sites have been identified so far. They have been localized and characterized initially on the basis of their affinity for 2-[^{125}I]-iodomelatonin, giving rise to high affinity ML_1 sites and low affinity ML_2 sites [12]. Melatonin exerts the major part of its aforementioned effects by the activation of two high affinity binding sites denoted as MT_1 [13] and MT_2 [14], which belong to the G-protein coupled receptor superfamily, and a low affinity binding site, referred to as MT_3, according to the IUPHAR nomenclature [15], which was recently identified as quinone reductase 2 (QR2 EC 1.6.99.2) [16,17], an enzyme closely related to the detoxifying enzyme quinone reductase 1. Moreover, there is also another high affinity binding site, the subtype Mel_{1c} that was first cloned from *Xenopus laevis* but not yet identified in mammals [18]. As the IUPHAR nomenclature concerns mammals only, Mel_{1c} has kept its original name. The MT_1 subtype is found in the retina, the kidneys and the brain, more particularly in the suprachiasmatic nuclei and thus should be implicated in the transmission of the circadian

Abbreviations: GPCR, G-protein coupled receptor; TM, transmembrane domain.

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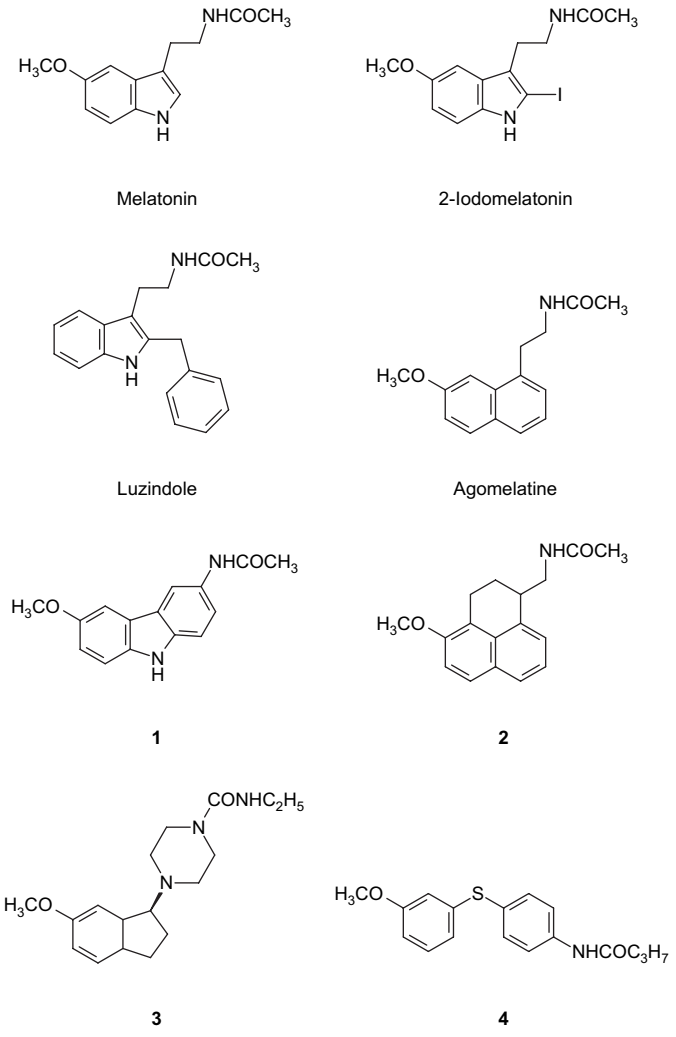

Fig. 1. Structures of melatoninergic ligands.

effects of melatonin and reproduction. This receptor could also be involved in peripheral vasoconstriction. The MT_2 subtype is found in the retina and the brain too, but contrary to MT_1, it does not appear in the suprachiasmatic nuclei. It could play a role in the physiology of the retina and in body temperature regulation [19,20]. Structurally, MT_1 is a 350 amino acid protein, presenting a sequence homology of 60% with the 362 amino acid MT_2 [14]. The GPCR class is an important target for drugs due to the great diversity of their ligands, ranging from photons to peptides or proteins. Some studies suggest that 30–50% of known drugs actually target a GPCR to produce their effects [21–23]. These receptors display a common characteristic fold composed of seven transmembrane alpha-helices. Moreover, they present highly conserved residues, such as the DRY sequence of the intracellular end of transmembrane domain 3 (TM3) implied in the G-protein activation. This sequence is replaced by an NRY motif for melatonin receptors, which plays exactly the same role by coupling the receptor and a G_i or Gq protein, leading to a decrease in cAMP [24,25]. However, GPCRs are extremely difficult to isolate and crystallize as their inclusion in the cell membrane contributes to their structural stability and renders them poorly purifiable [26]. This leads to the impossibility to determine directly their structure by standard means. The only way to cope with this problem is the construction of theoretical models based on the only GPCR crystallographic structure available, bovine rhodopsin, using a homology modeling approach [27,28]. However, it is assumed that the high-resolution X-ray structure of bovine rhodopsin is in an inactive state and cannot stand for agonist ligand activated GPCRs. This GPCR activation process can be simulated by rotation of some transmembrane domains as suggested by experimental data [29–31]. We report here the construction of a 3D structure for both human MT_1 and MT_2 receptors and their careful modification toward an activated state using mutational and ligand SAR data in an attempt to propose a valid melatonin binding site. Such models may be very helpful to facilitate the design and development of new selective ligands.

2. Results and discussion

2.1. Sequence alignment

The most important point in any homology modeling study, besides the choice of the reference, is the alignment of the sequences. The greatest attention was thus paid to the careful construction of a robust alignment. The transmembrane domains form the core of GPCRs, so it appeared safer to produce an alignment conserving the integrity of these domains (Figs. 2 and 3). The helices of melatonin receptors were believed to be superposable with the experimentally known helices of bovine rhodopsin. In this view, it therefore appeared important to create as few gaps as possible in the transmembrane domains. In consequence, there is only one gap in TM4 of MT_2 corresponding to the important Asn 175. In MT_1, both immediate neighbours of this residue are conserved in rhodopsin, whereas MT_2 sequence differs from that of the reference at this point, therefore resulting in a better alignment when leaving a gap in front of Asn 175. This naturally points to a peculiarity of this position between the subtypes of melatonin receptors. Two gaps were created in MT_1, in TM6 and TM7, for a similar reason. An interesting superposition was revealed from the comparison of the alignments: the couple of cysteines forming a disulfide bond in rhodopsin (Cys 110 and 187) were conserved in the three sequences. Even though the alignment in the TM4–TM5 range did not reveal at first a conservation of the second cysteine of the pair, the first one, in TM3, was conserved. Moreover, experimental data gathered on MT_2 showed such an interaction between Cys 113 and 190 [32]. We adjusted the alignment manually to superpose the latter with Cys 187 of rhodopsin. By checking the alignment of the melatonin receptors (Fig. 4), it was easily found that both cysteines are common to the two receptors and that the alignment of MT_1 versus rhodopsin also conserved the first cysteine. We therefore put the hypothesis that, by homology with MT_2 and rhodopsin, Cys 100 and 177 of MT_1 were involved in a disulfide bond. Comparing the alignments, it appears that MT_1 is slightly closer to the sequence of rhodopsin than MT_2, with about 23% of identity, while MT_2 shows about

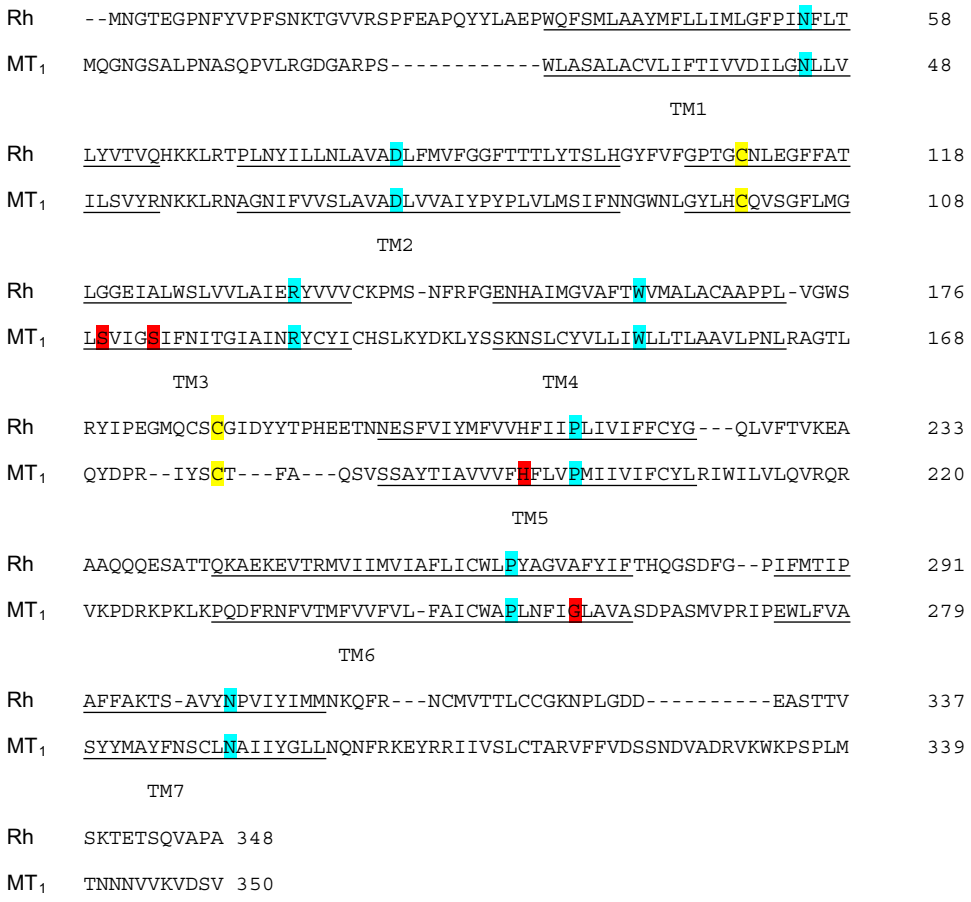

Fig. 2. Sequence alignments for bovine rhodopsin (Rh) and MT_1. Transmembrane (TM) helices (marked TM1–TM7) are underlined. The most conserved residues of each helix throughout the GPCR family are overtyped in cyan and residues known to be important for the binding are overtyped in red. The cysteines forming a disulfide bridge are overtyped in yellow. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

19% of identical residues. However, all the amino acids commonly conserved in the helices of GPCRs are present and well superposed as it is shown by a cyan overtyping in Figs. 2 and 3. Thus, we have not further adjusted the alignments.

2.2. Molecular modeling

The choice of the reference 3D structure is still very limited for the GPCRs. In fact, there is only one such protein for which crystallographic data are known. We have employed the highest resolution available at the moment for the bovine rhodopsin. The models achieved directly from these alignments were checked after a thorough energy minimization designed to reduce the steric clashes of the side chains without modifying the backbone of the protein to solve these contacts, in essence enabling the optimization of the geometry of the backbone with as least interference from the side chains as possible. After the optimization, both models were checked to assess the quality of their structure, resulting in the Ramachandran plots described in Fig. 5. No residue lies in the disallowed part of the plots and very few residues (2.9 and 1.1% of residues of MT_1 and MT_2, respectively) are in the less favourable regions. It appears that only two residues are outside the most favourable region for MT_2, and interestingly, one of them is Asn 175. This suggests a unique constraint around the binding site, although the extent of the discrepancy is fairly small and the conformation of the residue is still compliant with a correct structure. As this amino acid is inserted between two residues of the reference, however, this negligible difference with the ideal values is only natural. MT_1 has more residues outside the most favourable region, but all remain in the allowed space.

2.3. Hydrophobic moments

As another tool to assess the structural validity of the models, we have calculated the hydrophobic moments of the helices (Fig. 6). As felt intuitively, they should be oriented toward another lipophilic feature, such as another helix or the membrane [33]. Compared to rhodopsin, MT_1 has a very similar profile over the whole helix bundle. However, there are some differences. TM2 points more toward the membrane than its rhodopsin counterpart, which is pointing to TM1. It is still oriented fairly straightly to the same helix, thus bringing the net difference to a very minor one. Two major differences are apparent. First, TM3 has a hydrophobic moment directed toward TM5 for MT_1, while for the reference, it points between TM2 and TM4. Second, TM6 points toward

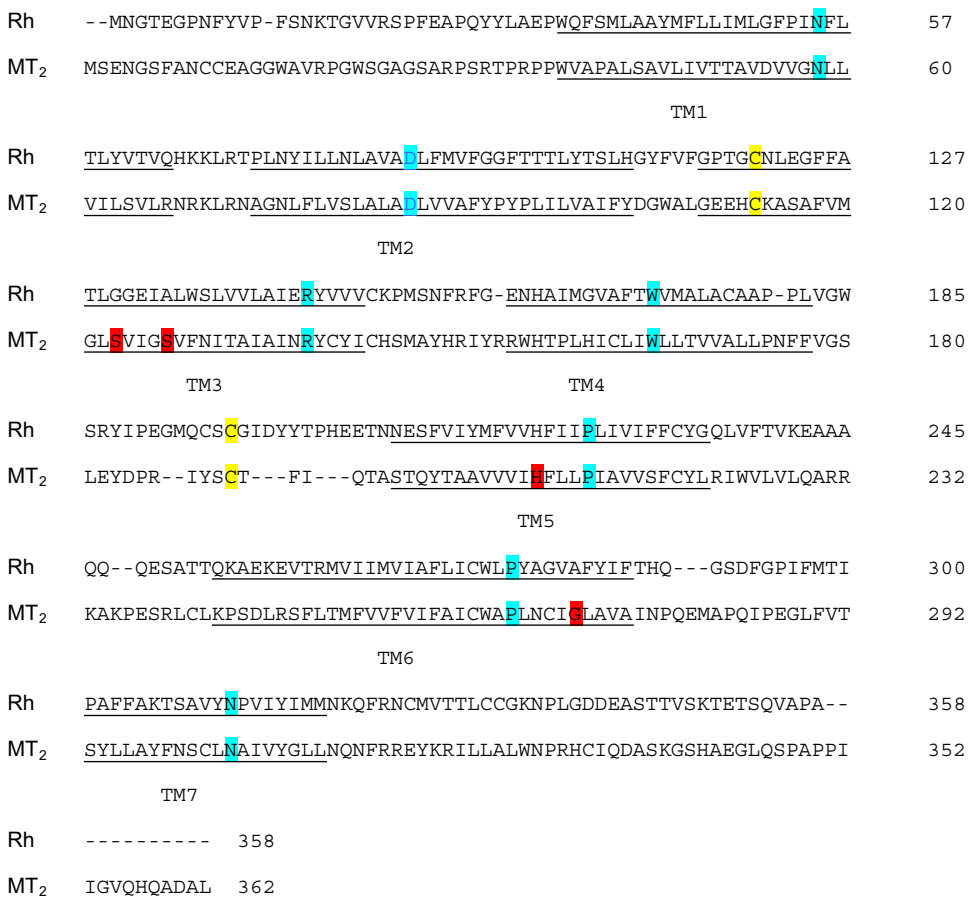

Fig. 3. Sequence alignments for bovine rhodopsin (Rh) and MT_2. Transmembrane (TM) helices (marked TM1–TM7) are underlined. The most conserved residues of each helix throughout the GPCR family are overtyped in cyan and residues known to be important for the binding are overtyped in red. The cysteines forming a disulfide bridge are overtyped in yellow. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

TM7 in our model, whereas it points toward the membrane in rhodopsin. Even if it is fairly important in terms of the angle, with about a 90° rotation between the two proteins, it is in fact of a low interest, as both moments are coherent with their theoretically allowed orientations. For MT_2, the same global agreement with the reference has been found. In contrast with MT_1, the moment of TM2 points to TM1 more sharply. Overall, there are only minor divergences between the models of the melatonin receptors, mostly slightly different orientations of the moments that do not affect their facing. Therefore, the biggest difference with rhodopsin for MT_2 is still the orientation of the hydrophobic moment of TM3.

2.4. Binding site identification

Using the site directed mutagenesis data available in the literature, we have identified the putative binding site of melatonin on both receptors. Interestingly, even if they lie in the same part of the receptor, in the immediate vicinity of TM5 and in the extracellular half of the transmembrane domain, they look clearly dissimilar (Fig. 7). On MT_1, the putative binding site seems to be relatively smaller than on MT_2, as it implies only TM3 and TM5, possibly also TM7, even if we cannot directly infer from mutagenesis data if Gly 258 is critical due to a direct interaction with the ligand or due to its role in maintaining the conformation of the receptor. On MT_2, four helices bear residues essential for the binding. A critical difference between the two receptors is that TM3 has not proved to be involved in the binding of the ligand in MT_2 whereas it bears two of the three important residues on MT_1. In fact, only the conserved histidine of TM5 (His 195 for MT_1 and 208 for MT_2) appears to be common to the binding site of the two subtypes. This is fairly surprising, as the residues important for ligand binding are conserved in both receptors, with the exception of Leu 295 of MT_2, which corresponds to Tyr 282 in MT_1 (Fig. 4). Selectivity between the two subtypes of melatoninergic receptors should therefore be studied carefully as the relatively few residues differing from one subtype to the other contribute to utterly modify the binding site.

2.5. Docking of melatonin

We have placed melatonin in the binding site according to the experimental data. In essence, its binding mode is fairly well described on MT_1. The oxygen of the 5-methoxy group should accept a hydrogen from histidine 195, while the carbonyl oxygen and the hydrogen carried by the nitrogen of its amide function should be engaged in two hydrogen bonds

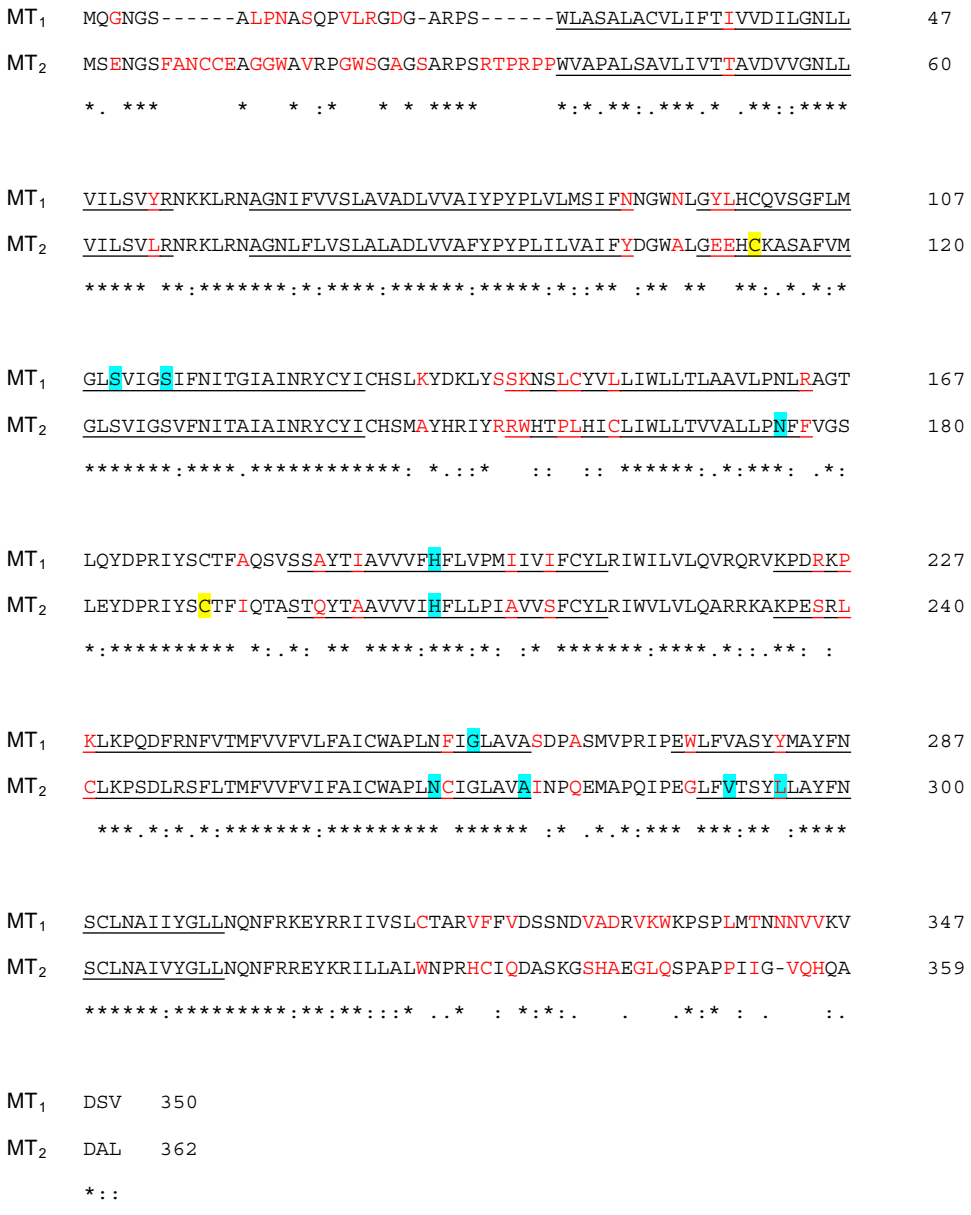

Fig. 4. Superposition of sequences of MT_1 and MT_2. Residues corresponding to the helices of rhodopsin are underlined, residues differing from one subtype to the other are in red. Residues critical for the binding of agonists are overtyped in cyan. Cysteines engaged in a disulfide bond are overtyped in yellow. In the sequences, an asterisk (*) indicates an identical or conserved residue, a colon (:) indicates a conserved substitution, a stop (.) indicates a semi-conserved substitution. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

with serines 110 and 114 (Fig. 8). However, it was impossible to form simultaneously all the required bonds as the serines were virtually unreachable due to the facing of TM3, exposing them on the side opposite to TM5. Therefore, the bulk of TM3 stood between His 195 and the serines, which could not be bound without disrupting the backbone of TM3. On MT_2, the situation is much less clear. There are two hypotheses validated by site directed mutagenesis data. In the first one, derived from data proposed by Gerdin et al. [34] two residues are critical for melatonin binding: Asn 175 and His 208. By similarity with MT_1, His 208 should probably bind the methoxy group, thus leaving the amide moiety to interact with Asn 175 (Fig. 9). In such a case, however, the conformation adopted by melatonin allowed both hydrogen bonds but was clearly not valid as it resulted in close contacts between the ligand and both residues. The second hypothesis stems from the data gathered by Mazna et al. [35,36]. In this interaction model, Asn 268 of TM6 and Tyr 298 of TM7 are critical. His 208 was not mutated in this study, but was nonetheless conserved in the immediate vicinity of the 5-methoxy group of 2-iodomelatonin. Although these interactions are possible, the experimental data could not afford enough insight to discriminate between a loss of binding due to an unfolding of the receptor impeding a proper interaction and a loss of binding due to the disappearance of a critical interaction. The most important residues in both site directed mutagenesis studies,

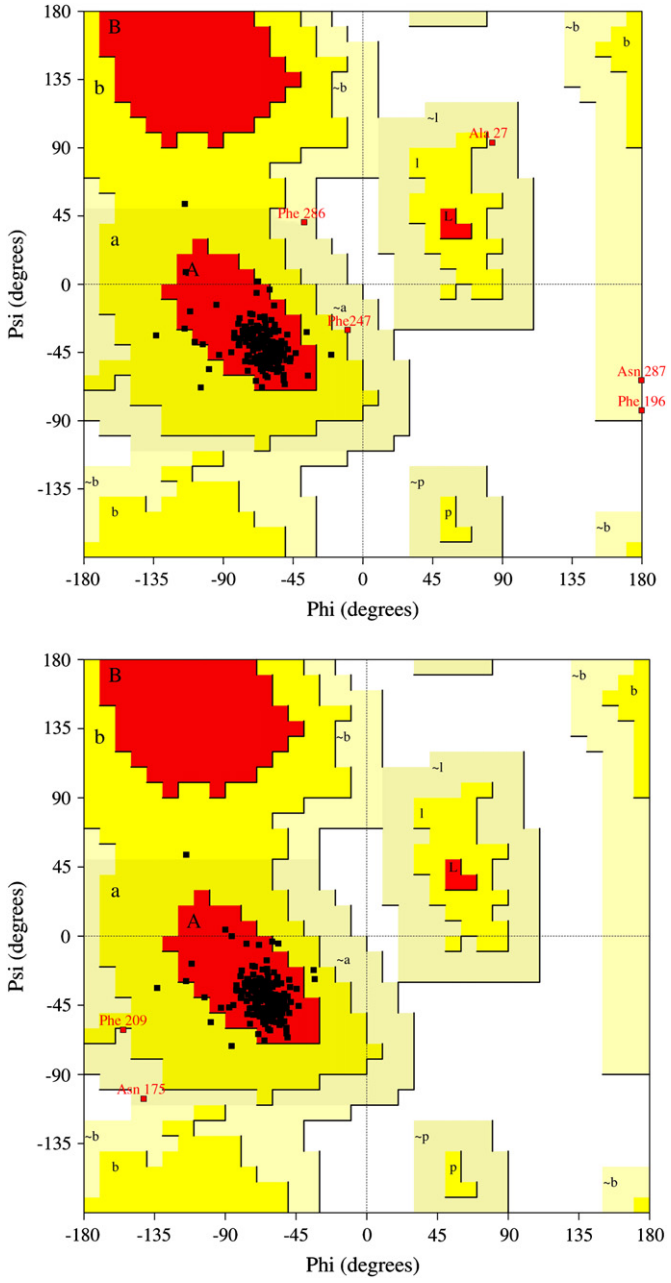

Fig. 5. Ramachandran plots obtained for the models of MT_1 and MT_2. The most favourable regions are in red, favourable regions in yellow, allowed regions in tan. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

that is those reducing the affinity of melatonin when modified, are Asn 175, Val 204, His 208, Asn 268, Leu 272, Ala 275, Tyr 298, Val 291 and Leu 295. Keeping the residues proposed in mind for further analysis of their effect, we nonetheless chose the Asn 175–His 208 hypothesis. These initial models were derived from the crystallographic data obtained from the dark adapted rhodopsin. They should therefore represent an inactive form of the receptors. In order to find a conformation more adequate to bind agonist ligands, we have adjusted the geometry of the transmembrane domains bearing the residues responsible for the hydrogen bonds. For MT_2, TM4 and TM5 were thus our first guess. However, considering the position of His 208 near the outside of the pocket, a counterclockwise rotation of TM5 (seen from the extracellular extremity) would be unfit. On the other hand, a clockwise rotation would lead His 208 in front of two other important residues (Ala 258 and 275 of TM6). TM4 can be rotated more freely to give some room to an agonist. Thus, we have rotated this helix until the receptor was able to accommodate melatonin without close contact between the ligand and the residues binding it. This was achieved for a 26° counterclockwise rotation of TM4. For MT_1, TM3 and TM5 were a natural choice. We first rotated TM3 to bring the serines closer to the histidine, but the resulting conformation was still unable to bind melatonin. For each of the rotations of TM3, we applied the same method to TM5, achieving a set of 129 conformations of the helix bundle. Only a few conformers were selected, based on the angles of rotation that brought His 195 and Ser 110 and 114 in a proper range of distance to permit the binding of melatonin. Manual docking of the natural ligand quickly showed that a clockwise rotation of 50° for TM3 and 20° for TM5 led to a correct position of melatonin.

2.6. Activation of the receptors

The two models achieved at this point should correspond to a putative activated form of the receptors. It is therefore of some interest to study the differences between the initial and the activated forms. For the ease of comparison, the hydrophobic moments of the activated models are illustrated in Fig. 6. Interestingly, on MT_1, the profiles of the two models are fairly similar, all the moments keeping roughly the same orientation. Although a 50° rotation was applied on TM3, its hydrophobic moment has not turned as much. This comes from the restricted environment of this central helix that severely limits the rearrangement of the side chains, therefore reducing the overall impact of the rotation on the hydrophobic moment. On the contrary, TM5 is free to move, as our model is not included in a membrane, thus explaining the larger reorientation of its moment. A slight tilting of TM2, compared to the initial model, was also observed. Its extracellular end is pushed toward TM6. This should compensate for the modification of the position of TM3. On MT_2, the rotation of 26° of TM4 had a higher impact on the helix bundle, although its own moment did not change. However, TM3 and TM5 are modified. In sharp contrast with MT_1, their moment turned in a counterclockwise manner. This result is interesting, as TM3 has a central position in the helical bundle and its intracellular extremity bears the NRY sequence involved in the stabilization of the inactive conformation of the receptor [37]. The reorientation of the vector of TM5 is also important, as this domain contains the conserved histidine binding melatonin. TM2 is tilted by the adjustment of TM4 exactly as in MT_1, but the modification is more sharply transmitted to TM1 than in the latter, resulting in an overall larger modification of the helix bundle in MT_2.

The hydrogen bonds between the helices changed too as a result of the modification of the helix bundle. In the inactive

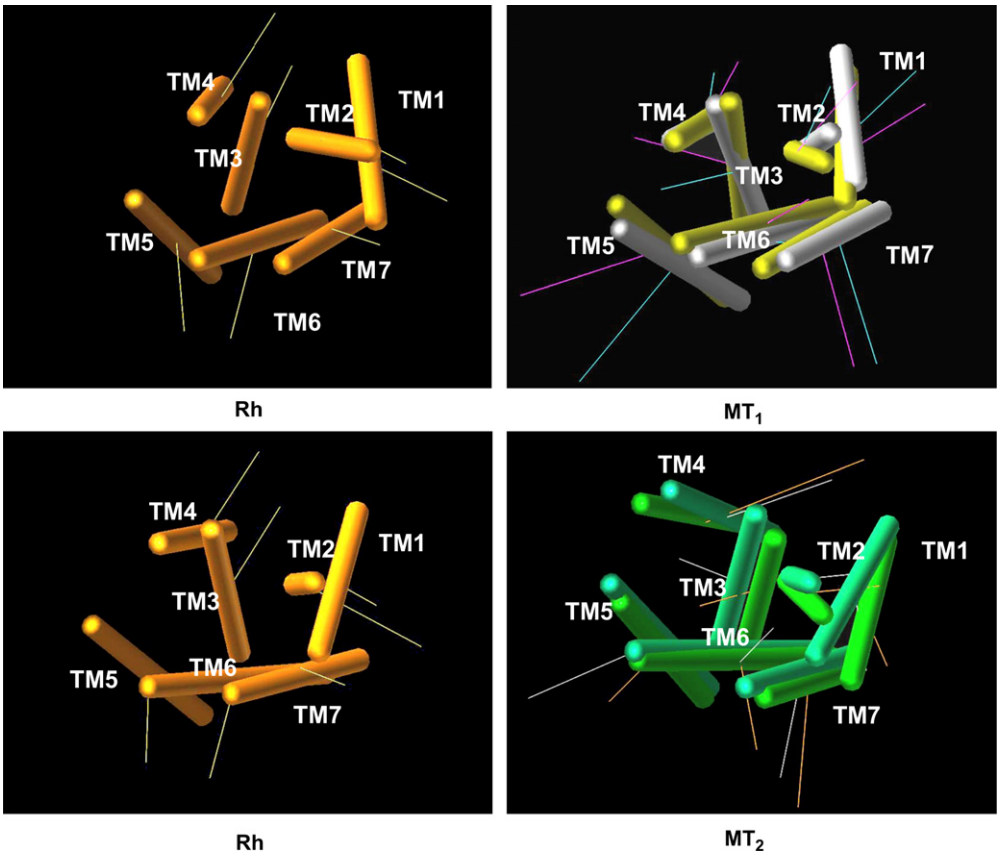

Fig. 6. Hydrophobic moments of MT_1 (the initial model is in white and the corresponding moments are indicated in blue) and MT_2 (the initial model is in green-blue with white vectors). For both receptors, bovine rhodopsin (Rh) is figured on the left. The helix bundle is seen from its extracellular end. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

model of MT_1 (Table 1), TM3 has a central role, as it is bound to four of the helices (TM2, TM5, TM6 and TM7). TM7 deserves a special attention due to the numerous interactions tying it to TM1 (two hydrogen bonds) and to TM2 (four hydrogen bonds). In particular, a strong interaction is formed between Asp 73 of TM2 and Asn 287 of TM7. On the other hand TM6 only forms one bond with TM3. The rotation of transmembrane domains 3 and 5 to render the activation of the receptor produces large changes in this bonding pattern (Table 2). Two groups of helices are formed. TM6 is linked to TM3 and TM5, while TM1, TM2, TM4 and TM7 are bonded together. The major change with the initial model is the disruption of the hydrogen bonds around TM3, which is now only linked to TM6 by its Ser 114. This residue has proved to be critical for agonist binding, thus the hydrogen bond it forms with Trp 251 of TM6 should probably be broken by the presence of a ligand. This opens the possibility for another bond between the ligand and Trp 251. Furthermore, there is a slight reduction of the magnitude of the bonding pattern of TM7, with the loss of one of its four links with TM2. MT_2 shows a fairly different bonding pattern in both its inactive and activated forms. However, a number of residue pairs are common to the two receptors. In its inactive conformation (Table 3), there are five hydrogen bonds that correspond exactly to those formed in MT_1 (Table 1) (TM1 Asn 58/45–TM7 Ser 301/288; TM2 Phe 78/65–TM3 Asn 130/117; TM2 Ser 81/68–TM4 Trp 165/152; TM2 Asn 76/63–TM7 Tyr 308/295; TM4 Ala 171/158–TM5 Tyr 200/187 in MT_2 and MT_1, respectively). This is a clear testimony of the closeness of their sequences. The common set of hydrogen bonds links TM1 and TM7, TM2 and TM3, TM2 and TM4, TM2 and TM7, TM4 and TM5. Strikingly, this pattern crosses the whole receptor, leaving only TM6 utterly different from one subtype to the other. The different stabilization of TM6 should be important in the activation mechanism. Contrary to MT_1, TM2 and TM7 are less tightly connected in MT_2. However, next to the conserved Asp–Ser interaction between TM1 and TM7, there is also a second hydrogen bond. The same is true between TM4 and TM5, where the Ala–Tyr link common to both subtypes is supplemented by another bond. Lastly, TM3 is not bound to TM5 or TM7 in MT_2, but TM6 is connected to TM7. Again, this interhelical bonding pattern is disrupted by the activation (Table 4). The activated form is characterized by a strong reinforcement of the bonds around TM3, that is doubly bound to TM2 and TM6, but in the meantime, the complete loss of interaction of TM4 and TM5. Whereas the activated MT_1 is divided in two blocks of helices (TM1, TM2, TM4, TM7 and TM3, TM5, TM6), there is one closely interacting group of transmembrane domains in MT_2 (TM1, TM2, TM3, TM6 and TM7) and two loose helices (TM4 and TM5).

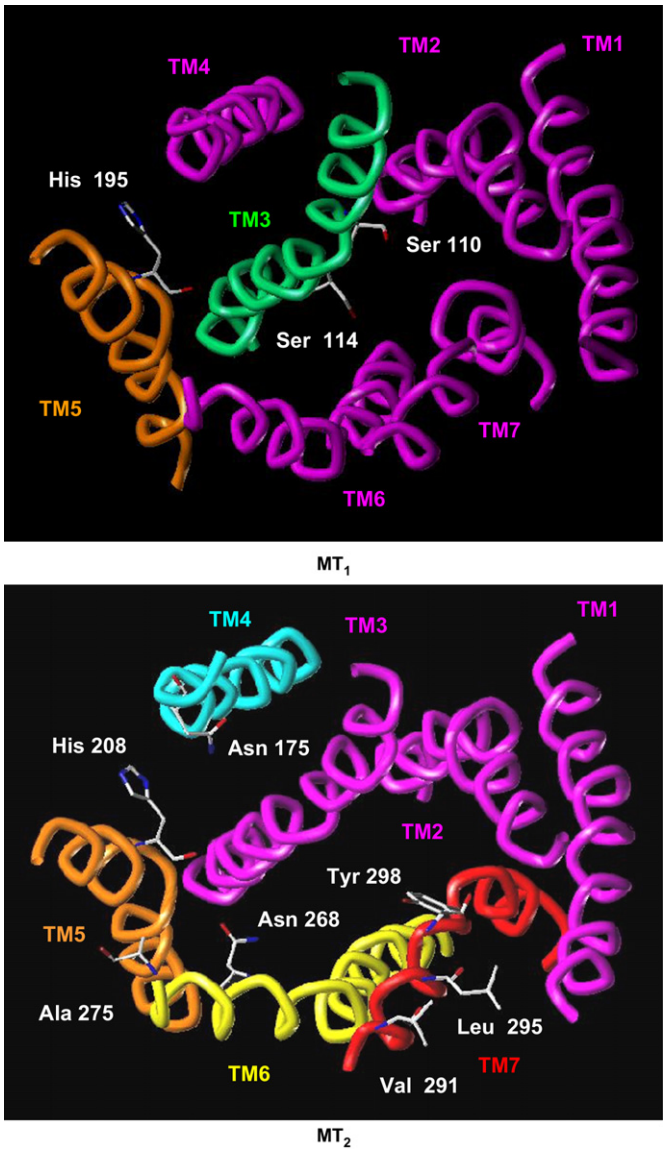

Fig. 7. Binding sites of MT_1 and MT_2.

Still, two hydrogen bonds between TM1 and TM7 and between TM2 and TM7 are conserved in the two receptors (TM1 Asp 41/54 and Asn 45/58–TM7 Ser 288/301; TM2 Asp 73/86–TM7 Asn 287/300 in MT_1 and MT_2, respectively). This conservation seems to indicate that TM7 has a common role in both receptors, probably intervening in the transduction rather than directly in the binding of the ligand.

2.7. Binding site

The pocket of the melatoninergic receptors has been derived from the available experimental data. We used melatonin as the reference compound to define more precisely the binding site and the interactions of the receptors in a putative active conformation with its ligands. A number of models have already been published. The earliest [38–40] lack the information derived from site directed mutagenesis or conformationally restricted analogs of melatonin and were performed

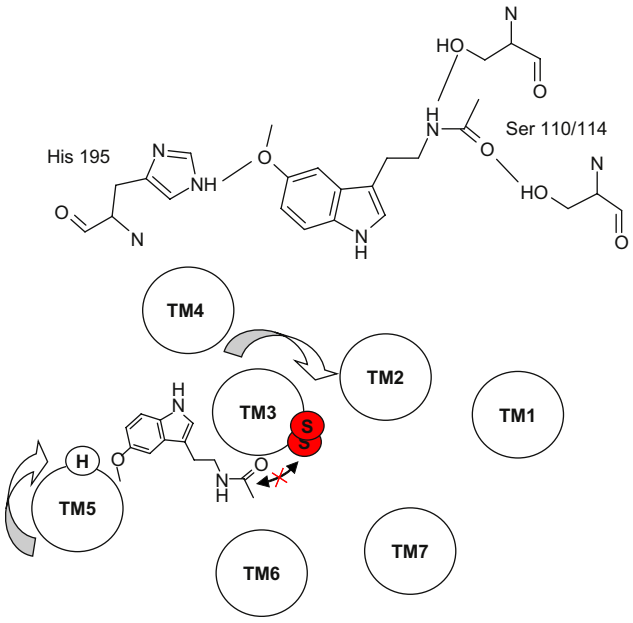

Fig. 8. Putative binding mode of melatonin in MT_1.

before the classification of the melatonin high affinity binding sites in two subtypes. However, despite their limitations, they collectively point piecemeal to serines of TM3, an asparagine of TM4 and a histidine of TM5 to explain the affinity of melatonin for what would become the MT_1 subtype. More recently, a new surge of models has been released [36,41–44]. They cover MT_1 as well as MT_2 but, to the best of our knowledge, no currently described model takes into account the possible modification of the spatial conformation of a GPCR upon activation, which is nonetheless fairly well documented now. Although some are purely a theoretical guess to supplement mutagenesis studies and as such include the binding of both agonists and antagonists, others have proved to be useful to explain structure–affinity relationships of antagonists or to hint at the potential binding mode of agonists. Another interesting point is that they are mostly built upon the crystallographic data of rhodopsin resolved at 2.6 Å, what means they are drawn from the same reference. The differences between the proposed models are therefore more due to several approaches than to divergences in the experimental data used, and, as such, should be seen as a proof of our still limited knowledge of GPCR modeling. In fact, defining a flawless model is clearly impossible and we can only guess at some insights into the interactions. There is a requirement as much of intuition as of experimental data in order to build a model by adding the already known facts together in a coherent ensemble. In our opinion, our model is of course probably a little bit closer to something of an active form of the receptors than previously published ones. They deliberately avoided the activation problem by focusing on antagonists or having a heuristic value to hint at the role of selected residues. In comparison, the model we propose here is still limited to one rigid view of a continuum of related but yet different conformations that form collectively the so-called activated state of a GPCR. As a general word of caution

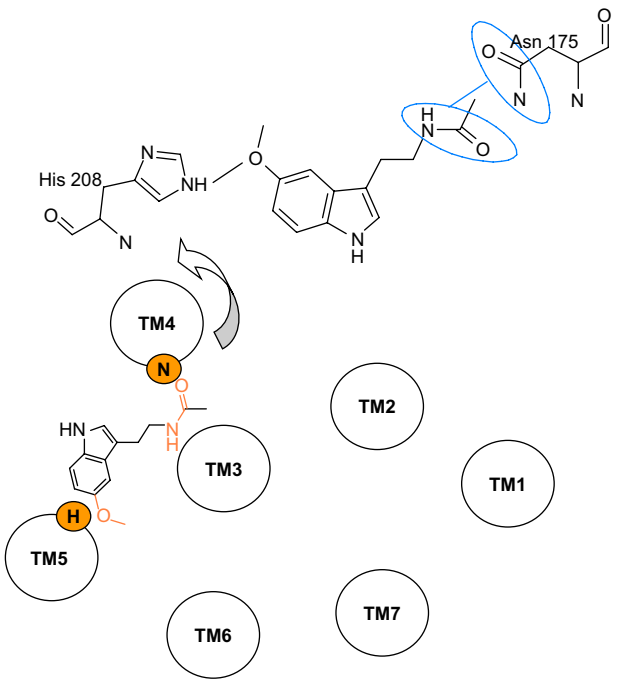

Fig. 9. Putative binding mode of melatonin in MT_2.

for homology modeling, liability to some biases, mainly coming from the chemical nature of the reference ligands chosen, is actually unavoidable.

2.8. Melatonin binding modes

In MT_1, melatonin binds to the receptor in such a way that the nitrogen of its indole ring points toward the extracellular side of the receptor (Fig. 10). It forms hydrogen bonds with the proximal hydrogen of His 195 by the oxygen of its 5-methoxy group, the carbonyl of its amide function binds to Ser 110, while the hydrogen carried by the nitrogen of the amide is accepted by Ser 114. Beyond these interactions, the environment of melatonin in MT_1 (Fig. 11) can be broken down in three regions corresponding to the fragments of the neurohormone. The methoxy can be stabilized by a hydrophobic interaction of the methyl with Phe 196. The indole is surrounded by hydrophobic residues (Val 111 and Met 107 behind the indole

Table 1
Interhelical hydrogen bonds in the inactive model of MT_1

TM1 Val 33 (BB C═O)	TM7 Tyr 285 (SC)
TM1 Asn 45 (SC)	TM7 Ser 288 (BB C═O)
TM2 Phe 65 (BB C═O)	TM3 Asn 117 (SC)
TM2 Ser 68 (SC)	TM4 Trp 152 (SC)
TM2 Asn 63 (BB C═O)	TM7 Tyr 295 (SC)
TM2 Asp 73 (SC)	TM7 Asn 287 (SC)
TM2 Tyr 81 (SC)	TM7 Ala 284 (BB C═O)TM7 Asn 287 (SC)
TM3 Arg 125 (SC)	TM5 Val 203 (BB C═O)
	TM5 Cys 206 (BB C═O)
TM3 Arg 125 (SC)	TM6 Phe 241 (BB C═O)
TM3 Ser 114 (SC)	TM7 Asn 291 (SC C═O)
TM4 Ala 158 (BB C═O)	TM5 Tyr 187 (SC)

BB: backbone; SC: side chain; C═O: carbonyl.

Table 2
Interhelical hydrogen bonds in the active model of MT_1

TM1 Trp 25 (BB NH)	TM2 Asn 90 (BB C═O)
TM1 Asp 41 (SC)	TM7 Ser 288 (SC)
TM1 Asn 45 (SC NH)	TM7 Ser 288 (BB C═O)
TM2 Ala 61 (BB C═O)	TM4 Ser 144 (SC OH)
TM2 Asn 63 (BB C═O)	TM7 Tyr 295 (SC)
TM2 Asp 73 (SC)	TM7 Asn 287 (SC)
TM3 Ser 114 (SC)	TM6 Trp 251 (SC)
TM5 Ser 184 (SC)	TM6 Ala 262 (BB C═O)
TM6 Asn 236 (SC)	TM7 Leu 298 (BB C═O)

BB: backbone; SC: side chain; C═O: carbonyl; NH: amide; OH: hydroxyl.

cycle, Leu 254 below its nitrogen) and the more hydrophilic Asn 255 which can create strong electrostatic interactions with the aromatic electrons of the indole. The acetamidoethyl chain binds to both Ser 110 and Ser 114, as already described, but also to Trp 251. As we had expected, the hydrogen bond between Ser 114 and Trp 251 is not maintained. The acetamide extremity lies in a cavity delimited by Leu 69, Phe 247 and Asn 291, thus presenting the ligand with a fairly hydrophobic environment and a strong possibility of hydrophilic or electrostatic interaction. The important Gly 258 does not interact directly with melatonin, but it is at the entrance of the binding site. Its mutation to a more voluminous residue will obviously affect adversely the ease with which the ligand can access to its binding site, and thus will decrease the observed binding values. The position of this residue in our model is in excellent agreement with the experimental data.

In MT_2, melatonin binds in a more closely packed conformation, with the oxygen of the 5-methoxy group bound to the distal hydrogen of His 208. The carbonyl of the amide function forms a hydrogen bond with Asn 175. However, the binding pocket is positioned much higher toward the extracellular extremity of the transmembrane helices. We had to rebuild the loops that were removed to rotate the helices. The loops from the initial model were copied and linked to the activated model, following a thorough energy minimization with the backbone of the helices constrained, thus forcing the loops to adapt to the new conformation of the transmembrane core of the protein. The final model did accept melatonin fairly well in a very similar conformation as previously mentioned. The amide group of the ligand is now able to form a second hydrogen bond with the backbone carbonyl of Thr 191 belonging to the E2 loop (Fig. 12). While melatonin is not in direct

Table 3
Interhelical hydrogen bonds in the inactive model of MT_2

TM1 Asp 54 (SC)	TM7 Ser 301 (SC)
TM1 Asn 58 (SC)	TM7 Ser 301 (BB C═O)
TM2 Phe 78 (BB C═O)	TM3 Asn 130 (SC NH_2)
TM2 Ser 81 (SC)	TM4 Trp 165 (SC)
TM2 Asn 76 (BB C═O)	TM7 Tyr 308 (SC)
TM3 Arg 138 (SC)	TM6 Phe 250 (BB C═O)
TM4 Ala 171 (BB C═O)	TM5 Tyr 200 (SC)
TM4 Asn 175 (SC)	
TM6 Trp 264 (SC NH)	TM7 Asn 300 (BB C═O)

BB: backbone; SC: side chain; C═O: carbonyl; NH_2: amine; NH: indole.

Table 4
Interhelical hydrogen bonds in the active model of MT_2

TM1 Asp 54 (SC)	TM2 Tyr 94 (SC)
	TM7 Ser 301 (SC)
	TM7 Cys 302 (BB NH)
TM1 Asn 58 (SC)	TM7 Ser 301 (SC and BB C=O)
TM2 Phe 78 (BB C=O)	TM3 Asn 130 (SC)
TM2 Ser 81 (SC)	TM3 Asn 130 (SC C=O)
TM2 Asn 76 (BB C=O)	TM6 Ser 249 (SC)
TM2 Asp 86 (SC)	TM7 Asn 300 (SC)
TM3 Arg 138 (SC)	TM6 Asp 246 (SC and BB C=O)
TM3 Ile 142 (BB C=O)	TM6 Lys 243 (SC)

BB: backbone; SC: side chain; C=O: carbonyl; NH: amide.

contact with all the residues bordering the binding site, it is interesting to note that a number of residues described as important for the binding of ligands lie on the fringe of the pocket in our model (Fig. 13). This is the case for the prominent Asn 268 and Tyr 298. Melatonin *per se* is interacting with several residues of TM3: Ala 117, blocking the conformation of its acetamidoethyl side chain, Gln 121 that is permitting the positioning of the indole cycle due to its small size, Val 124 and Ile 125 that are just below the indole and can form hydrophobic contacts. On TM4, apart from Asn 175, Leu 172 also helps in stabilizing melatonin by restricting the conformation of the 5-methoxy group. TM5 strongly contributes to the binding of melatonin, with His 208 and Tyr 200, which could form a second hydrogen bond with the methoxy. Therefore, an alternate binding of melatonin, which, however, was not found in our study, could be characterized by the hydrogen bonding of the amide with Tyr 200 and His 208, while the methoxy group could be bound to Asn 175. As our model is still only a theoretical guess at the exact binding mode of the ligand, it is not impossible that conformations other than the one we present here can be closer to the biologically relevant binding mode. Lastly, the acetamidoethyl chain is held in place by several residues of the extracellular loop E2, namely Leu 181, in hydrophobic contact with the terminal methyl, Tyr 183 and

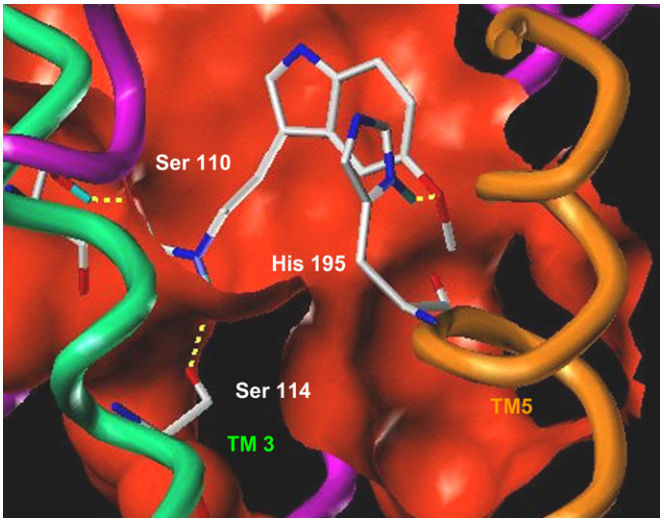

Fig. 10. Binding mode of melatonin in MT_1.

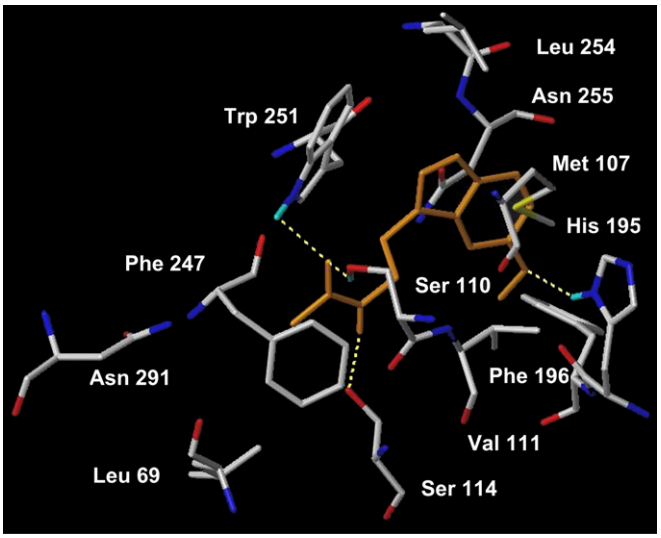

Fig. 11. Environment of melatonin in MT_1.

Phe 192 firmly binding it in an aromatic–hydrophobic–aromatic sandwich. The binding site itself contains Met 120 on TM3, Val 204 and 205 of TM5. For TM6, Trp 264, Leu 267, Asn 268, Ile 270 and Gly 271 contribute to the binding site. Lastly, TM7 completes the pocket, with Phe 290, Ser 293, Tyr 294 and Tyr 298. Compared to MT_1, the pocket of MT_2 appears to restrict less the placement of the indole ring of melatonin as it is more globular than its MT_1 counterpart. However, the addition of the loops strongly reduces the apparent volume of the binding site of MT_2.

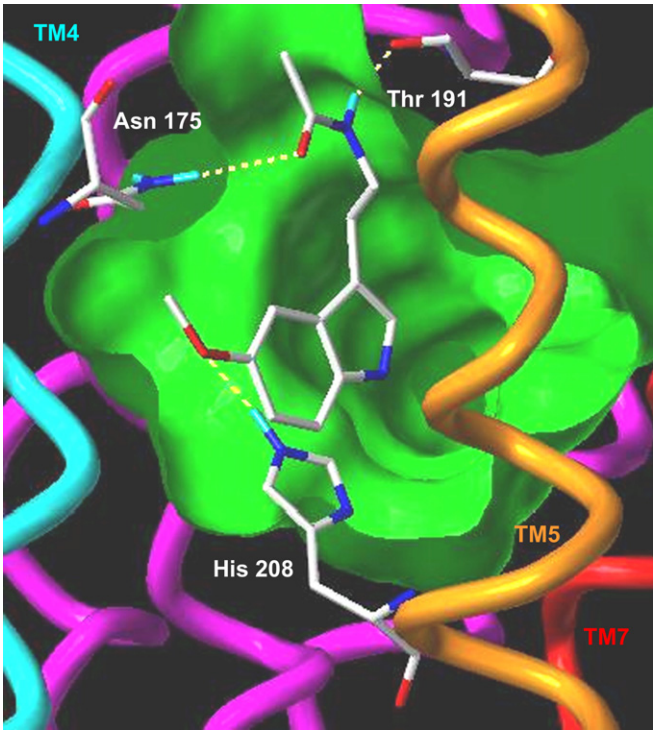

Fig. 12. Binding mode of melatonin in MT_2.

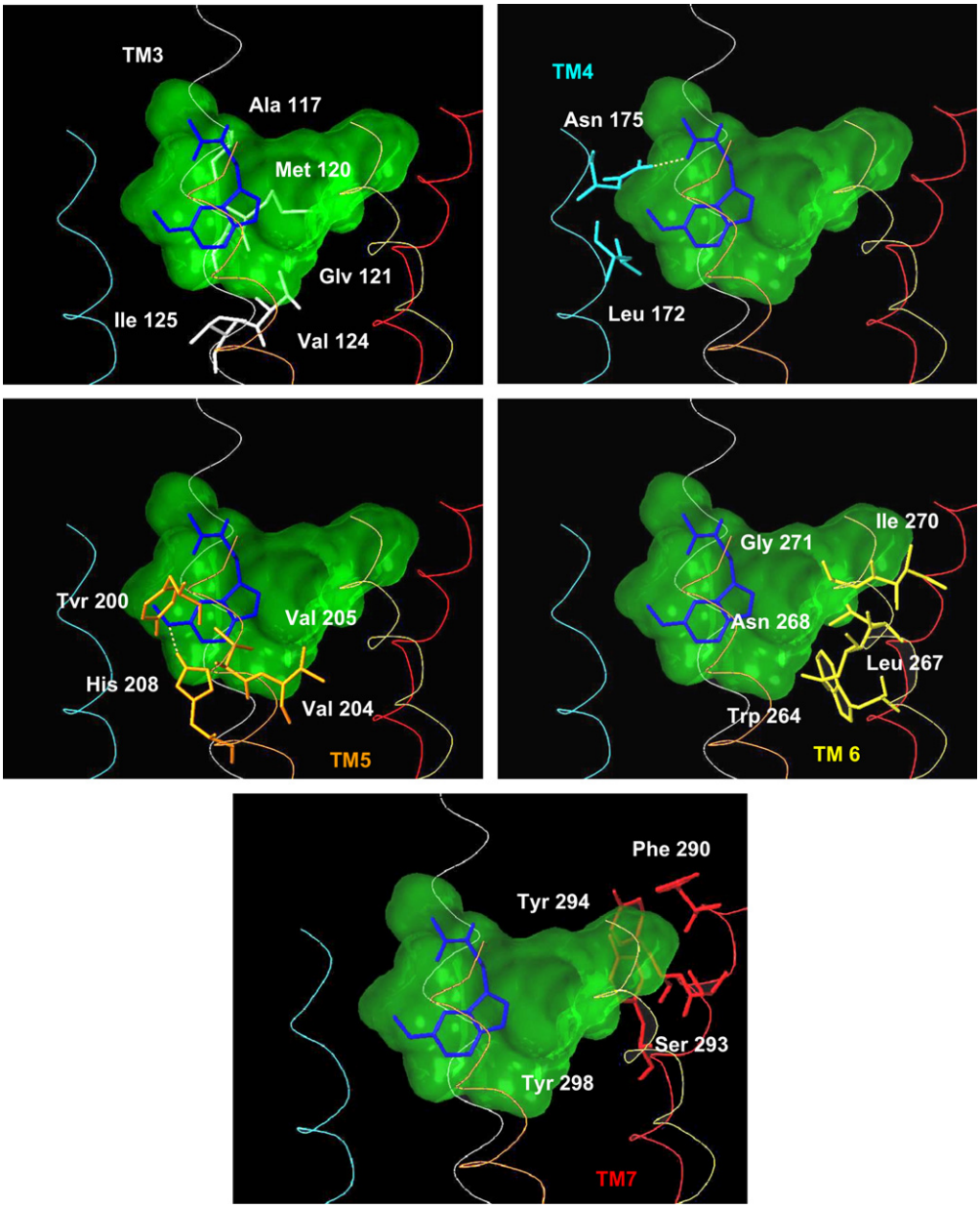

Fig. 13. Environment of melatonin in MT_2.

2.9. Other ligands' binding mode

A number of other ligands have been selected from the literature on the basis of their structural diversity and docked in our models (Fig. 1). In order to verify their capacity to discriminate between agonists and antagonists, we have first tried to dock luzindole [45], as this compound is widely used as a reference antagonist in binding studies. It was not possible to position it correctly in either subtypes due to high strains, even though it looks much like melatonin without its 5-methoxy group. This result is in agreement with the already known importance of the hydrogen bond accepting capacity of the oxygen of this group. Moreover, the indolic nitrogen was unable to compensate for the loss of the 5-methoxy interaction due to the steric bulk of the adjacent benzyl moiety. As we have focused on an agonist activated form of the melatoninergic receptors, this was considered a fairly encouraging finding. Another reference compound is the agonist 2-iodomelatonin used as the base radioligand in binding assays [12,46]. It occupies the pocket of both receptors as melatonin (Fig. 14). Compared to the non-iodinated ligand, it is somewhat more constrained by the higher volume of its halogen in position 2, but nonetheless forms all the interactions of melatonin. In particular, in MT_1, the iodine atom is engaged in a hydrophobic interaction with the indolic cycle of Trp 251, while there is no such interaction in MT_2, where the iodine is lying in the middle of the pocket, thereby potentially explaining the slight difference of affinity between the two receptors that has been reported. A number of rigidified analogs of melatonin have been published. Such molecules are of a great interest either

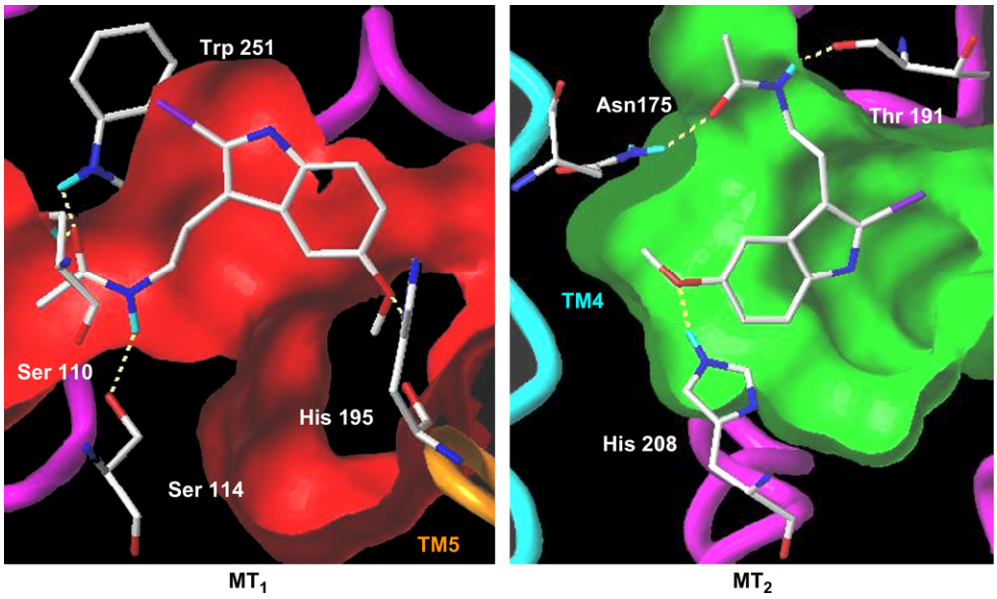

Fig. 14. Docking of 2-iodomelatonin in MT_1 and MT_2.

to draw SAR for a receptor or to test the plausibility of a given model of the receptor, as they point to essential conformational restrictions. In this way, compound **1** displays a restriction of the conformational space of the acetamidoethyl chain of melatonin [47]. This ligand has been described as an agonist, although the tests were performed on quail optic tecta, without prior knowledge of the exact subtypes of melatoninergic receptor expressed. We were thus docking this compound with only scarce information on the possible results, hinting at an agonist activity on MT_1 most probably, but also maybe on MT_2. Moreover, due to the nature of the biological assay, the affinity of this compound is hardly comparable to that of other compounds we have docked. In MT_1, compound **1** is positioned much like melatonin, with the exception of its amide. The roles of the carbonyl and the hydrogen carried by the nitrogen are inverted compared to melatonin. Our first attempt to dock the ligand was therefore a failure, as GOLD does not have yet the capacity to modify the binding site. We had to adjust manually the orientation of both serines to find an acceptable position of this molecule (Fig. 15). It then binds to Ser 110, accepting the hydrogen of the amide, and 114, sharing its hydrogen with the carbonyl of the amide, and the methoxy group at its other extremity forms the third required hydrogen bond with His 195. On the contrary, its very straight conformation hinders its placement in MT_2. Only one hydrogen bond is formed between the methoxy and Asn 175, with the methyl pointing upward in the crevice occupied by the methyl of the acetamide of melatonin. The rest of the structure lies

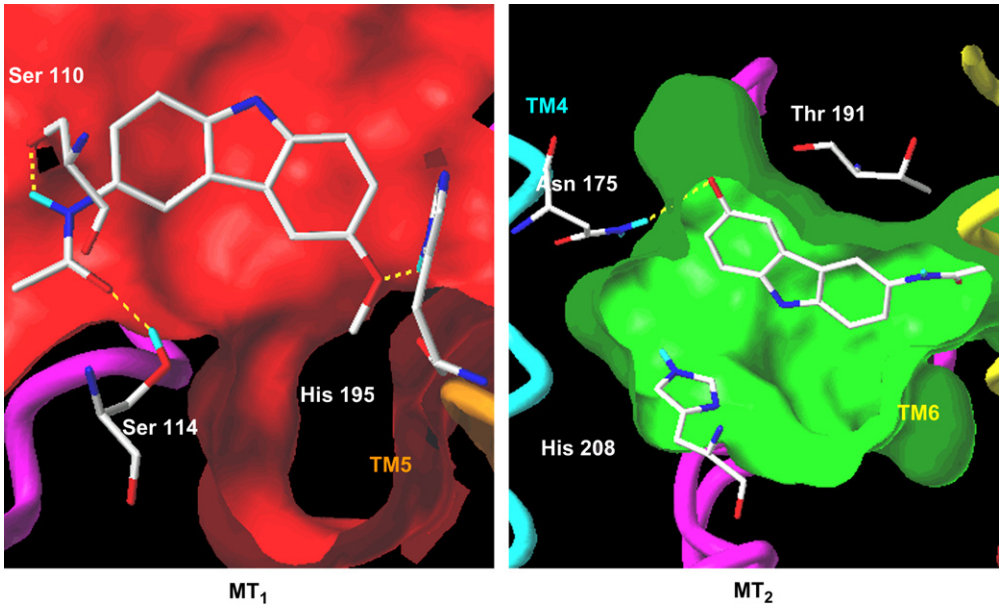

Fig. 15. Docking of compound **1** in MT_1 and MT_2.

perpendicular to melatonin, thus occupying the pocket with few strains but not interacting with His 208 or any other residue, whether found important to the binding or not. This result could indicate two things. First, the MT_2 model can be biased toward more flexible molecules and its conformation might not be the right one to accommodate ligands unable to fold in the same way as melatonin. Second, it could be that this particular compound is just unable to bind to MT_2. In the absence of experimental data, this issue remains unsolvable. A second possibility of conformational restriction of the acetamidoethyl of melatonin has led to compound **2** [48]. This tricyclic molecule is more bent than compound **1** and has been described as an agonist on both MT_1 and MT_2, although the molecule has been tested as a racemic mixture. Following a preliminary inspection, two of the four possible conformers generated by varying the conformation of the saturated cycle and the configuration of the asymmetric carbon of this ligand were found geometrically unavailable due to steric contacts. The two remaining are those in which the hydrogen of the asymmetric carbon and the neighbouring sp^3 carbon are eclipsed. In essence, the configuration of the asymmetric carbon induces the conformation of the saturated cycle, leaving us with *R* and *S* enantiomers. The *R* enantiomer was found more easily amenable to a docking study, as its acetamidoethyl chain points in the same direction as that of melatonin. Accordingly, it was placed in MT_1 as melatonin (Fig. 16). In MT_2, on the other hand, it forms two hydrogen bonds of the amide, with Asn 175 and Thr 191, but is unable to reach His 208 with its methoxy, creating only a stacking with its naphthyl cycle. Interestingly, the *S* enantiomer behaves exactly in an opposite way, binding fairly worse than its enantiomer on MT_1, where it loses one of the interactions of the amide, as its nitrogen is dragged away from Ser 114 to permit the formation a hydrogen bond between its methoxy and His 195 (Fig. 17). On MT_2, the initial runs were disappointing, and the orientation of His 208 had to be adjusted to help the docking. The resulting solutions were quite good, with all three hydrogen bonds formed, even though the necessity to reorient the histidine clearly shows that the models are somewhat biased and can't be used to dock any compound without some caution. Another series of compounds was provided by more recently developed ligands. We have chosen three of them, each bearing little structural similarity with the others. This point was viewed as important to assess the dependence of our models on the chemical family of the ligand to dock. In fact, only the methoxyphenyl at one end and the amide at the other are common, that is the putative pharmacophore of the melatonin receptors. The first, agomelatine, was synthesized in our laboratory [49,50]. It is the direct naphthalenic analog of melatonin. This compound has a similar mode of binding to melatonin in either receptor (Fig. 18). In MT_1, however, the hydrogen bond with Trp 251 is replaced with a stacking between the naphthyl cycle of agomelatine and the indolic cycle of the tryptophan, whereas in MT_2, there is no difference in interaction. As agomelatine is structurally very close to melatonin, these results were expected, only hinting at a plausible conformation of the activated models. We therefore selected a fairly different scaffold, based on indanylpiperazine, with compound **3** [51]. It has been described as a good affinity agonist on MT_2, and displays 100 times lower affinity for MT_1, even though its functional activity has not been determined on this subtype. In these conditions, its docking in MT_1 was somewhat challenging, as we could not ascertain what the result should be like. The conformations achieved did not bond to any residue, except for a hydrogen bond with Ser 114 that was found in about one third of the solutions. The piperazine cycle, in particular, was always found lying in the pocket with no contacts with the receptor. In consideration with its larger steric requirements than an aromatic cycle, it could not be placed between the serines but should have nonetheless been positioned closer to the histidine than

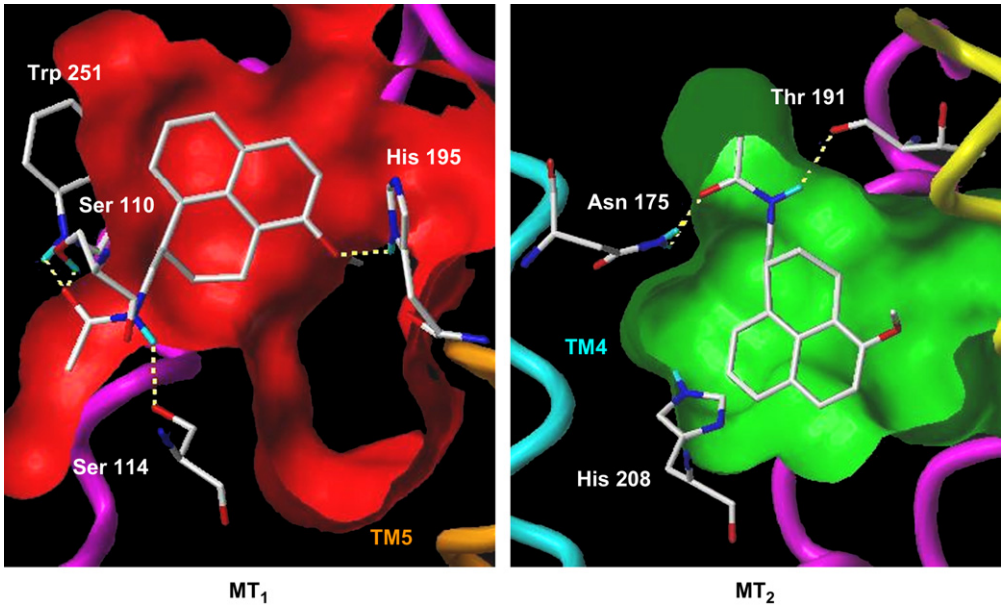

Fig. 16. Docking of compound **2** in *R* configuration in MT_1 and MT_2.

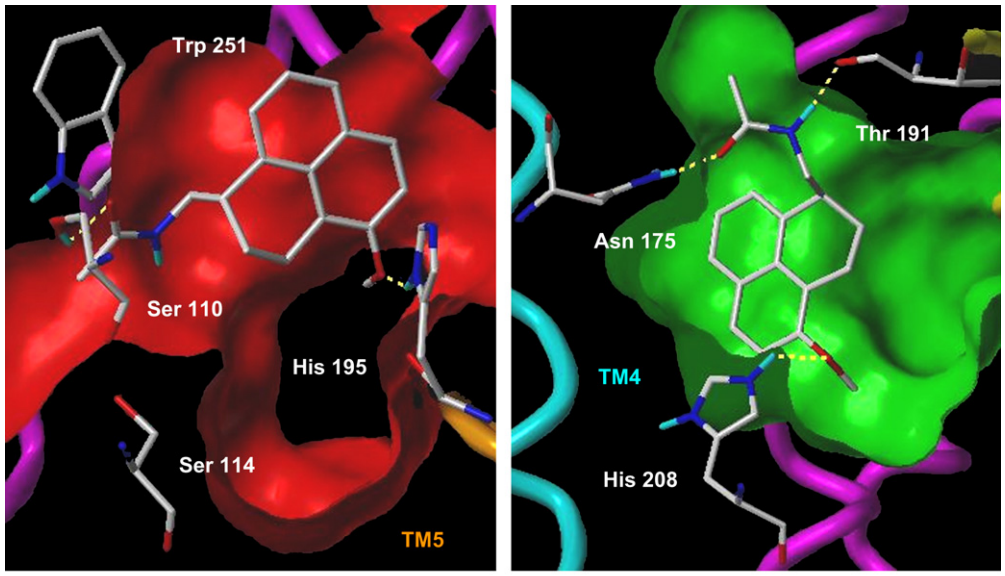

Fig. 17. Docking of compound **2** in *S* configuration in MT_1 and MT_2.

it was. This compound could as well be docked as an antagonist due to a flaw in our model hindering a correct placement of this particular scaffold. Again, in the absence of more detailed data, we assumed that an antagonist activity was more relevant. On the contrary, in MT_2, its conformation was relatively closer to that of melatonin (Fig. 19). Following a similar hydrogen bonding pattern for its amide function, it is linked to Asn 175 and Thr 191. Nevertheless, the second half of compound **3** binds in another way than melatonin. Instead of folding in the same direction as Asn 175 and binding to His 208, it is oriented toward the other end of the pocket and its methoxy accepts the donatable hydrogen of the side chain of Ser 189. Such a binding mode is fairly interesting as it might indicate a different conformation of the receptor. In fact, the relative position of His 208 and Asn 175 does not permit creation of a ligand–receptor complex supporting an interaction between the methoxy of the ligand and the histidine. A slightly more important rotation of TM4 would probably accommodate compound **3** with the same interactions as melatonin. Thus, in our opinion, this result is a clue to the adaptation of a GPCR to its ligand. On the other hand, as the rotation of TM4 is still maintained by the hydrogen bonds formed by the ligand in its current conformation, it could also be an alternate binding mode for melatoninergic ligands in MT_2, which could explain in part the unclear image that stems from the site directed mutagenesis data pointing to a fair number of

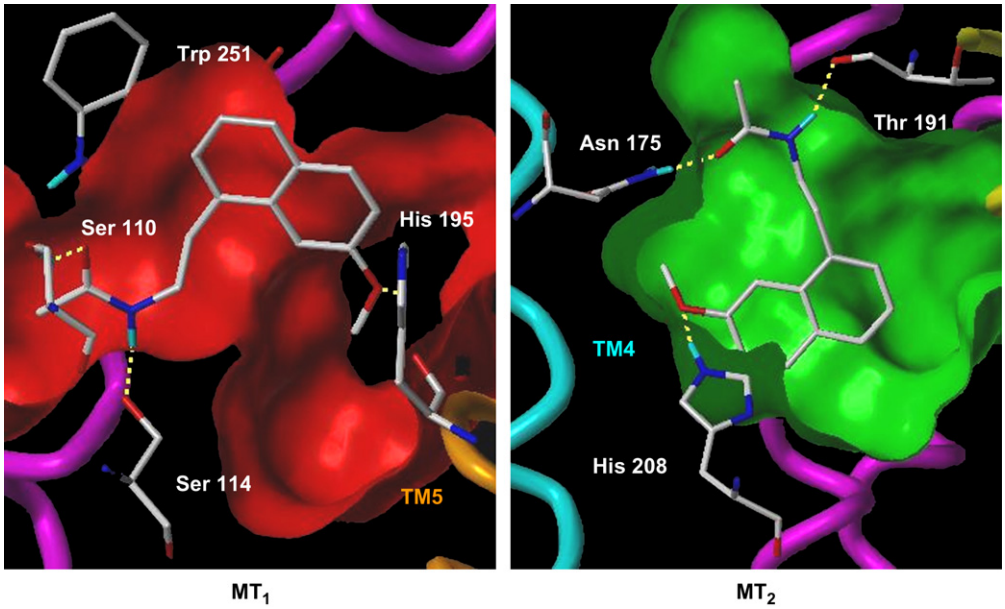

Fig. 18. Docking of agomelatine in MT_1 and MT_2.

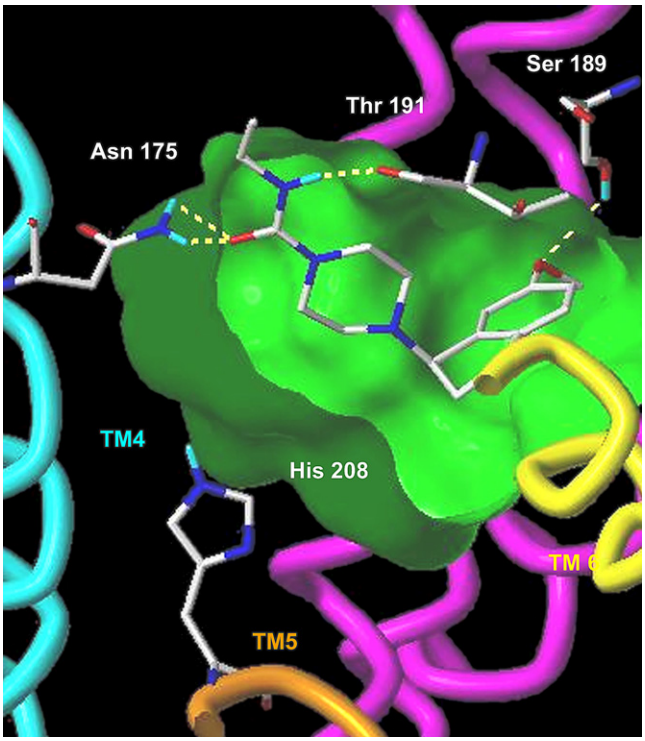

Fig. 19. Docking of compound **3** in MT_2.

important residues bordering the whole cavity. We docked another molecule with a very simple structure, compound **4** [52], to determine if its flexibility could be an advantage to fit in both receptors over the more rigid compound **3**. Compared to any other ligands we had docked, compound **4** only bears the basic functional groups necessary to bind to the melatoninergic receptors, without any attempt to restrict its conformation. We therefore found it interesting to test it in our models, even though, as compound **3**, its functional activity as an agonist was assessed only on MT_2. Contrary to compound **3**, the conformation of compound **4** in MT_1 is characterized by the formation of the same hydrogen bonds as with melatonin (Fig. 20). The sulfur points sharply toward the extracellular side of the protein, leading to a slightly different conformation of the methoxy group of this ligand, compared to melatonin. However, the amide functions of both are positioned in the same way. Surprisingly, it binds in a completely different conformation in MT_2. While the amide of melatonin binds to Asn 175, compound **4** forms a hydrogen bond with this residue by its methoxy moiety. Very much like compound **3**, it does not fold to reach His 208 but extends in the depth of the binding site, forming a second hydrogen bond with the backbone nitrogen of Gly 271 that has proved to play a dubious but important role in the activation of MT_2 [36]. When it is replaced with a threonine, the mutant becomes non-saturable. Interestingly, in MT_1, the counterpart of this residue is Gly 258 that probably plays important role in the early stage of the ligand recognition as it is located at the entry of the binding site, accordingly reducing the affinity of the ligands when replaced with a bulkier residue. This was utterly unexpected and despite our best attempts to adjust the geometry of the residues to favour an interaction with the histidine, this was also the unique pose discovered. Two possibilities are available to explain such a conformation. The more simple one is that our model may require a more advanced modification of its geometry to bind compound **4** in a similar interaction pattern as melatonin, probably needing as for compound **3** a larger rotation of TM4. The more sophisticated one is that several binding modes are available in the pocket of MT_2, depending heavily on the nature of the ligand, thus explaining the still complex view of the residues dubbed important for ligand affinity. Moreover, compounds **3** and **4** can be superposed fairly well (Fig. 21). The position of the interaction point with Asn 175 is perfectly conserved across all the ligands we docked, either

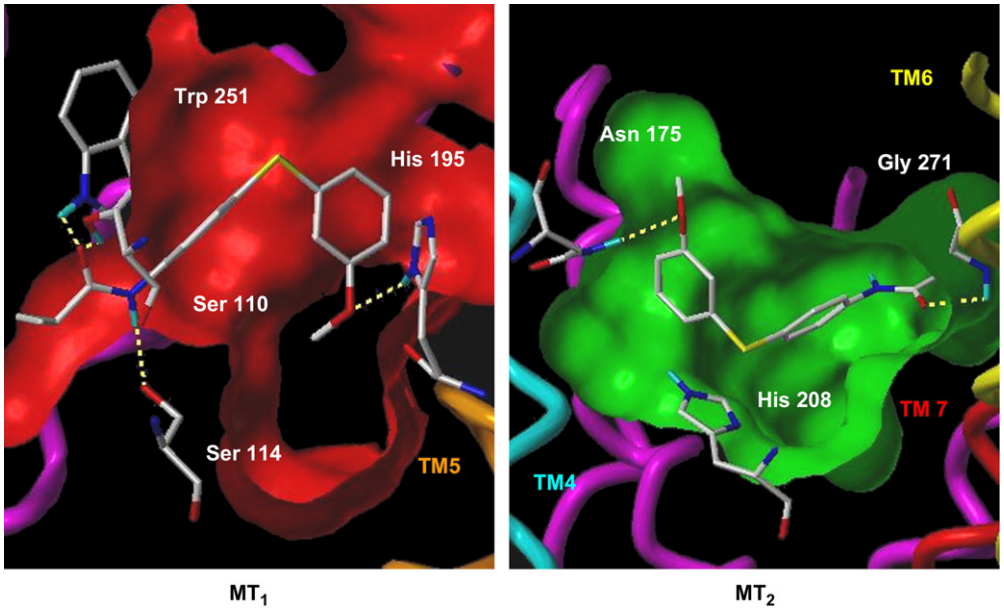

Fig. 20. Docking of compound **4** in MT_1 and MT_2.

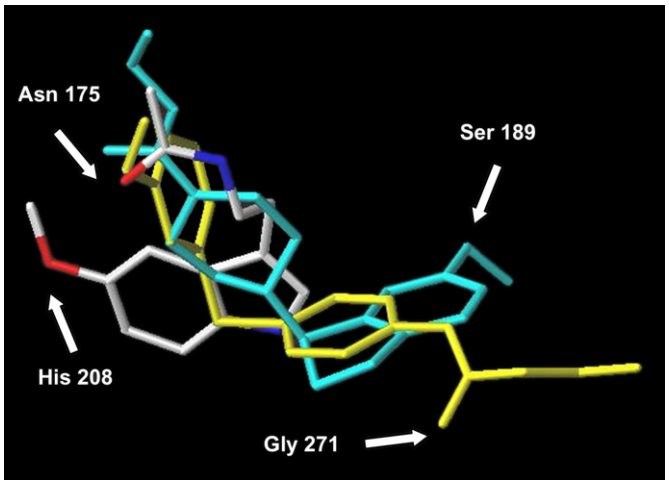

Fig. 21. Superposition of melatonin (coloured by atom types), compound **3** (cyan) and compound **4** (yellow) in MT_2. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

by the carbonyl of the amide function of melatonin and compound **3** or the methoxy of compound **4**, while the aromatic not bearing the methoxy of compound **4** is superposed with the inden ring of compound **3**. The binding conformation of the two last molecules is therefore very similar, although their scaffold and flexibility greatly differ. Compared to melatonin, where the interaction points are carried out by a single cyclic system, they both have two distinct cycles each bearing one of the interacting groups. Nonetheless, they point their second group in opposite directions. The shorter length of compound **4** does not let it contact Ser 189, while the less flexible compound **3** cannot fold to form a bond with Gly 271. The aromatic cycle of melatonin or agomelatine therefore seems to be a directing factor in the exact positioning of the ligand, driven by hydrophobic contacts with Met 120 and Val 204 of MT_2 that have proved to be important for the affinity of 2-iodomelatonin, while it is not the motor of the receptor activation.

2.10. Selectivity

Although we have tested the discrimination between agonists and antagonists of our models on a reference compound, it is completely another thing to define the selectivity of a ligand toward one of the melatoninergic receptors on the basis of its docking into either subtypes. Such a task can be a tricky bet. The conformations of the receptors are different and, more importantly, the binding sites and the critical residues appear to be also divergent. In the early stages of our study, we had thought that Ser 123 and 127 of MT_2 that are superposed with Ser 110 and 114 of MT_1, known to be required for agonist binding, would play a role similar to their MT_1 counterpart. Rotations of TM3 and TM5 of MT_2 to permit these residues to interact with melatonin were inconclusive, as the bonds were effectively achieved, but the overall feeling of the docking was not as good as on MT_1, in the way that the complex MT_2–melatonin needed much larger optimization of its geometry. In our mind, this meant a too high energy barrier between the ground state of the receptor and the stable state of the complexed receptor. These clear difficulties to position correctly even melatonin, but nonetheless not to the point of impossibility, led us to think that our initial hypothesis was wrong and we therefore sought another potential binding site. The hypothesis we chose was based on site directed mutagenesis, without any bias due to an *a priori* knowledge of another melatoninergic receptor. This difference of binding site should be related to the strain induced by the relatively few amino acids not common to the two subtypes that orient the modification of the helix bundle during the activation. Interestingly, the most different transmembrane domain from one receptor to the other is the fourth, closely followed by TM5 and TM6. It should seemingly play a fairly important role in the different behaviour of the melatoninergic receptor subtypes sensing a ligand. As a side note, there is a clear difference in the interhelical bonding pattern around these three helices. Nevertheless, the activation mechanism is dynamic and it is therefore quite difficult to explain it through two static views of its end points. If we accept the bias of the chemically aware binding sites of the activated receptors, dissimilarities between the conformations of melatonin in both subtypes can address at least a part of this selectivity issue. Apart from being bound in a different orientation in the two subtypes of receptors, melatonin also adopts a different binding conformation. When the indole ring of melatonin bound to both subtypes is superposed (Fig. 22), it appears that the 5-methoxy chain is roughly in the same orientation, with a lag of a few degrees only. The methoxy is pointing slightly more outward from the indole ring plane in MT_1 than in MT_2. Although the ethyl linkers of the acetamidoethyl chain are perfectly superposable, with a 90° kink from the plane of the indole, the major difference comes from the acetamide. In MT_1, it is in a deployed conformation, with the amide bond directed away from the indole and parallel to it. In MT_2, on the contrary, this bond is directed toward the indole and forms an angle of about 30° with the plane of the aromatic. This is a critical conformational difference that allows the more flexible compounds to fit in both receptors, while being sufficiently stringent to hinder an agonist binding to some compounds unable to adopt one of the two conformations. This is the case of compound **1**, which is rigidly held in a straight conformation. However, melatonin itself only gives a few ideas about the conformational requisites for an agonist activity. Another source of knowledge is the number of restricted analogs synthesized. The docking of some such molecules has been discussed earlier in this paper. Strikingly, both rigidifications of the acetamide side chain (compounds **1** and **2**) were able to bind to MT_1 in a melatonin-like mode, even if the orientation of the serines had to be modified to exchange their respective role in the hydrogen bonding pattern of compound **1**. It is noteworthy that only one of the two enantiomers of compound **2** was able to bind to both receptors, with a slight adjustment of the position of the critical His 208, while the other was less than perfect on both subtypes as well. For these two ligands, the exact conformation of the receptor had to be

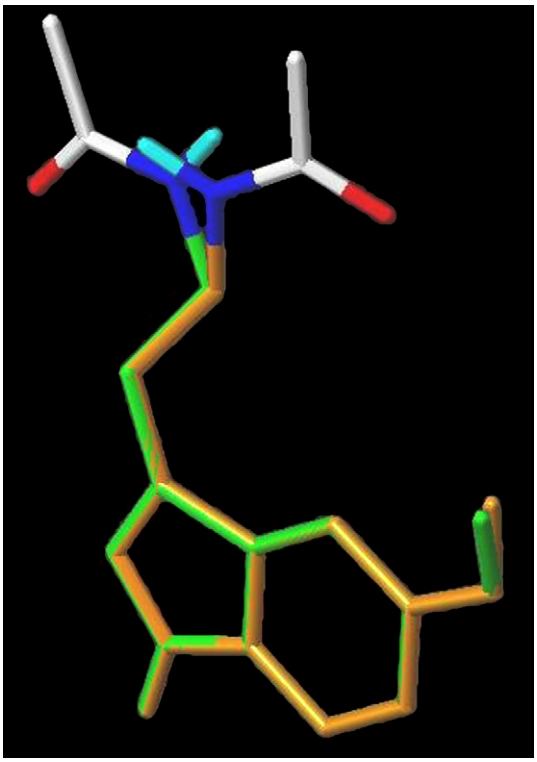

Fig. 22. Conformations of melatonin bound to MT_1 (green) and to MT_2 (orange). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

slightly modified, showing that the side chain of the residues involved in ligand binding must be viewed more as the sum of their possible conformations than as static anchoring points. Compounds **3** and **4** go even beyond by suggesting either an adaptation of the conformation of the whole transmembrane domains of MT_2 to the ligand while leaving the receptor in a still active conformation or multiple activating binding conformations in the site, with a central role of Asn 175.

3. Conclusion

We report the construction of homology models of both subtypes of human melatonin receptors from the crystallographic structure of bovine rhodopsin. The resulting models should represent an inactive form of the receptors. As our interest was focused on agonists, we had to modify the arrangement of the helix bundle in order to create a putative active conformation of the melatoninergic receptors. Taking into account the data gathered by site directed mutagenesis studies, we have rotated two helices of MT_1, TM3 and TM5, to place the three critical residues identified (Ser 110, Ser 114, His 195) in proper position to bind melatonin. For MT_2, the literature hinted at two possible binding sites, centered around Asn 175 and His 208 for the first, Asn 268 and Tyr 298 for the second. The rotation of TM4, bearing Asn 175, led to formation of a site bordered by both series of residues. To assess the validity of our activated models, we have docked seven well-known agonists and a reference antagonist to test the capacity of the activated receptors to discriminate it from the agonists. The results were fairly good, as the antagonist was unable to bind to either receptor. In the meantime, however, the difficulties encountered with compounds **3** and **4** also show the adaptation of the receptors to their ligand, making an absolute definition of selectivity on the basis of a simple docking an uneasy one, more specially when considering the low sequence dissimilarity between the two subtypes and their neatly different binding sites. We have moreover limited these investigations to the acetamidoethyl side chain restriction, leaving aside the elucidation of the 5-methoxy group conformation, which is present and freely rotatable in all the ligands we have docked. Keeping these limits in mind, these models are a tool to explore at an atomic level the different requirements of the melatoninergic receptors.

4. Experimental protocols

4.1. Sequence alignment

Models were constructed using full length sequence alignment of the human MT_1 [13] and MT_2 [14] receptors (PIR entries I38848 and I38990, respectively) with bovine rhodopsin [53] (PIR entry OOBO) (http://www-nbrf.georgetown.edu/pirwww/dbinfo/pirpsd.html) [54]. In order to favour pertinent superimposition of the residues conserved throughout the GPCR family, the alignment of the sequences was broken down in two steps, allowing for a more flexible inspection of the results and easier corrections. First, the sequences of the three receptors were subjected to a pairwise alignment achieved with ClustalX [55,56]. Second, the helices from bovine rhodopsin as defined in the original description of the crystallographic structure [57] and the corresponding residues of the melatonin receptors were examined to verify the correspondence of conserved amino acids. Then each interhelical alignment was refined separately without altering the rest of the superimposition using the selected residue range realignment feature of ClustalX.

4.2. Homology modeling

The above described sequence alignment files were used as input for the Nest program of the Jackal protein structure modeling package [58] with the high-resolution crystal structure of bovine rhodopsin [59] available in the RSCB Protein Data Bank (PDB entry 1L9H) [60] as reference for the 3D structure. This procedure is based on the idea that transmembrane helices are much less flexible than loops, thus permitting to produce a sounder core alignment if the integrity of the helices is conserved. On the contrary, the more volatile loops can bear the more important difference between the coordinates of the reference and the model.

4.3. Molecular modeling

Molecular modeling studies were performed using SYBYL software version 6.9.1 [61] running on Silicon Graphics

workstations. The output structures were iteratively energy-minimized using the Powell method available in Maximin2 procedure with the AMBER force field [62,63] including the electrostatic term, a dielectric constant set to 4 and a 10 Å non-bonded cutoff. Energy of the system was minimized in three stages. First, clashes in the side chains were softened by 200 steps of conjugated gradients, followed by 1000 iterations with the backbone of the loops being free to move. Lastly, the whole protein was allowed to move until the gradient value was smaller than 0.01 kcal mol^{-1} $Å^{-1}$. The stereochemical quality of the models was assessed by running the PROCHECK software [64]. Three-dimensional models of ligands were built from a standard fragment library, and their geometry was subsequently optimized using the Tripos force field [65] including the electrostatic term calculated from Gasteiger and Hückel atomic charges. The method of Powell available in Maximin2 procedure was used for energy minimization until the gradient value was smaller than 0.001 kcal mol^{-1} $Å^{-1}$.

4.4. Hydrophobic moment calculation

The mean hydrophobic moments (μH) were calculated for each transmembrane helix by assigning each amino acid residue a vector whose magnitude is proportional to its hydrophobicity [66] and its direction is determined by the orientation of the side chain about the helix axis. The mean hydrophobic moment (μH) is the vectorial sum of the individual hydrophobic moments (H) [67]. The hydrophobic moments (H) were calculated using the program HMOMENT [68].

4.5. Binding site identification

The binding sites of melatonin in both human MT_1 and MT_2 receptors were identified by site directed mutagenesis studies. The fully conserved His 195 residue in TM5 was proposed to interact with the 5-methoxy group of melatonin [25,69,70] while Ser 110 and Ser 114 in TM3 were identified as critical for agonist binding to the human MT_1 receptor [71]. Gly 258 in TM6 was also reported as a critical residue required for ligand binding and receptor function [72,73]. In the same way, His 208 in TM5 and Asn 175 in TM4 were shown critical for melatonin binding to the human MT_2 receptor [34]. Other residues, such as Asn 268 and Ala 275 in TM6, Val 291 and Leu 295 in TM7, were also essential for melatonin binding to the human MT_2 receptor [35].

4.6. Docking studies

Melatonin was then docked manually into the putative binding site for each receptor model. To provide better agreement with the geometric constraints on agonist ligand binding state imposed by mutagenesis data, rotations about the helix axis of TMs were performed using an ''in house'' macrocommand written in Sybyl Programming Language (SPL). In order to evaluate the capacities of the activated models to bind known agonist ligands, a series of compounds belonging to structurally different families were docked into the receptors' active sites (Fig. 1). Flexible docking was performed using the GOLD 2.2 software [74]. The most stable docking models were selected according to the best-scoring conformation predicted by the GoldScore [74] and X-Score scoring functions [75]. The complexes were energy-minimized using the Powell method available in Maximin2 procedure with the Tripos force field and a dielectric constant of 4.0 until the gradient value reached 0.01 kcal mol^{-1} $Å^{-1}$.

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