ELSEVIER

European Journal of Pharmacology 304 (1996) 173–183

ejp

A rhodopsin-based model for melatonin recognition at its G protein-coupled receptor

Cecil Navajas [a,*], Tarja Kokkola [b], Antti Poso [a], Nina Honka [b], Jukka Gynther [a], Jarmo T. Laitinen [b]

[a] *Department of Pharmaceutical Chemistry, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland*
[b] *Department of Physiology, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland*

Received 12 October 1995; revised 5 February 1996; accepted 6 February 1996

Abstract

The recent elucidation of the primary structures of different melatonin receptors as well as the deduction of the secondary structure of rhodopsin has allowed us to construct a model for melatonin recognition at its G protein-coupled receptor. To achieve this, we have used the quantum mechanics method Austin model 1 to fully optimize the structures of melatonin and several analogs. We also synthesized three compounds and used the three-dimensional analysis comparative molecular field analysis (CoMFA) to generate a model for the structure-activity relationships of melatonin and 27 melatonin-like compounds. This model predicted with good accuracy the affinities of the synthesized compounds for the melatonin receptor. We propose that recognition of the functional moieties of melatonin occurs through specific interaction of these moieties with fully conserved amino acid residues present in transmembrane helices V, VI and VII of the melatonin receptor. These residues are not found in other members of the G protein-coupled receptor family. The rhodopsin-based model can explain the importance of some structural features of melatonin and related active compounds.

Keywords: Melatonin receptor; Comparative molecular field analysis; Quantitative structure-activity relationship model; Austin model 1; Binding site; G protein-coupled receptor

1. Introduction

Melatonin is synthesized and secreted from the pineal gland during the hours of darkness (Reiter, 1991). In mammals, pineal melatonin secretion is driven by the biological clock in the hypothalamic suprachiasmatic nuclei (Reiter, 1991). Through the suprachiasmatic nuclei, the prevailing light-dark cycle entrains the daily rhythm of melatonin secretion. The melatonin signal serves as a chemical expression of darkness, thus providing information of the duration and intensity of ambient light which critically participates in the synchronization of circadian and circannual rhythms, such as seasonal reproduction (Reiter, 1991). In addition, melatonin enhances immune function (Guerrero and Reiter, 1992) and has growth-inhibitory actions on human breast tumor cells (Molis et al., 1995). Many of the actions of melatonin are believed to be mediated through specific receptors. The development of a radioiodinated melatonin agonist 2-[^{125}I]iodomelatonin (Vakkuri et al., 1984) allowed the identification and characterization of melatonin receptors from various anticipated target areas (Krause and Dubocovich, 1991; Stankov et al., 1991; Dubocovich, 1985). These receptors belong to the superfamily of G protein-coupled receptors. Full or partial amino acid sequences for the amphibian, avian and mammalian melatonin receptors were recently obtained by molecular cloning (Ebisawa et al., 1994; Reppert et al., 1994; Kokkola et al., 1995). Moreover, various studies have addressed the structural requirements of the melatonin receptor. These studies have demonstrated the importance of both 5-methoxy and *N*-acetyl moieties for both ligand binding and biological activity (Morgan et al., 1989; Laitinen and Saavedra, 1990a; Sugden and Chong, 1991; Yous et al., 1992; Garratt et al., 1995; Sugden et al., 1995).

According to currently accepted concepts, the G protein-coupled receptors consist of seven transmembrane helices (TM I–VII) joined together by extracellular and

* Corresponding author. Tel.: +358-71-162462; fax: +358-71-162456; e-mail: cecil.navajas@uku.fi.

PII S0014-2999(96)00114-8

intracellular loops, with the N-terminus in the extracellular side and the C-terminus in the cytoplasm. All members of the G protein-coupled receptor superfamily are thought to have the same basic structure in the membrane-embedded part, mainly because of sequence similarities and their common ability to activate G proteins to initiate signal transduction (Findlay and Eliopoulos, 1990; Hibert et al., 1991; Trumpp-Kallmeyer et al., 1992). A hydrophilic pocket formed by the seven transmembrane helices is thought to be a docking site for small neurotransmitter and hormone ligands such as dopamine, adrenaline, noradrenaline, acetylcholine, histamine, adenosine and serotonin. Determinants for the binding of these ligands to their appropriate binding pockets have been studied in some detail, mainly by using site-directed mutagenesis of amino acid residues thought to be involved (Jackobson et al., 1992; Ostrowski et al., 1992; Savarese and Fraser, 1992).

It is widely assumed that the G protein-coupled receptor family of receptors have the same structure as bacteriorhodopsin, an integral membrane protein with seven helices. However, bacteriorhodopsin is not coupled to G proteins and its sequence shows none of the distinctive features of the G protein-coupled receptor family (Baldwin, 1993). A substantial advance in our knowledge was made when the projection map of rhodopsin at 0.9 nm (9 Å) resolution was obtained by electron cryo-microscopy of two-dimensional crystals (Schertler et al., 1993). The original work was further extended to provide the three-dimensional structure of rhodopsin from tilted two-dimensional crystals (Unger and Schertler, 1995). Rhodopsin is a member of the G protein-coupled receptor family and it mediates light-evoked activation of 11-*cis* retinal to the retinal G protein transducin (Lolley and Lee, 1990). In addition, rhodopsin is known to have seven transmembrane α helices (Lolley and Lee, 1990). Based on the rhodopsin projection map, Baldwin has recently provided a general model for the G protein-coupled receptor family of receptors which differs considerably from the previously constructed models based on the structure of bacteriorhodopsin (Baldwin, 1993).

Using the rhodopsin-based model proposed by Baldwin, we suggest how the pineal indole melatonin may be docked at its binding pocket in the G protein-coupled melatonin receptor, based on the probable arrangement of the seven helices in rhodopsin. Our model docks melatonin in its minimized energy conformation at a hydrophilic binding pocket where the recognition of the functional moieties of the indole occurs through interaction with fully conserved amino acid residues present in helices V, VI and VII of the melatonin receptor, but not in other members of the G protein-coupled receptor family. We have also applied the three-dimensional quantitative structure-activity relationship method, comparative molecular field analysis (CoMFA) (Cramer et al., 1988a), to create a model of the structural properties needed for the binding to the melatonin binding site.

2. Materials and methods

CoMFA methodology is based on the assumption that the differences in ligand-receptor interaction are reflected by differences in steric and Coulombic 'potentials' surrounding molecules. Furthermore, molecular mechanics force fields can account for a great variety in these interactions, which are calculated on a regularly spaced grid using an appropriate probe atom (for example, sp^3 carbon with a charge of +1) and its force field. Besides conventional steric and electrostatic fields, the corresponding fields calculated with oxygen sp^3 and hydrogen as probe atoms were created to include the effect of hydrogen bonding in CoMFA. The quantitative structure-activity relationship (QSAR) table of CoMFA contains more columns than rows and the system is strongly underdetermined. Therefore, a proper multivariate method, partial least squares (PLS) regression (Cramer et al., 1988b) with cross-validation and bootstrapping, is needed for a valid QSAR model. The CoMFA analyses of this study were done with the SYBYL molecular modeling program (version 6.0) running on an IBM RISC/6000 320H workstation using appropriate options. The Tripos force field (Clark et al., 1989) was used in field calculations. The inverse logarithm of K_i values was used as dependent variable in CoMFA. The molecules were superimposed by using the root-mean-square-fitting routine (RMS FIT) of the SYBYL software. C_5, N_1 (C_1 in naphthalene derivatives) and the amide N were the reference points selected, with the melatonin molecule as the parent. The CoMFA grid spacing for every field was set at 0.2 nm (2 Å). The standard deviation thresholds (Minimum, Sigma) for exclusion of columns were kept at the default value, 8.37 kJ/mol (2 kcal/mol). The structures and atomic charges for each molecule were calculated using the MOPAC program package (QCPE no. 455, version 5.0) with Austin model 1 (AM1) parametrization (Stewart, 1990). All geometric variables were optimized for each compound (using NOMM keyword), and the lowest energy conformations were used in the CoMFA. All the regions were created by using the automatic procedure of SYBYL. Cross-validation in PLS was carried out using the leave-one-out method. Bootstrapping was done with 50 groups. The optimum number of PLS components for the final, non-validated analysis depended on the smallest S_{PRESS} value from the cross-validated analysis.

The three compounds were synthesized as described for melatonin-D_4 by Gynther (1988). The reactions were carried out by gently refluxing 5-chlorotryptamine hydrochloride (Lancaster, England) with acetic anhydride to yield 5-chloro-*N*-acetyltryptamine, or by refluxing 5-benzyloxytryptamine hydrochloride (Sigma, USA) with acetic anhydride to obtain 5-benzyloxy-*N*-acetyltryptamine, or by refluxing 6-methoxytetrahydro-β-carboline, obtained from 5-methoxytryptamine hydrochloride (Sigma, USA) as described by Gynther et al. (1985), with acetic anhydride to

produce 6-methoxy-*N*-acetyltetrahydro-β-carboline. The main characteristics of each synthesized compound are as follows:

- *5-chloro-N-acetyltryptamine*: MW = 236; m.p. = 128–130°C; MS, m/z (rel. int.%) = 236 (2, M), 178 (32), 176 (100), 161 (22), 163 (64)
- *5-benzyloxy-N-acetyltryptamine*: MW = 308; m.p. = 110–112°C; MS, m/z (rel. int.%) = 266 (5, M), 265 (27), 236 (77), 235 (81), 145 (100), 144 (48)
- *6-methoxy-N-acetyl tetrahydro-β-carboline*: MW = 244; m.p. = 177–179°C; MS, m/z (rel. int.%) = 244 (26, M), 199 (37), 198 (96), 183 (100), 173 (25), 155 (34)

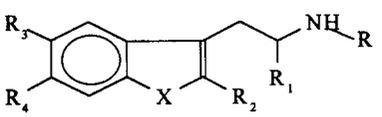
The structure-activity relationship was calculated as the relative affinity of melatonin analogs at the melatonin receptor of sheep pars tuberalis. The relative affinity was calculated as the rounded-up quotient (K_i analog/K_i melatonin) from the papers published by Sugden and Chong (1991) and Yous et al. (1992). The K_i values were determined from the Fig. 3 of the paper by Sugden and Chong (1991). The relative potencies of the three synthesized compounds compared to that of melatonin in displacing 2-[^{125}I]iodomelatonin binding (60–70 pmol radioligand concentration) from chicken retinal membranes at 22°C, as previously described (Morgan et al., 1989; Laitinen and Vakkuri, 1993).

The amino acid sequences (Fig. 2) were compared by using the genetics computer group (GCG) sequence analysis software package (version 8.0). Receptor sequences were extracted from the Swiss-Prot protein sequence data bank and aligned manually. All vertebrate receptors for melatonin and biogenic amines in Swiss-Prot (release 31.0) and Swiss updates (1366 entries on 7.4.1995) were included. The conserved amino acid residues are presented as a one-letter codes whereas other symbols are used at sites where conservation is incomplete. Amino acids are classified as being in one of the two groups described by Baldwin (1993). The first group includes the amino acids which are usually regarded as hydrophobic (A, C, F, G, I, L, M, P, V, W) and those that are able to satisfy their hydrogen binding potential by bonding to the main chain of a helix (S, T, Y). These residues could be in contact with the lipid membrane. The second group includes the remaining amino acids which are either charged (D, E, H, K, R) or are capable of forming more than one hydrogen bond (N, Q) and are thus not expected to be in contact with the lipid membrane.

3. Results

The different structures used in this study are represented in Tables 1 and 2, together with the reported biological activities calculated from the cited works (Sugden and Chong, 1991; Yous et al., 1992).

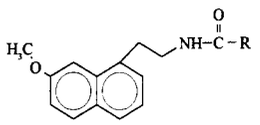
Table 1
Structures of compounds 1–18 used in this study with their relative affinities (K_i) in sheep pars tuberalis at 37°C



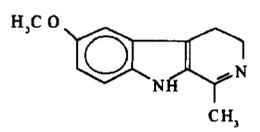
Compound	R	R_1	R_2	R_3	R_4	X	K_i (nmol) [a]
1	$COCH_3$	H	Cl	OCH_3	H	NH	0.06
2	$COCH_3$	H	I	OCH_3	H	NH	0.1
3	COC_4H_9	H	H	OCH_3	H	NH	0.3
4	COC_3H_7	H	H	OCH_3	H	NH	0.9
5 (Mel. [c])	$COCH_3$	H	H	OCH_3	H	NH	1
6	$COCH_3$	H	H	OCH_3	Cl	NH	1.3
7	$COCH_3$	H	H	OCH_3	H	S	7
8	$COCH_3$	H	H	OCH_3	OH	NH	8
9	COC_5H_{11}	H	H	OCH_3	H	NH	40
10	$CO(i)C_4H_9$ [b]	H	H	OCH_3	H	NH	60
11	$COCH_3$	H	H	OCH_3	OCH_3	NH	200
12	COC_4H_9	H	H	H	H	NH	200
13	COC_3H_7	H	H	H	H	NH	300
14	$COCH_3$	H	H	OH	H	NH	600
15	$COCH_3$	H	H	H	H	NH	700
16	$CO(i)C_4H_9$ [b]	H	H	H	H	NH	700
17	H	H	H	OCH_3	H	NH	4000
18	H	CH_2OH	H	OCH_3	H	NH	6000

[a] Relative affinity. [b] Isobutanoyl. [c] Mel. = melatonin.

Table 2
Structures of compounds 19–28 used in this study with their relative affinities (K_i) in sheep pars tuberalis at 37°C



Compound	R	K_i (nmol) [a]
19	C_2H_5	0.24
20	CH_3	1.1
21	$(i)C_3H_7$ [b]	25
22	Cyclobutyl	260
23	Cyclohexyl	1700
24	C_5H_{11}	3100
25	C_6H_{13}	25000
26	Phenyl	55000
27	$(m)C_6H_3Cl_2$ [c]	120000



Compound 28 (6-methoxy harmalan) K_i (nmol) [a] = 5000

[a] Relative affinity. [b] Isopropyl. [c] *m*-dichloro phenyl.

3.1. CoMFA model for the structure-activity relationships

From the results of the different CoMFA models presented in Table 3, we chose the H bond O probe model (model 5) as the best one because of the better statistical results obtained with it. This model is the one which best represents the structure-activity relationships of the studied compounds.

The CoMFA models can also be visually represented as graphic three-dimensional coefficient contour maps. In these maps (calculated with the model 5), the polyhedra surround lattice points where the scalar products of the QSAR coefficient and the standard deviation of all values in the corresponding column of the QSAR table are higher (or lower) than a specific user-defined value. These lattices show regions where higher (or lower) values would strengthen the binding affinity. From the electrostatic map illustrated in Fig. 1a, it is possible to observe three regions in which a decrease in negative density increases biological activity. These regions are near the substituent at position 6; over the bridge of two methylene residues and the exocyclic nitrogen, and near the hydrocarbon substitution on the carbonyl group attached to the exocyclic nitrogen. This electrostatic map also indicates two regions in which an increase in the negative density increases biological activity. These regions are located near the carbonyl oxygen in the direction of the C=O bond and near the methoxy group in the direction of the $O–C_{methyl}$ bond.

The steric influences shown in Fig. 1b can be obtained from the same model. In a clearly defined region near the methoxy group in position 5, an increase in bulk increases biological activity. A similar effect, although comparatively weak, is present near the substituent at position 2. For other regions, the model provides no clear information.

As the affinity of melatonin for the melatonin receptor is linearly correlated between sheep pars tuberalis and chicken brain and retina (Dubocovich, 1985; Sugden and Chong, 1991), we generated a new model in a similar way to the best one obtained (model 5) but this model also included the three newly synthesized compounds (Table 3).

This model yields good results and its predictive ability was better than that of the model which did not include these compounds (Table 4). This improvement in the prediction ability of the model is achieved by the introduction of new structural differences that were not present in the first set of compounds. The CoMFA maps for this new model have the same characteristics as the previous model.

3.2. The G protein-coupled melatonin receptors

The amino acid residues forming the seven transmembrane helices of the receptors for melatonin and biogenic amines are shown in Fig. 2. Fig. 3 depicts the putative spatial arrangement of the seven transmembranes helices in the melatonin receptor, as viewed from three different horizontal levels. The numbering of the residues is identical to that used by Baldwin (1993) to facilitate direct comparison with the original model. In our model of the

Table 3
Results from the different CoMFA analysis of the melatonin like compounds (all the models with five components)

Model #	Type of field	R^2_{cv} (SE) [a]	R^2_{nv} (SE) [b]	R^2_{bs} (SE) [c]	F [d]
1	CoMFA	0.697 (1.106)	0.947 (0.461)	0.974 (0.012)	79.256
2	Steric	0.656 (1.177)	0.941 (0.488)	0.970 (0.014)	70.014
3	Electrostatic	0.497 (1.424)	0.918 (0.575)	0.947 (0.025)	49.342
4	H bond H probe	0.656 (1.178)	0.954 (0.430)	0.975 (0.009)	91.759
5	H bond O probe	0.763 (0.978)	0.966 (0.373)	0.981 (0.009)	123.176
6	H bond O probe new [e]	0.662 (1.107)	0.960 (0.381)	0.975 (0.012)	120.168

[a] Cross-validated. [b] Non-validated. [c] Bootstrap. [d] F ratio. [e] Model generated including the three synthesized compounds.

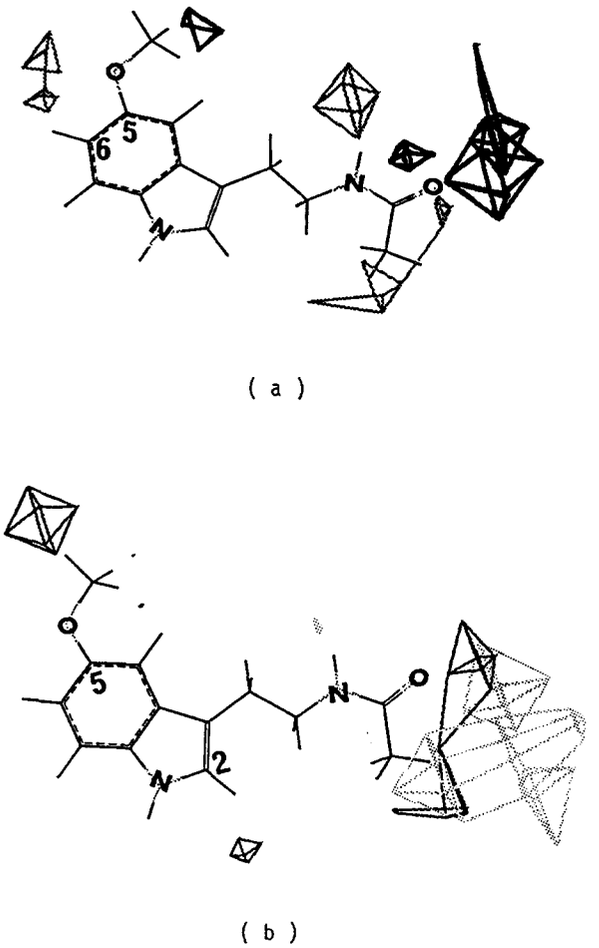


Fig. 1. a: CoMFA map for the model 5 (electrostatic). Light gray (20 kcal/mol) indicates an area in which a decrease in negative density increases affinity. Dark gray (80 kcal/mol) indicates an area in which an increase in negative density increases affinity. b: CoMFA map for the model 5 (steric). Light gray (20 kcal/mol) indicates an area in which a decrease in bulk increases affinity. Dark gray (80 kcal/mol) indicates an area in which an increase in bulk increases affinity.

melatonin receptor, most of the hydrophobic residues point towards the lipid bilayer and are indicated by the black triangles (Fig. 3).

In transmembrane (TM) helix I, threonine T10 and aspartic acid D14 are unique for the melatonin receptor sequences whereas glycine G17, asparagine N18 and valine V21 are all well conserved in most of the G protein-coupled receptors, including the melatonin receptor (Fig. 2). According to the model (Fig. 3), T10 points towards helix VII while D14 points towards the hydrophilic pocket.

In TM II, tyrosine Y20, proline P21 and tyrosine Y22 are fully conserved in all cloned melatonin receptors but are not found in other G protein-coupled receptors; the model places these residues close to the extracellular end of the helix. The conserved motif N-x-x-x-x-S-L-A-x-x-D, which occupies residues 4 through 14, is present in several G protein-coupled receptors including the melatonin receptor (Fig. 2). Previous studies have established aspartic acid D14 as an important residue mediating G protein activation as well as allosteric regulation of ligand binding to various G protein-coupled receptors by sodium ions (Trumpp-Kallmeyer et al., 1992; Savarese and Fraser, 1992; Hutchins, 1994). The presence of this residue in the melatonin receptor was anticipated from its behavior, before any sequence data were available (Laitinen and Vakkuri, 1993; Laitinen and Saavedra, 1990b). Site-directed mutagenesis studies have shown that D14 is probably not directly involved in ligand binding but rather participates in the ligand-induced conformational change that results in G protein activation (Trumpp-Kallmeyer et al., 1992; Savarese and Fraser, 1992).

TM III possesses several amino acid residues that are fully conserved in all cloned melatonin receptors, including the sequence S-G-F-L-M-G-L-S-V-I-G which occupies residues 3 through 13. In all cloned receptors for the biogenic amines, with the exception of adenosine receptors, aspartic acid D7 is fully conserved and its carboxylate side chain is thought to be involved in an ion-ion interaction with the ammonium group of biogenic amines (Hibert et al., 1991, Trumpp-Kallmeyer et al., 1992; Ostrowski et al., 1992; Savarese and Fraser, 1992). Threonine T12 is fully conserved in all cloned catecholamine and histamine receptors. Threonine T19 in the melatonin receptor is replaced by cysteine in the catecholamine receptors and by phenylalanine in histamine receptors (Fig. 2). This residue points towards the hydrophilic pocket in the model (Fig. 3). Serine S14 is fully conserved in all G protein-coupled receptors which bind biogenic amines but is also found in other G protein-coupled receptors (Hibert et al., 1991;

Table 4
Activities of the synthesized compounds evaluated in chicken retina and prediction using two different CoMFA models

Compound	Predicted [a] K_i (nmol) [d]	Predicted [b] K_i (nmol) [d]	Measured [c] K_i (nmol) [d]
Melatonin	1.2	1.1	1
5-Chloro-*N*-acetyltryptamine	44	40	45
5-Benzyloxy-*N*-acetyltryptamine	3.4	770	562
6-Methoxy-*N*-acetyl tetrahydro-β-carboline	> 1000	> 1000	> 1000

[a] Predicted using model 5 (in sheep pars tuberalis at 37°C). [b] Predicted using model 6 (sheep pars tuberalis at 37°C and chicken retina at 22°C). [c] In chicken retina at 22°C. [d] Relative affinity.

Trumpp-Kallmeyer et al., 1992). Interestingly, the triplet motif D-R-Y, which occupies residues 24 through 26 at the intracellular face of helix III, is replaced by a fully conserved triplet N-R-Y in all cloned melatonin receptors (Fig. 2). In other G protein-coupled receptors, this region has been implicated in the interaction with G proteins (Trumpp-Kallmeyer et al., 1992).

In TM IV, tryptophan W11 is fully conserved in all G protein-coupled receptors whereas cysteine C5 and tyrosine Y6 are present in the melatonin receptors but not in other members of the G protein-coupled receptor family (Fig. 2). The model has both amino acids pointing towards the lipid bilayer. Proline P20 and asparagine N21 are fully conserved in the melatonin receptors and proline is also frequently found at either position among other G protein-coupled receptors. The model places these residues close to the extracellular face of helix IV. In general, the amino acid residues in TM IV exhibit the least conservation among different families of G protein-coupled receptors, suggesting that this helix is not crucial for a direct interaction with agonists.

Several unique amino acid residues are fully conserved in the TM V of all cloned melatonin receptors, including threonine T3, valine V7, histidine H10 and cysteine C21 (Fig. 2). All of these face towards the hydrophilic pocket; T3 is located close to the extracellular end, V7 and H10 in the middle, whereas C21 lies at the intracellular face of TM V (Fig. 3). Serines S6 (or S7) and S10 are conserved as a pair only in adrenergic and dopamine receptors; these residues are thought to form hydrogen bonds with the *para-* and *meta*-hydroxyl groups of the catecholamine moieties in noradrenaline, adrenaline and dopamine (Hibert et al., 1991; Trumpp-Kallmeyer et al., 1992; Ostrowski et al., 1992; Savarese and Fraser, 1992). In muscarinic acetylcholine receptors, muscarinic agonists are thought to interact with the fully conserved threonines T3 and T6 (Wess et al., 1991). In the rhodopsin-based model, residues 3, 7 and 10 point towards the hydrophilic pocket whereas residue 6 points towards the lipid bilayer (Fig. 3). All cloned serotonin receptors have a conserved threonine T7 or serine S7; these amino acid residues are thought to form hydrogen bonds with the 5-hydroxyl group of serotonin (Hibert et al., 1991, Trumpp-Kallmeyer et al., 1992). All melatonin receptors have a conserved hydrophobic residue, valine V7, at this position. Phenylalanine F11, proline P14 and tyrosine Y22 are well conserved in all G

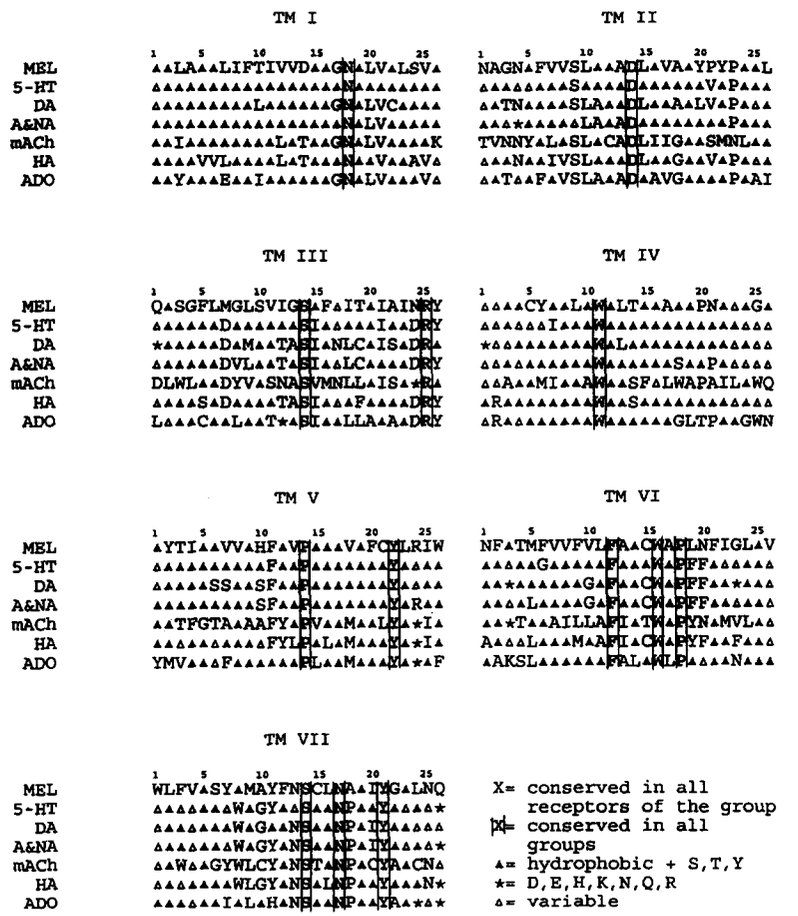


Fig. 2. Amino acid sequences of the putative seven transmembrane helices of the receptors for melatonin and biogenic amines. MEL = melatonin receptors, 5-HT = serotonin (5-hydroxytryptamine) receptors, DA = dopamine receptors, A and NA = adrenergic receptors, mACh = muscarinic acetylcholine receptors, HA = histamine receptors, ADO = adenosine receptors. Sequences were obtained as described in Materials and methods.

protein-coupled receptors, including the melatonin receptor (Fig. 2).

In TM VI, the rhodopsin-based model accommodates two fully conserved amino acid residues pointing towards the hydrophilic pocket in the melatonin receptor: methionine M5 and phenylalanine F9 (Fig. 3). The motif F-x-x-C-W-x-P, which occupies residues 12 through 18, is well conserved among all G protein-coupled receptors, whereas phenylalanines F19 and F20 are fully conserved in the receptors for serotonin and catecholamines (Fig. 2). The melatonin receptor has leucine L19 and asparagine N20.

The TM VII of the melatonin receptors contains a pair of fully conserved amino acid residues close to the extracellular face of the helix: serine S6 and alanine A10, at a

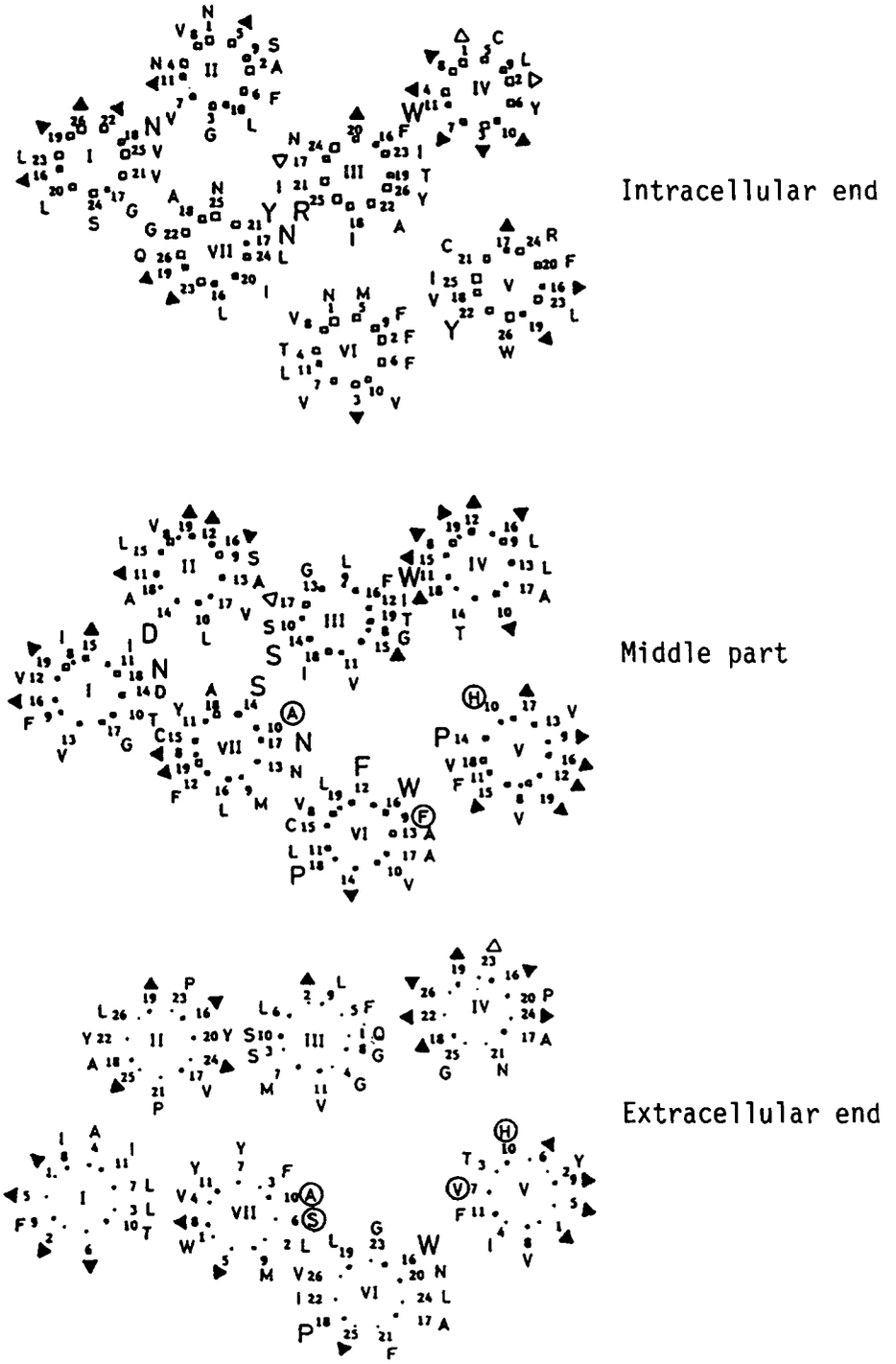


Fig. 3. Probable arrangement of the transmembrane helices of the G protein-coupled melatonin receptor viewed from the intracellular surface. Top: 11 residues in the intracellular end of each helix. Middle: 12 residues in the middle. Bottom: 11 residues in the extracellular end of each helix. The amino acid residues which are proposed to recognize melatonin are circled. Adapted from Baldwin (1993). x = conserved in all receptors of the group; **X** = conserved in all groups; ▲ = hydrophobic + S, T, Y; ★ = D, E, H, K, N, Q, R; △ = variable.

distance of approximately one helical turn from each other and both pointing towards the hydrophilic pocket and helices IV–VI (Fig. 3). Alanine A10 lies just upstream of the motif Y-x-N-S-x-x-N-P-x-x-Y (residues 11 through 21), which is highly conserved in several G protein-coupled receptors. However, all cloned melatonin receptors have alanine A18 instead of the highly conserved proline found in other G protein-coupled receptors. Tyrosine Y11 is replaced by histidine in all cloned adenosine receptors (Fig. 2) and H11 has been suggested to be involved in ligand binding in the adenosine A_1 receptor (Jackobson et al., 1992).

4. Discussion

4.1. Strategy for the development of a new molecular model of a putative binding site for melatonin

In a recent paper, Sugden et al. (1995) proposed a molecular model of a putative binding site for melatonin based on the predicted sequence of the cloned *Xenopus laevis* melanophore melatonin receptor (Ebisawa et al., 1994) and of the correlation of binding affinities with some energetic and electronic parameters. The geometry of the melatonin structure used by Sugden et al. (1995) is different to ours in the fact that our geometry of melatonin is 0.63 kJ/mol (0.15 kcal/mol) lower in energy than theirs (using both NOMM or MMOK keywords for the AM1 optimization). More importantly, the orientation of the methoxy and amide moieties in our model is completely opposite to that in the melatonin model of Sudgen (Fig. 4).

Taking these facts into consideration, we planned the synthesis of some derivatives that could provide us with some additional evidence to test the proposed receptor binding site model. The compounds synthesized were 5-chloro-*N*-acetyltryptamine, 5-benzyloxy-*N*-acetyltryptamine and 6-methoxy-*N*-acetyl tetrahydro-β-carboline and were obtained as described in Materials and methods.

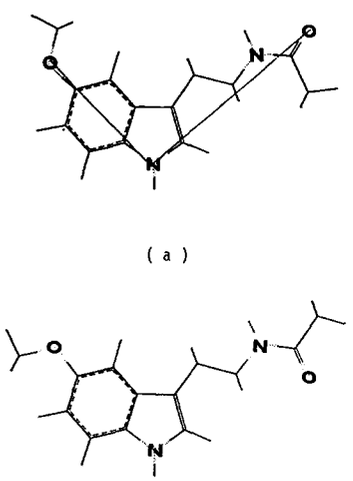


Fig. 4. Structures of (a) our geometry for melatonin (lines indicate the tilted L-form), and (b) Sugden's geometry.

The 5-chloro derivative was used to confirm the importance of the methoxy group for binding to the receptor site, by placing a different electronegative substituent with H-bond acceptor characteristics. The purpose of the 5-benzyloxy derivative was to provide a structure with a large substituent in position 5 to see if a bigger hydrophobic group can be attached to the oxygen atom. Finally, 6-methoxy-*N*-acetyl tetrahydro-β-carboline was used to introduce a compound with a different geometry around the amide moiety, but with the methoxy group in the same position. These compounds would enable us to confirm the predictive ability of our CoMFA model and to gain clues about the location and type of interaction of the binding sites at the receptor.

As presented in Table 4, we confirmed that the methoxy group in position 5 is necessary for high binding activity. Thus the formation of a hydrogen bond with the binding site is more likely to occur with the oxygen from the methoxy group than with the chloro moiety, as theoretically predicted. In addition, a relatively large group attached to the oxygen in the same position sterically hinders the interaction between the oxygen and the binding site. The difference between the predicted and observed K_i values for the 5-benzyloxy-*N*-acetyltryptamine (Table 4, prediction with model 5) can be explained by the fact that the original set of compounds did not include any structure with such a substituent at position 5. With respect to the amide moiety, it seems that the position of the carbonyl oxygen has to be the same as in the optimized melatonin structure and in other derivatives with high affinity for the binding site. This indicates that the interaction between the carbonyl oxygen and the binding is defined by the angle O(methoxy)-N(indole)-O(carbonyl) ≈ 90°; as it is with almost all of the structures presented in Tables 1 and 2. This also indicates that the distance between the oxygen of the methoxy group and the oxygen of the carbonyl group must be around 1.08 nm (10.8 Å), as it is in all the melatonin- and naphthalene-related structures analyzed.

4.2. Docking melatonin at its recognition site

Four criteria are expected to be fulfilled when melatonin occupies its receptor. First, the 5-methoxy group of melatonin is specifically recognized and selectively differentiated from the 5-hydroxyl group of *N*-acetyl serotonin, a molecule whose structure is otherwise identical to that of melatonin but which has approximately 600-fold lower affinity for the melatonin receptor (Table 1, compound 14). In addition, a bulky hydrophobic substituent at the 5-position is not tolerated, as demonstrated by the relatively low binding affinity of the analog in which the

5-methoxy group was replaced by a 5-benzyloxy group. Also the presence of a 5-chloro instead of the 5-methoxy group decreases binding affinity. This indicates that the oxygen in position 5 has to be selectively recognized, together with the methyl group attached to it.

Second, the oxygen of the *N*-acetyl group is recognized specifically and this recognition is expected to take place at a distance of about 1.08 nm (10.8 Å) from the recognition site of the 5-methoxy group. Variation of this distance and/or a change in the relative position of the *N*-acetyl group produces a decrease in binding affinity, as demonstrated by the low potency of 6-methoxy-*N*-acetyltetrahydro-β-carboline.

Third, an aromatic interaction between the receptor and the indole moiety of melatonin stabilizes the docking of melatonin at its receptor. That this interaction occurs is supported by the high potency of the naphthalenic melatonin analogs for the receptor. Finally, as the energetically minimized conformation of melatonin occupies a tilted L form (Fig. 4), the 5-methoxy and *N*-acetyl groups are recognized in a plane which is clearly outside the plane of the aromatic interaction.

Based on the above criteria, we propose a model in which melatonin in its relaxed conformation fits to the hydrophilic binding pocket formed by the seven transmembrane helices. Specific recognition of the functional groups in the melatonin molecule occurs in a small cleft formed by the extracellular ends of helices V and VII and the middle part of helix VI (Fig. 5a and Fig. 5b).

The 5-methoxy group has a docking site in TM V, where a fully conserved histidine (H10) points towards the hydrophilic pocket thereby allowing hydrogen bonding with the oxygen atom of the methoxy group. A fully conserved valine (V7), located approximately one helical turn upwards and pointing in the same direction, provides the aliphatic milieu required for the methyl group attached to the 5-oxygen. This cooperation between H10 and V7 can explain why replacement of the 5-methoxy group of melatonin with the 5-hydroxy group, as in *N*-acetylserotonin (Table 1, compound 14), results in such a dramatic loss of activity. This could also explain why a bulky group over the 5-oxygen, as in 5-benzyloxy-*N*-acetyltryptamine, is not tolerated. If the 5-methoxy group is docked at the indicated residues in helix V, then the *N*-acetyl group would point towards helix VII, where the fully conserved serine (S6), which points towards the hydrophilic pocket, would allow specific hydrogen bonding with the carbonyl oxygen. A fully conserved alanine (A10), located approximately one helical turn downwards and pointing in the same direction, could be an additional anchor for the methyl group attached to the carbonyl group. This cooperation between S6 and A10 could explain why alkyl groups bigger than four carbon atoms attached to the carbonyl group of the amide are not tolerated. An aromatic interaction could take place between the indole ring and the phenylalanine (F9) in TM

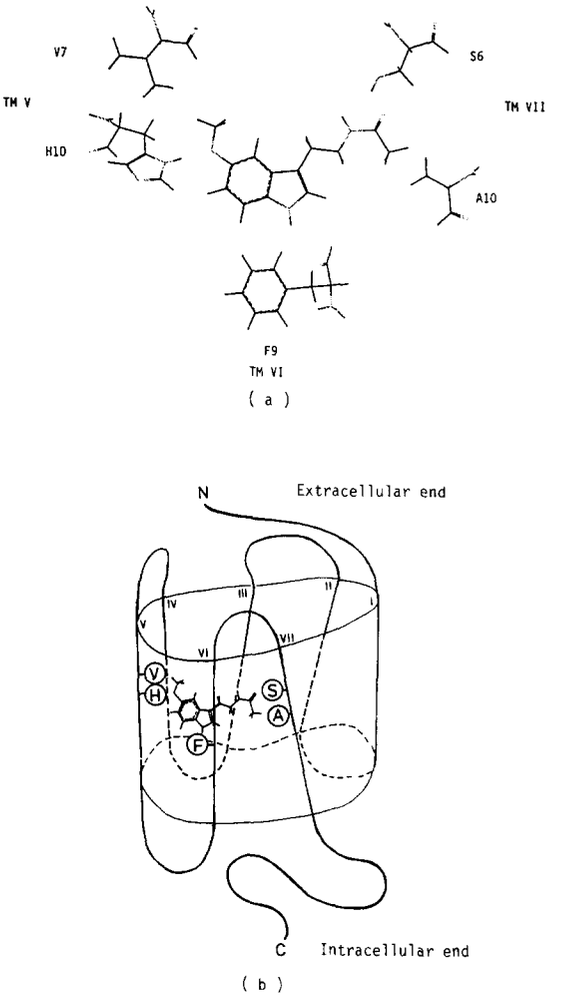


Fig. 5. a: Proposed amino acid residues that recognize the functional groups of melatonin at its receptor. b: Schematic three-dimensional presentation of melatonin docking at its receptor. The cylinder shows which part of the receptor is surrounded by the lipid membrane. The receptor has an extracellular N-terminus and a rather long, intracellular C-terminus.

VI, which is at a proper distance and is conserved in all of the cloned melatonin receptors.

Although it has not been tested in any way, it is possible that some compounds interact with the methionine 7 of transmembrane helix III. This interaction could occur with the nitrogen of the amide moiety instead of the carbonyl oxygen, also by the formation of an hydrogen bonding. The methionine residue is at the same level as the serine 6 of transmembrane helix VII, and also faces the pocket. This possibility could occur instead of or together with the interaction of the carbonyl oxygen of the melatonin analog with the serine 6 of transmembrane helix VII, and it would also comply with the tilted L-form of our melatonin model.

Our model places all essential interactions in such a way that the acceptors for the 5-methoxy and *N*-acetyl groups reside in the same horizontal plane close to the extracellular face of the pocket, whereas the aromatic

interaction takes place deeper in the binding pocket (Fig. 5b). This arrangement would be fully compatible with the tilted L-form of melatonin in its energetically minimized conformation (Fig. 4). Our model is also compatible with the structure-activity relationships obtained by Garratt et al. (1995) using 2-phenyltryptamines as melatonin analogs. Moreover, the geometry of our melatonin structure is similar to the geometry of the melatonin structure in their paper.

Based on the helical structure of bacteriorhodopsin and the melatonin receptor sequence from *Xenopus laevis* melanophores, Sugden and coworkers have recently proposed a model for melatonin recognition at this receptor (Sugden et al., 1995). This model differs considerably from our model, which is based on helical arrangement of rhodopsin and on the amino acid sequences of several cloned melatonin receptors. The model of Sugden and coworkers proposed that the 5-methoxy group is recognized by serine S10 in helix III and isoleucine I25 in helix II, and the *N*-acetyl group was suggested to be recognized by asparagine N21 and valine V24, both in helix IV. Moreover, the model proposed an aromatic interaction between the indole moiety of melatonin and tryptophan W16 in helix VI, which is conserved in all G protein-coupled receptors. As already discussed by Baldwin (1993), the spatial arrangement of the seven α helices in G protein-coupled receptors differs considerably between the bacteriorhodopsin and rhodopsin models. When placed in the rhodopsin-based model (Fig. 3), many of the specific amino acid residues proposed by Sugden and coworkers (1995) point towards the lipid bilayer and other helices rather than towards the hydrophilic pocket, suggesting that these residues may not be able to interact with the functional groups of the melatonin molecule. Moreover, tryptophan residue W16 is highly conserved in all G protein-coupled receptors regardless of the type of ligand they bind, suggesting that this residue is critical for the general arrangement of helix VI and may not belong to a receptor's binding site.

With site-directed mutagenesis it should be now possible to directly assess the contribution of each indicated amino acid residue to the recognition of melatonin in both models.

Note added in revision

A recent paper with full or partial sequence information from 15 different G protein-coupled melatonin receptors, spanning four vertebrate classes, indicates that the amino acid residues proposed by us to recognize the functional moieties of melatonin are fully conserved in all 15 sequences (Reppert et al., 1995).

Acknowledgements

The data in this paper have been submitted in an abstract form (Laitinen et al., 1995). Miss Raija Holopainen is thanked for drawing Figs. 3 and 5b. The study was supported by the Academy of Finland (J.T.L., T.K. and N.H., grant #17845).

References

Baldwin, J.M., 1993, The probable arrangement of the helices in G protein-coupled receptors, EMBO J. 12, 1693.

Clark, M., R.D. Cramer III, and N. van Opdenbosch, 1989, Validation of the general purpose Tripos 5.2 Force Field, J. Comput. Chem. 10, 982.

Cramer III, R.D., D.E. Patterson, and J.D. Bunce, 1988a, Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins, J. Am. Chem. Soc. 110, 5959.

Cramer III, R.D., J.D. Bunce, D.E. Patterson, and I.E. Frank, 1988b, Crossvalidation, bootstrapping, and partial least squares compared with multiple regression in conventional QSAR studies, Quant. Struct.-Act. Relat. 7, 18.

Dubocovich, M.L., 1985, Melatonin receptors: are there multiple subtypes?, Trends Pharmacol. Sci. 16, 50.

Ebisawa, T., S. Karne, M.R. Lerner, and S.M. Reppert, 1994, Expression cloning of a high affinity melatonin receptor from *Xenopus* dermal melanophores, Proc. Natl. Acad. Sci. USA 91, 6133.

Findlay, J. and E. Eliopoulos, 1990, Three-Dimensional modeling of G protein-linked receptors, Trends Pharmacol. Sci. 11, 492.

Garratt, P.G., R. Jones, and D.A. Tocher, 1995, Mapping the melatonin receptor. 3. Design and synthesis of melatonin agonists and antagonists derived from 2-phenyltryptamines, J. Med. Chem. 38, 1132.

Guerrero, J.M. and R.J. Reiter, 1992, A brief survey of pineal gland-immune system interrelationships, Endocr. Res. 18, 91.

Gynther, J., 1988, EI and CI mass fragmentation of tryptamine, tetrahydro-β-carboline and some of their derivatives, Acta Chem. Scand. B 42, 433.

Gynther, J., P. Peura, and S. Salmi, 1985, Electron impact and chemical ionization fragmentation of 5-methoxytryptamine and some of 6-methoxy-β-carbolines, Acta Chem. Scand. B 39, 849.

Hibert, M.F., S. Trumpp-Kallmayer, A. Bruinvels, and J. Hoflack, 1991, Three-Dimensional models of neurotransmitter G-binding protein-coupled receptors, Mol. Pharmacol. 40, 8.

Hutchins, C., 1994, Three-Dimensional models of the D1 and D2 dopamine receptors, Endocr. J. 2, 7.

Jackobson, K.A., P.J.M. Van Galen, and M. Williams, 1992, Adenosine receptors: pharmacology, structure-activity relationships, and therapeutic potential, J. Med. Chem. 35, 407.

Kokkola, T., J.M. Hyttinen, and J.T. Laitinen, 1995, Molecular cloning of the G protein-coupled melatonin receptors from chick brain and retina, Soc. Neurosci. Abstr. 21, 183.

Krause, D.N. and M.L. Dubocovich, 1991, Melatonin receptors, Annu. Rev. Pharmacol. Toxicol. 31, 549.

Laitinen, J.T. and J.M. Saavedra, 1990a, Characterization of melatonin receptors in the rat suprachiasmatic nuclei: modulation of affinity with cations and guanine nucleotides, Endocrinology 126, 2110.

Laitinen, J.T. and J.M. Saavedra, 1990b, Differential sensitivity to cations of the melatonin receptors in the rat area postrema and suprachiasmatic nuclei, J. Neurochem. 55, 1450.

Laitinen, J.T. and O. Vakkuri, 1993, Intracellular sodium regulates agonist-melatonin receptor interactions in the chick retina, in: Melatonin and the Pineal Gland – From Basic Science to Clinical Application, eds. Y. Touitou, J. Arendt and P. Pevet (Elsevier, Amsterdam) p. 137.

Laitinen, J.T., C. Navajas, T. Kokkola, A. Poso, N. Honka, J. Gynther, and K.S.M. Laitinen, 1995, A rhodopsin-based model for melatonin recognition at its G protein-coupled receptor, Soc. Neurosci. Abstr. 21, 183.

Lolley, R.N. and R.H. Lee, 1990, Cyclic GMP and photoreceptor function, FASEB J. 4, 3001.

Molis, T.M., L.L. Spriggs, Y. Jupiter, and S.M. Hill, 1995, Melatonin modulation of estrogen-regulated proteins, growth factors, and protooncogenes in human breast cancer, J. Pineal Res. 18, 93.

Morgan, P.J., L.M. Williams, G. Davidson, W. Lawson, and H.E. Howell, 1989, Melatonin receptors on ovine pars tuberalis: characterization and autoradiographical localization, J. Neuroendocrinol. 1, 1.

Ostrowski, J., M.A. Kjelsberg, M.G. Caron, and R.J. Lefkowitz, 1992, Mutagenesis of the β_2-adrenergic receptor: how structure elucidates function, Annu. Rev. Pharmacol. Toxicol. 32, 167.

Reiter, R.J., 1991, Pineal melatonin: cell biology of its synthesis and of its physiological interactions, Endocrine Rev. 12, 151.

Reppert, S.M., D.R. Weaver, and T. Ebisawa, 1994, Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses, Neuron 13, 1177.

Reppert, S.M., D.R. Waever, V.M. Cassone, C. Godson, and L.F. Kolakowski Jr, 1995, Melatonin receptors are for the birds: molecular analysis of two receptor subtypes differentially expressed in chick brain, Neuron 15,1003.

Savarese, T.M. and C.M. Fraser, 1992, In vitro mutagenesis and the search for structure-function relationships among G-protein-coupled receptors, Biochem. J. 283, 1.

Schertler, G.F.X., C. Villa, and R. Henderson, 1993, Projection structure of rhodopsin, Nature 362, 770.

Stankov, B., F. Fraschini, and R.J. Reiter, 1991, Melatonin binding sites in the central nervous system, Brain Res. Rev. 16, 245.

Stewart, J.J.P., 1990, MOPAC: A semiempirical molecular orbital program, J. Comp.-Aid. Mol. Des. 4, 1.

Sugden, D. and N.W.S. Chong, 1991, Pharmacological identity of 2-[^{125}I]iodomelatonin binding sites in chicken brain and sheep pars tuberalis, Brain Res. 539, 151.

Sugden, D., N.W.S. Chong, and D.F.V. Lewis, 1995, Structural requirements at the melatonin receptor, Br. J. Pharmacol. 114, 618.

Trumpp-Kallmeyer, S., J. Hoflack, A. Bruinvels, and M. Hibert, 1992, Modeling of G-protein-coupled receptors: application to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors, J. Med. Chem. 35, 3448.

Unger, V.M. and F.X. Schertler, 1995, Low resolution structure of bovine rhodopsin determined by electron cryo-microscopy, Biophys. J. 68, 1776.

Vakkuri, O., E. Lämsä, E. Rahkamaa, H. Ruotsalainen, and J. Leppä-Luoto, 1984, Iodinated melatonin: preparation and characterization of the molecular structure by mass and ^{3}H NMR spectroscopy, Anal. Biochem. 142, 284.

Wess, J., D. Gdula, and M.R. Brann, 1991, Site-directed mutagenesis of the m3 muscarinic receptor: identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding, EMBO J. 10, 3729.

Yous, S., J. Andrieux, H.E. Howell, P.J. Morgan, P. Renard, B. Pfeiffer, D. Lesieur, and B. Guardiola-Lemaitre, 1992, Novel naphthalenic ligands with high affinity for the melatonin receptor, J. Med. Chem. 35, 1484.