TARJA KOKKOLA

Mapping the Structure and Function of the Human MT1 Melatonin Receptor

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L22, Snellmania building, University of Kuopio, Friday 14th November 2003, at 12 noon

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ABSTRACT

G protein-coupled receptors are an essential group of membrane-associated signalling molecules. The pineal hormone melatonin binds to and activates G protein-coupled melatonin receptors, which form a distinct subgroup in the rhodopsin-like G protein-coupled receptor family. In humans and other mammals, only two melatonin receptor subtypes, MT1 and MT2, have been found to exist. An additional Mel subtype exists in lower vertebrates. Compared to most other hormone receptors, only few investigations on the function and structure of melatonin receptors exist so far. This study aimed at obtaining more information about how melatonin binds to and activates its G protein-coupled receptors.

A rhodopsin-based model of melatonin recognition by specific amino acids in the melatonin receptor transmembrane domains was constructed. The model was tested by site-directed mutagenesis of the human MT1 melatonin receptor and recombinant expression of the wild-type and mutant receptors in yeast and mammalian cells. The effects of mutations in some conserved amino acids assumed to be important in melatonin receptor activation and G protein coupling were also studied. Functional properties, ligand binding characteristics, and subcellular localization of the wild-type human MT1 receptor and a total of 12 mutant receptors were assessed.

Site-directed mutagenesis studies revealed that a conserved histidine H195 (His345) in transmembrane domain V is involved in ligand binding and activation of the human MT1 melatonin receptor. Two other melatonin recognition sites suggested by the rhodopsin based receptor model were not found to be important in MT1 receptor function. The results of the empirical studies thus indicated that the theoretical receptor model presented in this thesis would need to be reconstructed. In transmembrane domain VI, proline P253 (Pro520) was shown to have a role in maintaining the receptor in the correct conformation. Mutations in N124 (Asn36) at the end of the transmembrane domain III were harmful to receptor function; functional properties of the receptor were affected more in the mammalian expression system than in the yeast. Two conserved cysteines (C127, Cys352 and C130, Cys355) in the interphase between transmembrane domain III and the second intracellular loop of the human MT1 melatonin receptor were found to be involved in G protein coupling of the receptor, suggesting that these cysteines are important for the interaction between melatonin receptors and associated signalling partners.

Melatonin is involved in a wide range of physiological functions. The results from studies like this can provide us with a better understanding on the molecular basis of these functions regulated by melatonin and help building improved models of melatonin receptors.

Universal Decimal Classification: 577.17, 575.224.22, 577.112
National Library of Medicine Classification: QV 38, QU 55, WK 185
Medical Subject Headings: receptors, cell surface; GTP-binding proteins; melatonin; mutagenesis, site-directed; point mutation; gene expression; Saccharomyces cerevisiae; COS cells; CHO cells; S-nitrosothiols; cysteine; quantitative structure-activity relationship; models, molecular
THIVISTELMÄ


Kohdenetuissa mutagenesitutkimuksissa selvisi, että konservoitunut hisidiin H195 (H195<sup>46</sup>) MT1 melanoniireseptorin transmembraaniosassa V osallistuu ligandin sitoutumiseen ja reseptoriaktivointoon. Kaksik muuta rodopiinipohjaisen reseptorimalliin ehdotamaa melanoniinin tunnistuskohtaa eivät kuitenkaan osoittautuneet tärkeiksi MT1-reseptorin toiminnalle. Kokeiluinen tutkimusten tulokset siis osoittivat, että tämän vastoskirjan alussa esitettyjen teoreettisten mallien olisi syytä kehittää edelleen. Transmembraaniosat VI protiini P253 (P<sub>253</sub>) osoittautui tärkeäksi reseptorin ulkoiseen rakenteen kannalta. Mutaatio transmembraaniosan III lopussa sijaitsevassa asparaginisissa N124 (Asn3,49) olisi haitallinen reseptorin toiminnalle; mutaation vaikutus olivat suurempia jos resitoreja tuotettiin niskässoluisissa verrattuna hiivasoluisissa tuotettuihin reseptoreihin. Transmembraaniosan III loppuosan ja toisen solunsisäisen lenkin rajakohdassa sitäjäsevien konservoituneiden kysyminen (C127, Cys<sub>127</sub> ja C130, Cys<sub>130</sub>) havaittiin vaikuttavan reseptorien kytkeytymiseen G-proteineihin. Tästä voitiin päätellä, että nämä kysyimet ovat tärkeitä melanoniireseptorien ja niiden signalointipartiojen välisissä vuorovaikutuksissa.

Melatoniini osallistuu moniin fysiologiisiin toimintoihin. Tämänkaltaisten tutkimusten tulokset auttavat ymmärtämään näitä toimintoja molekyylitasolla ja kehitämään parempia melanoniireseptorimalleja.
ACKNOWLEDGEMENTS

This work was carried out predominantly at the Department of Physiology, University of Kuopio, Finland. An important part of the studies was conducted at the GlaxoSmithKline Medicines Research Centre, Stevenage, England.

I wish to thank all the people who have helped and supported me during this project. I wish to express my deepest gratitude to my principal supervisor, Docent Jarno Laitinen. He introduced me to the fascinating world of science. His deep and broad knowledge of neuroscience and G protein-coupled receptors is something I can only wish to achieve some day. I thank my supervisor Professor Juhani Jänne for help with the molecular biological methods, especially in the initiating phase of my research project. I owe my thanks also to Professor Osmo Häkkinen, Head of the Department of Physiology, for providing me the facilities for my work in his department and for his support.

I appreciate the valuable advice and constructive criticism of the official referees of this thesis, Professor Peter Morgan and Professor Mika Scheinin.

The efforts of all co-authors are greatly appreciated. Thank you for the fruitful and pleasant collaboration.

I am deeply grateful to the present and former members of the GPCR research team for sharing the good and the bad times with me and for the inspiring discussions: Mr. Juha Savimänen, M.Sc., Ms. Kati Mönkkönen, M.Sc., Ms. Retja Heikkinen, Dr. Kaisa Kurkinen, and Ms. Nina Koponen, M.Sc. It has been a privilege to know you and to work with you. I want to thank all the colleagues and friends at the Department of Physiology for providing a pleasant work atmosphere throughout the years. Special thanks belong to Ms. Taina Vihavainen, B.Eng. and Ms. Tiina Räsänen for their skilful technical assistance.

I owe my thanks for the members of the Receptor Systems laboratory and the 'Yeast group' at the GlaxoSmithKline Medicines Research Centre. The time that I spent in Stevenage was not very long, but the months were happy and full of work. I wish to thank Dr. Marie-Ange Watson, Dr. Steven Foord, Dr. Dianne Cousens, Dr. Simon Dowell, Dr. Julia White, Dr. Andrew Brown, and the others for teaching me so many things about science, and for their friendship.

Finally, I want to thank my dearest ones; my husband Harri for his love and support through so many years, and our sons Roni and Tuukka for showing me what is really important in life. I also wish to thank my parents, Meeri and Reino Keskitalo and my brother Toni Keskitalo, my relatives and all my friends for their encouragement and support.

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Kuopio, October 2003

Tarja Kokkola
ABBREVIATIONS

123I-MeI  2-[123I]iodomelatonin
[35S]GTPγS  guanosine-5’-O-[(3-[35S]-thio)triphosphate
5-HT  5-hydroxytryptamine (serotonin)
5-MCA-NAT  5-methoxycarbonylamino-N-acetyltrypamine
ADA  adenosine deaminase
AMP  adenosine monophosphate
cAMP  cyclic adenosine monophosphate
cGMP  cyclic guanosine monophosphate
BAI  brain-specific angiogenesis inhibitor
Bmax  maximal binding capacity (receptor density)
Cadherin EGF LAG receptors  cadherins involved in the regulation of planar cell polarity
CCK  cholecystokinin
CHO  Chinese hamster ovary
CoMFA  comparative molecular field analysis
COS-7  transformed African green monkey kidney cell line
CPRG  chlorophenol red-β-d-galactopyranoside
CRF  corticotropin releasing factor
CysNO  nitrosocysteine
DMEM  Dulbecco’s modified Eagle’s medium
EC50  concentration resulting in 50% of the maximal response
EGF  epidermal growth factor
FSH  follicle stimulating hormone
GABA  gamma-amino butyric acid
GAP  GTPase-activating protein
GDP  guanosine diphosphate
GHRH  growth hormone-releasing hormone
GPR50  G protein-coupled receptor 50 (melatonin-related receptor)
G protein  guanine nucleotide binding protein
GSNO  nitrosoglutathione
GTP  guanosine triphosphate
GTPase  guanosine triphosphatase
GPCR(s)  G protein coupled receptor(s)
HA  hemagglutinin
HEK  human embryonic kidney
IC  intracellular loop
IgG  immunoglobulin G
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>I-Mel</td>
<td>2-iodomelanotonin</td>
</tr>
<tr>
<td>IUPHAR</td>
<td>International Union of Pharmacology</td>
</tr>
<tr>
<td>$K_d$</td>
<td>equilibrium dissociation constant</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>MCH</td>
<td>melanin-concentrating hormone</td>
</tr>
<tr>
<td>MOPAC</td>
<td>package of semi-empirical molecular orbital programs</td>
</tr>
<tr>
<td>NAS</td>
<td>N-acetylserotonin</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>PACAP</td>
<td>pituitary adenylate cyclase activating polypeptide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RGS</td>
<td>regulators of G protein signalling</td>
</tr>
<tr>
<td>SAD</td>
<td>seasonal affective disorder</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SDY102</td>
<td><em>Saccharomyces cerevisiae</em> yeast strain</td>
</tr>
<tr>
<td>SYBYL</td>
<td>molecular modelling environment</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane (domain)</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin-releasing hormone</td>
</tr>
<tr>
<td>lSH</td>
<td>thyroid-stimulating hormone</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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</table>
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following original publications referred to in the text by their corresponding Roman numerals:


IV:  Tarja Kokkola, Outi Salo, Antti Poso, Jarmo T. Laitinen. The functional role of cysteines adjacent to the NRY motif of the human MT1 melatonin receptor. *Manuscript*. 
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1. INTRODUCTION

G protein-coupled receptors (GPCRs) are an essential group of membrane-associated signalling molecules. Each receptor is a single polypeptide chain, which spans through the cell membrane seven times. The seven transmembrane alpha helices are closely packed together in a barrel-like structure, which for some, such as the rhodopsin-like receptors, the largest group of GPCRs, forms the ligand binding pocket. In some other receptor groups, ligand binding also involves extracellular parts of the receptor. The intracellular parts of GPCRs are responsible for G protein coupling. The activation of the receptor leads to G protein activation, which conveys the physiological effects of receptor ligands.

The pineal hormone melatonin binds and activates G protein coupled melatonin receptors, which form a distinct subgroup in the rhodopsin-like GPCRs. In humans and other mammals, only two melatonin receptor subtypes, MT1 and MT2, have been found to exist. An additional Mel1c subtype exists in lower vertebrates. The pharmacological profiles of melatonin receptor subtypes are very similar and only recently the development of ligands with some subtype-selectivity has been accomplished. All recombinant melatonin receptor subtypes couple to Gi proteins to inhibit cyclic AMP accumulation in the cell.

Compared to most other hormone receptors, the primary structure of melatonin receptors was resolved only relatively recently. Therefore few investigations on the structure and function of melatonin receptors exist so far. This study was aimed at better understanding of how melatonin binds and activates its G protein-coupled receptors. A model of melatonin recognition by specific amino acids in the melatonin receptor transmembrane domains was constructed. The model was tested by site-directed mutagenesis of the human MT1 receptor and comparison of the recombinant expression of the wild-type (WT) and mutant receptors in yeast and mammalian cells. The effect of mutagenesis of some conserved amino acids assumed to be important in melatonin receptor activation and G protein coupling was also studied. The objective of the study was to obtain more information about how melatonin binds and activates its G protein-coupled receptors.
2. REVIEW OF THE LITERATURE

2.1. Signal transduction

The cells that form a multicellular organism must be able to communicate with each other. Sometimes the communication needs to be rapid, as in the case of neuronal signalling, and sometimes slower and possibly prolonged responses are desirable (Uings and Farrow 2000). In most known cases, communication occurs with the help of signal molecules that interact with receptor proteins in the cells. Receptors were traditionally classified into four categories according to their functional properties. This classification has been validated by the cloning of receptor genes, which supports the existence of four major receptor groups (Table 1). The opening and closing of an ion channel receptor is the most rapid signal transduction mechanism, which responds in milliseconds to the changing concentration of signal molecules. Ion channel receptors are required for neural transmission and they also regulate muscular contraction (Sheng and Pak 2000). The effects conveyed by G protein-coupled receptors (GPCRs) typically appear in seconds, while the responses to tyrosine kinase receptor or nuclear receptor activation usually take from hours to days to appear. Tyrosine kinase receptors mediate their signals through phosphorylation of tyrosine residues in specific target molecules. Many growth factor receptors belong to this class (Uings and Farrow 2000). Steroid hormones and some other lipophilic molecules act through nuclear receptors. When activated, these receptors bind to regulatory elements in the DNA to regulate the expression of target genes (Bourguet et al. 2000). Another type of signalling is mediated by gases like NO and CO. They diffuse freely across cell membranes and affect their target molecules directly, without depending on classical receptors (Maines 1997).

<table>
<thead>
<tr>
<th>Location</th>
<th>Ion channel receptors</th>
<th>GPCRs</th>
<th>Tyrosine kinase receptors</th>
<th>Nuclear receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effector</td>
<td>Ion channel</td>
<td>Enzyme / ion channel</td>
<td>Enzyme</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Time scale</td>
<td>milliseconds</td>
<td>seconds - minutes</td>
<td>minutes - hours</td>
<td>hours - days</td>
</tr>
<tr>
<td>Examples</td>
<td>Nicotinic receptors,</td>
<td>Adrenergic receptors,</td>
<td>Insulin receptor,</td>
<td>Steroid receptors,</td>
</tr>
<tr>
<td></td>
<td>GABA&lt;sub&gt;4&lt;/sub&gt; receptors</td>
<td>rhodopsin</td>
<td>EGF receptor</td>
<td>vitamin D receptor</td>
</tr>
</tbody>
</table>

2.2. G protein-coupled receptors

G protein-coupled receptors are integral cell membrane proteins that convey extracellular signals into intracellular events. When activated, the receptors activate heterotrimeric guanyl
nucleotide binding proteins, G proteins; a property after which they have been named. The 1994 Nobel Prize in Physiology or Medicine was awarded to Alfred G. Gilman (born 1941) and Martin Rodbell (1925-1998) "for their discovery of G proteins and the role of these proteins in signal transduction in cells" (Raju 2000). Today, three decades after the initial discovery of G proteins and two decades after the cloning of the first GPCRs, the importance of these molecules in cellular signalling is well recorded. Genome projects have revealed that the GPCRs form the largest class of receptors with more than 900 members in the human genome (Fredriksson et al. 2003).

The receptors play hundreds of roles in our body. Several sensations (vision, taste, smell) are mediated by the action of the GPCRs. They are also important in the regulation of cell growth, metabolism, and differentiation. Defects in GPCR signalling are the cause for several diseases (Spiegel 1996, Farrel et al. 1999). On the other hand, about 30% of commonly prescribed medicines work through GPCRs (Wise et al. 2002). Nevertheless, only 10% of GPCRs are currently targeted by these medicines, emphasizing the potential of the remaining 90% of the GPCR superfamily for the treatment of human disease (Vassilatis et al. 2003).

2.2.1. Structure of G protein-coupled receptors

GPCRs have several common structural features (Figure 1). The receptors have an extracellular N-terminus, seven membrane-spanning transmembrane (TM) domains connected by extra- and intracellular loops, and an intracellular C-terminus. Based on the structural features of the GPCRs this receptor group is also called seven-transmembrane or serpentine receptors. The existence of seven hydrophobic TM alpha helices was anticipated with secondary structure predictions and hydrophobicity plots long before the three-dimensional receptor structure was known (Donnelly et al. 1989). The receptors are classified into approximately 100 subfamilies according to sequence homology, ligand structure, and receptor function. To date, the crystal structure of only one prototypical GPCR, rhodopsin, has been resolved (Palczewski et al. 2000). Because of the difficulties in obtaining good crystals of membrane proteins, no other receptors have been crystallized and the structural modelling of other GPCRs relies on the rhodopsin template. The elucidation of the structure of rhodopsin was not easy. Several steps including projection structures of two-dimensional crystals were taken before the three-dimensional structure of rhodopsin in its inactive (ground state, dark) form was resolved (Schertler et al. 1993, Schertler and Hargrave 1995, Unger and Schertler 1995, Unger et al. 1997, Krebs et al. 1998, Palczewski et al. 2000 Teller et al. 2001). The three-dimensional structure of metarhodopsin II (light-activated state, bleached) has been resolved with molecular modelling studies only (Choi et al. 2002).
The extracellular N-terminus of the receptor may be glycosylated and varies in length from 7 to 595 amino acids. The seven TM helices form a barrel-like structure surrounding a hydrophilic pocket (Figure 2). Each helix is generally composed of 24 - 39 amino acids. They are connected by three extracellular loops and three intracellular loops (2 - 230 amino acids long). The intracellular C-terminus varies in size from 12 to 359 amino acids. A fourth intracellular loop is often formed when a cysteine in the C-terminus is anchored to the lipid membrane by palmitoylation. Many small ligands probably have their binding sites in the hydrophilic pocket, whereas many peptide hormones and glycoproteins bind to the extracellular domains (N-terminus and the loops) of the receptor. (Bockeert and Pin 1999) The C-terminus and other intracellular domains may be phosphorylated, with important consequences for receptor desensitization and internalization. A common feature in nearly all GPCRs is a disulphide bond between cysteines in the first and the second extracellular loop of the receptor. This disulphide bond is expected to constrain the loops and receptor, pulling the second extracellular loop over and blocking the extracellular opening of the hydrophilic pocket (Ji et al. 1998).
2.2.2. Variety of G protein-coupled receptors: the receptor classes

GPCRs act as receptors for a multitude of different signals (Vassilatis et al. 2003). Chemosensory GPCRs are receptors for sensory signals of external origin that are sensed as odors, pheromones or tastes (Mombaerts 1999). Most other GPCRs respond to endogenous signals such as hormones, peptides, lipids, neurotransmitters or nucleotides (Ji et al. 1998). Not only members of the animal kingdom use the seven-transmembrane receptor system for signal transduction. Members of this receptor family have been found in plants (Arabidopsis thaliana, Plakidou-Dymock et al. 1998), yeast (Dohlman et al. 1991), protozoa (slime mold Dictyostelium discoideum, Devreotes 1994), and the earliest diploblastic metazoa (jellyfish, sea anemones, New et al. 2000).

Various classifications have been used to characterize the GPCRs. The first systems grouped the receptors according to the ligands they bind (e.g. muscarinic receptors, adrenergic receptors). The elucidation of the primary sequences of the receptors allowed classification according to structural features. For several years, the most frequently used classification system used receptor families A, B, C, D, E, and F, which covered all organisms (Kolakowski 1994). Receptors from all families do not exist in humans or other vertebrates; for example family E contains only slime mold cAMP receptors. A novel classification system called GRAFS groups human GPCRs into five main families (Fredriksson et al. 2003). These families are G (glutamate), R (rhodopsin), A (adhesion), F (frizzled/taste2), and S (secretin) (Table 2.). The rhodopsin family is by far the largest, with at least 701 members. Some examples of receptor subfamilies from each group are listed in Table 2.
Review of the literature

**Table 2.** G protein-coupled receptor families according to the GRAFS classification system (Fredriksson et al. 2003). The rhodopsin family is divided into four main groups (α, β, γ, and δ).

<table>
<thead>
<tr>
<th>Receptor family</th>
<th>Examples of receptor subfamilies</th>
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<tbody>
<tr>
<td>G - glutamate</td>
<td>metabotropic glutamate receptors&lt;br&gt; (15 receptors)&lt;br&gt;GABA&lt;sub&gt;3&lt;/sub&gt; receptors&lt;br&gt;calcium-sensing receptor&lt;br&gt;TAS1 taste receptors</td>
</tr>
<tr>
<td>R - rhodopsin</td>
<td>α (89 receptors)&lt;br&gt;prostaglandin receptors&lt;br&gt;amine (e.g., adrenergic receptors, muscarinic receptors...)&lt;br&gt;opsin receptors (rhodopsin, opsins, melanopsin...)&lt;br&gt;melatonin receptors&lt;br&gt;melanocortin receptors, cannabinoid receptors, adenosine receptors&lt;br&gt;β (35 receptors)&lt;br&gt;gonadotropin-releasing hormone receptors&lt;br&gt;neuropeptide Y (NPY) receptors&lt;br&gt;oxytocin receptor&lt;br&gt;cholecystokinin (CCK) receptors&lt;br&gt;thyrotropin-releasing hormone (TRH) receptor&lt;br&gt;γ (59 receptors)&lt;br&gt;somatostatin receptors&lt;br&gt;opioid receptors&lt;br&gt;melanin-concentrating hormone (MCH) receptors&lt;br&gt;chemokine receptors&lt;br&gt;angiopeptin receptors&lt;br&gt;bradykinin receptors&lt;br&gt;δ (58 receptors, plus approximately 460 olfactory receptors)&lt;br&gt;MAS oncogene receptors&lt;br&gt;follicle stimulating hormone (FSH) receptors&lt;br&gt;thyroid-stimulating hormone (TSH) receptors&lt;br&gt;lutinizing hormone (LH) receptors&lt;br&gt;purine receptors&lt;br&gt;thrombin receptors</td>
</tr>
<tr>
<td>A - adhesion</td>
<td>LGR-like module containing receptors&lt;br&gt;(24 receptors)&lt;br&gt;brain-specific angiogenesis inhibitor (BAI) receptors&lt;br&gt;lectomedin receptors&lt;br&gt;cadherin EGF LAG receptors</td>
</tr>
<tr>
<td>F - frizzled/taste2</td>
<td>frizzled receptors&lt;br&gt;(24 receptors)&lt;br&gt;TAS2 taste receptors</td>
</tr>
<tr>
<td>S - secretin</td>
<td>secretin receptors&lt;br&gt;(15 receptors)&lt;br&gt;vasoactive intestinal peptide (VIP) receptor&lt;br&gt;calcitonin receptor&lt;br&gt;parathyroid hormone (PTH) receptors&lt;br&gt;corticotropin releasing factor (CRF) receptors&lt;br&gt;growth hormone-releasing hormone (GHRH) receptor&lt;br&gt;pituitary adenylate cyclase activating polypeptide (PACAP) receptors</td>
</tr>
</tbody>
</table>

Orphan receptors are receptors that have been cloned but are still without any known ligand or function. At the moment there are about 140 human orphan receptors (Vassilatis et al. 2003).
Most of the orphan receptors can be classified to be members of one of the GPCR subfamilies based on structural similarities (Fredriksson et al. 2003).

2.2.3. Mechanisms of G protein-coupled receptor signal transduction

2.2.3.1. Receptor activation

The information obtained from the extensively studied activation process of rhodopsin provides valuable insight into the activation processes of other GPCRs. The change from the inactive to the active conformation in rhodopsin molecule is associated to a change in the relative orientation of TM III and TM VI, which unmask G protein binding sites (Bockaert and Pin 1999, Dunham and Farrens 1999). It seems that several receptor conformations are capable of exposing crucial intracellular domains to activate G proteins (Kenakin 2003). The activation process of the rhodopsin molecule includes a 30° clockwise rotation of TM VI and its movement away from TM III. The conformations of the second and the third intracellular loops, directly linked to TM III and TM VI, are also changed. These intracellular loops constitute one of the key sites of G protein recognition and activation (Bockaert and Pin 1999, Yamashita et al. 2000). In rhodopsin like GPCRs, a conserved D R Y or E R Y tripeptide at the end of TM III and a conserved aspartic acid are important for receptor activation (Gether and Kobila 1998). It has been proposed that the C-terminal end (last 4-5 residues) of the G protein α-subunit binds in a pocket constituted by these intracellular loops in the various GPCR families.

The classical view of receptor activation (Figure 3) describes agonist (A) binding to an inactive receptor (R) to form a complex (AR) that, because of the efficacy of the agonist, internalizes the receptor to the active conformation (AR*). Because GPCR signalling contains an essential third partner, namely the G protein, an improved model, called the ternary complex model, was developed (De Lean et al. 1980). In this model (Figure 3), the activation of the receptor is followed by binding of the receptor in active conformation (AR*) to the G protein (G). The ternary complex (AR*G) then evokes the functional response. It was later realized that the theoretical framework of the ternary complex model was not able to explain some experimental findings, and a modified version of the ternary complex model was developed (Samama et al. 1993). The extended ternary complex model allows for the spontaneous formation of a receptor in active conformation independent of the presence of an agonist (R*) (Figure 3). Activation of the receptor either spontaneously or through ligand binding modifies the affinity of the receptor for the G protein. It is this modified affinity of the receptor for the G protein that is the expression of efficacy. In a further extension of the model, the cubic ternary complex model, both the inactive and active conformations of the receptor can couple to G protein (Weiss et al. 1996, Figure 3). The coupling of the receptor in
inactive conformation with the G protein forms a non-signalling complex (AR*G), which can be relevant in some receptor systems (Kenakin 1999, Kenakin 2003).

**Figure 3.** Models for GPCR systems. A = agonist; R = receptor in inactive conformation; R* = receptor in active conformation; G = G protein. (Modified from Kenakin 2002)

According to current opinion, receptors are flexible, constantly oscillating molecules, which exist in numerous conformations (Kenakin 2002, Kenakin 2003). Some of these conformations are 'active', meaning that they are capable of producing a pharmacological effect. Ligands selectively stabilize the conformations for which they have the highest affinity. Different receptor conformations have varying affinity for different G proteins and are capable of initiating different signal transduction pathways (Kenakin 2003). Other receptor conformations may, in addition, promote receptor desensitization, internalization or interaction with other membrane proteins (Brady and Limbird 2002). This means that ligands that bind to a certain receptor and stabilize different receptor conformations can also induce different events in the cell.

Ligands that reversibly bind to GPCRs were classically thought to either possess efficacy (cause an effect, agonists) or to lack efficacy (antagonists). The intrinsic activity of full
agonists is defined as 1; drugs that belong to this class evoke a maximal functional response (100%) in a given system (Figure 4). Neutral antagonists have intrinsic activity equal to 0, and partial agonists have intrinsic activity that ranges between 0 and 1 (De Lean et al. 1980, Figure 4). In the recent years, along with the discovery of constitutively active receptor systems and increased understanding of GPCR behaviour in the cells, the classical view has had to be changed. Constitutively active GPCRs spontaneously adopt active conformations and activate G proteins in the absence of agonists (Parnot ct al. 2002). In such a system, ligands (agonists) that have negative intrinsic activity are called inverse agonists. A full inverse agonist would theoretically have its intrinsic activity defined as -1 (De Lean et al. 1980, Figure 4). In systems without spontaneous receptor activity an inverse agonist appears to behave like a neutral antagonist. It seems that true neutral antagonists hardly exist and most antagonists are actually very weak partial agonists or inverse agonists. A true neutral antagonist would need to have equal affinity for all various receptor conformations, whereas most drugs prefer certain receptor conformations and possess at least some efficacy, either positive or negative (Kenakin 2002).

![Figure 4](image)

**Figure 4.** Theoretical dose-response curves for different types of receptor ligands in a system where constitutive receptor activation exists. Intrinsic activities in this figure are set to 1 (full agonist, functional response 100%), 0.5 (partial agonist), 0 (neutral antagonist), -0.5 (partial inverse agonist) and -1 (full inverse agonist, functional response -100%). Ligand concentrations are arbitrary values.

### 2.2.3.2. G protein activation cycle

For signal transduction across the plasma membrane, three components are classically required; the receptor itself, the G protein, and an effector. The latter is typically an enzyme or an ion channel. The receptors and effectors are transmembrane glycoproteins whereas heterotrimeric G proteins are associated with the cytoplasmic side of the plasma membrane. Conformational changes follow agonist binding and the activation of a GPCR. These changes act as a switch to relay the signal to G proteins that in turn evoke further intracellular responses. Some intracellular regions of the receptor, including the transmembrane domain III
/intracellular loop 2 interphase and the third intracellular loop, are considered to be important for receptor/G protein interaction (Bockaert and Pin 1999).

The first step after receptor activation that is accompanied by structural changes in the receptor is the activation of a G protein. G proteins interacting with GPCRs are often called heterotrimeric G proteins, because they are composed of three distinct subunits, alpha (α), beta (β), and gamma (γ). Under physiological conditions, the βγ subunit acts as a heterodimer, and the subunits β and γ can be dissociated from each other only in denaturing conditions. In its inactive state, the guanine nucleotide-binding site of the G protein alpha subunit is occupied by GDP (guanosine diphosphate). When activated by a receptor, the G protein exchanges GDP for GTP (guanosine triphosphate), present in the cell in higher concentrations than GDP, and the Gα subunit dissociates from the Gβγ subunit. Both dissociated subunits can have separate effects on effectors inside the cell. The Gα subunit has an intrinsic GTPase activity; this means it hydrolyses GTP, converting it to GDP. This event is accompanied by the re-association of the Gα and Gβγ subunits, which returns the G protein into its inactive state. The cycle will begin again when a receptor again activates the G protein (Hamm 1998).

<table>
<thead>
<tr>
<th>Gα family</th>
<th>Members of the family</th>
<th>Effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>α1</td>
<td>Adenylate cyclase ↑</td>
</tr>
<tr>
<td></td>
<td>αoff</td>
<td>Adenylate cyclase ↑</td>
</tr>
<tr>
<td>αi</td>
<td>αi</td>
<td>Adenylate cyclase ↓</td>
</tr>
<tr>
<td></td>
<td>α4</td>
<td>cGMP phosphodiesterase ↑</td>
</tr>
<tr>
<td></td>
<td>α5</td>
<td>Adenylate cyclase ↓</td>
</tr>
<tr>
<td></td>
<td>αartic</td>
<td>cGMP phosphodiesterase ↑</td>
</tr>
<tr>
<td></td>
<td>αs</td>
<td>Adenylate cyclase ↓</td>
</tr>
<tr>
<td>αq</td>
<td>αq</td>
<td>Phospholipase C ↑</td>
</tr>
<tr>
<td></td>
<td>αq1</td>
<td>Phospholipase C ↑</td>
</tr>
<tr>
<td></td>
<td>αq15, 16</td>
<td>Phospholipase C ↑</td>
</tr>
<tr>
<td>α12</td>
<td>α12, 15</td>
<td>Na+/H+ exchanger ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c-jun amino-terminal kinase ↑</td>
</tr>
</tbody>
</table>

αoff = olfactory Gα; αi = α-transducin (visual Gα); αartic = α-gustducin (taste-specific Gα); cGMP = cyclic guanosine monophosphate; c-jun = one of the members of the AP-1 transcription factor family. Modified from Hamm and Gilchrist 1996 and Neves et al 2002.

G proteins are classified according to their Gα subunit. Each group of α subunits affects distinct second messenger systems (Table 3). GPCRs are sometimes able to couple to several
different G proteins, which mediate different cellular responses. The multistep signalling cascade also enables signal amplification: One receptor is able to activate dozens of G proteins, each of which in turn activates several effectors, each of which produces numerous second messenger molecules (Lodish et al. 1995). To date, more than 20 α, 6 β, and 12 γ G protein subunits are described in the literature, resulting in dozens of possible combinations of the heterotrimeric G proteins. To make the signal transduction network even more complicated, the Gβγ subunit can in many cases also act as a transducer to activate or inhibit effectors (Hamm 1998).

Along with the recent discoveries of various regulators of G proteins and the GPCRs, it has become clear that GPCR signalling is far from simple. Activated receptors must be inactivated and the signal terminated in a timely manner for the cell to function properly. Receptor signalling is constantly regulated on several levels in order to maintain optimal sensitivity. Desensitization and resensitization processes adjust the availability of functional receptors all the time (Pierce et al. 2002). A diverse family of proteins, called regulators of G protein signalling (RGSs) acts as GTPase-activating proteins (GAPs) attenuating GPCR signalling by enhancing the GTPase activity of Gα subunits (Neubig and Siderovski 2002). Activators of G protein signalling (AGS) are proteins that have potential to activate G protein signalling systems in the absence of classical GPCR (Blumer and Lanier 2003). The components of signalling pathways are often clustered into microdomains in the cell membrane. The closeness of the signalling components in such domains facilitates rapid signal initiation and provides specificity of signalling, since excluding certain components from these domains can inhibit signal initiation (Insel 2003). Traditionally, the GPCRs have been thought to function as monomers. This view has changed, and there are several convincing examples of GPCRs function as dimers (White et al. 1998, Li et al. 2002). Even the possibility that oligomeric receptors could exist has to be taken into account. On the other hand, many (if not most) GPCRs most likely function as monomers (Milligan 2001). Moreover, an increasing number of studies report that several other proteins can also interact with GPCRs either directly or via scaffolding proteins (Milligan and White 2001).

### 2.3. Melatonin receptors

#### 2.3.1. Melatonin

Over three centuries ago, the French philosopher René Descartes (1596-1650) described the pineal gland as "the seat of the soul". In 1958, the major hormonal product of the pineal gland was identified (Lerner et al. 1958, Lerner et al. 1959) and was given the name melatonin (mel from melatin and tonin meaning "to contract") because of its blanching effect on frog _Xenopus laevis_ dermal melanophores (Lerner et al. 1958, von Gall et al. 2002). Melatonin is secreted during the hours of darkness to regulate the biological rhythms of various endocrine and non-
endocrine tissues. The nocturnal surge in pineal melatonin production gives information about the prevailing light/dark conditions to the rest of the body. Melatonin is also reported to have hypnotic actions and to have a role in sleep initiation as the trigger for opening the circadian "sleep gate" (von Gall et al. 2002, Arendt 2003). Other actions of melatonin include enhancement of the immune system function, vasoregulatory activity, inhibition of dopamine release from retina, and effects on cell growth (reviewed by Morgan 1994, Vanceck 1998, Borjigin et al. 1999, von Gall et al. 2002). Melatonin treatment may be useful in synchronization of disturbed circadian rhythms in jet lag, in blind persons, or in persons with sleep disorders (Cajochen et al. 2003). Melatonin might also have therapeutic potential in cancer therapy (Vijayalaxmi et al. 2002), in the treatment of seasonal affective disorder (SAD, Leppäniemi et al. 2003), and as an enhancer of immune function (Maestroni 1998).

![Chemical structure of melatonin](image)

**Figure 5.** The chemical structure of melatonin (chemical names N-acetyl-5-methoxytryptamine and N-[2-(5-methoxy-1H-indol-3-yl)ethyl]acetamide).

The synthesis of melatonin is regulated through the master biological clock in the hypothalamic-suprachiasmatic nuclei (SCN), which is synchronized by the daily light-dark cycle with light signalling through a direct retinal pathway to the SCN (Reiter 1991). The clock signals through a multisynaptic neural pathway to the pineal gland to regulate the activity of the enzymes which convert serotonin (5-hydroxytryptamine) into melatonin (N-acetyl-5-methoxytryptamine, Figure 5). The circadian rhythm of the enzyme activities creates the circadian rhythm of melatonin production. Melatonin is also synthesized in the retina and possibly in some other extrapineal sites of some species. The pineal gland seems to be the only source of circulating melatonin, whereas melatonin synthesized in the retina acts in a local fashion to regulate various dark adaptive functions (Cahill and Besharse 1995, Vanceck 1998).

### 2.3.2. Melatonin receptors belong to the G protein-coupled receptor family

Melatonin is a small, relatively lipophilic molecule that is thought to easily penetrate cell membranes. Despite this and the notion that specific binding of melatonin can be found in the nuclear fraction of some animal tissues (Acuna-Castroviejo et al. 1994), many of the established effects of physiological concentrations of melatonin have been shown to be mediated via high-affinity cell membrane receptors which form a subfamily within the family of rhodopsin-like receptors.
GPCRs (Kokkola and Laitinen 1998). Membrane-associated melatonin receptors have been found in all animals investigated so far. The cloning of several G protein-coupled melatonin receptor genes has revealed that three melatonin receptor subtypes exist, two of which have been found in mammals. The mammalian melatonin receptor subtypes are currently termed MT1 (previous names Mel₁₆ or ML₁₆(A)) and MT2 (previous names Mel₁₈ or ML₁₈(B)) (Reppert et al. 1994, Reppert et al. 1995a) according to the recommendation by IUPHAR (Dubocovich et al. 1998). A third melatonin receptor subtype, Mel₁₅c, has been found to exist in amphibians, fish and birds, but not in mammals (Ebisawa et al. 1994, Reppert et al. 1995b). The diversity in subtype nomenclature arises from the fact that IUPHAR classifies and names only mammalian receptors for which the receptor protein in native tissues and the genes are known, thus necessitating the use of the older nomenclature for the non-mammalian Mel₁₅c receptor (Hunt et al. 2001, von Gall et al. 2002).

2.3.3. Primary structure of melatonin receptors

Melatonin receptors share some common features with other GPCRs. They consist of a single polypeptide chain, which has seven TM domains connected by intra- and extracellular loops, an extracellular N-terminus, and an intracellular C-terminus plus a few amino acid residues conserved throughout the superfamily. On the other hand, certain fingerprints clearly distinguish the melatonin receptors from other members of the rhodopsin-like GPCRs. At the end of TM III, melatonin receptors have a N-R-Y motif instead of D-R-Y (or E-R-Y) present in other receptors. In other rhodopsin-like GPCRs, this region is thought to be important for the interaction with G proteins. A highly conserved N-P-x-x-Y motif (x corresponds to a non-conserved amino acid) that is found in TM VII of nearly all the other GPCRs is replaced by N-A-x-x-Y in melatonin receptors. The human melatonin receptor subtypes MT1 and MT2 show about 60% amino acid identity when the whole receptor molecules are compared. If only TM domains are compared, the amino acid identity of the subtypes increases to 73%. The length of the intra- and extracellular loops is also exceptionally well conserved; only the length of the third intracellular loop shows slight variation. The melatonin receptor proteins are 346 - 420 amino acids long with calculated molecular weights of 39 - 47 kDa. The receptors have 1 - 2 potential glycosylation sites in their N-terminus and several potential protein kinase C phosphorylation sites, which may participate in the regulation of receptor function (Kokkola and Laitinen 1998).

2.3.4. Melatonin receptor genes

Full-length MT1 receptors have been cloned from human, mouse, sheep, Djungarian hamster, chicken, and rainbow trout (Reppert et al. 1994, Reppert et al. 1995b, Roca et al. 1996, Barrett et al. 1997, Mazurais et al. 1999). MT1 receptor fragments have been cloned from capuchin monkey, rat, rabbit, golden hamster, the diurnal rodent Arvicantbus unguiculatus, pig, cow, goat, elephant, the frog Xenopus laevis, and zebrafish (Reppert et al. 1994, Reppert et al. 1995b,
Messer et al. 1997, Migaud et al 2002, Torres-Farfan et al. 2003). At least two different MT1 receptors exist in the zebrafish (Reppert et al. 1995b). Some investigators have suggested the *Xenopus laevis* melatonin receptor fragment X2.0 to represent a putative new subtype, Mel1a (Shiu et al. 1998), but it is usually classified as the Mel1a (MT1) subtype (Reppert et al. 1996b). The MT1 receptor subtype is likely to mediate some circadian and reproductive responses to melatonin in mammals, as it is expressed in mammalian SCN and pars tuberalis of the pituitary gland (Reppert et al. 1994), and as hamsters naturally lacking MT2, the other functional melatonin receptor, show normal seasonal reproductive and circadian responses to melatonin (Weaver et al. 1996). Human MT1 mRNA is detectable in the whole brain, SCN, and retina but not in the pituitary, liver, spleen or white blood cells (Reppert et al. 1995a, Weaver and Reppert 1996, Mazzucchelli et al. 1996, Thomas et al. 2002). As several human brain areas were mapped for MT1 mRNA expression, the following order of abundance was established: cerebellum > occipital cortex > temporal cortex > thalamus > frontal cortex > hippocampus (Mazzucchelli et al. 1996, Al-Ghoul et al. 1998). The MT1 receptor is also found in human and rat cerebral arteries (Savaskan et al. 2001, Chucharoen et al. 2003) and rat caudal arteries (Masana et al. 2002), human myometrium (Schlabritz-Loutsevitch et al. 2003), and ovaries of various species (Niles et al. 1999, Soares et al. 2003). In rat, mouse, hamster, and sheep, pars tuberalis of the pituitary gland expresses MT1 receptor (Hazerigg 2001, Schuster et al. 2001, Poirel et al. 2003). Spleen, adipose tissue, adrenal gland, testes, and kidney are also sites of MT1 expression (Drew et al. 1998, Li et al. 1998, Peschke et al. 2000, Brydon et al. 2001, Slominski et al. 2003, Torres-Farfan et al. 2003).

A full-length MT2 receptor has been cloned from human, rat, mouse, and pike (Reppert et al. 1995a, Jin et al. 2003). Receptor fragments have also been cloned from rat, mouse, Campbell’s hamster, golden hamster, Djungarian hamster, pig, rhesus monkey, capuchin monkey, chicken, and *Xenopus laevis* (Reppert et al. 1994, Liu et al. 1995, Reppert et al. 1995a, Reppert et al. 1995b, Weaver et al. 1996, Torres-Farfan et al. 2003). Furthermore, two different MT2 receptor fragments have been cloned from zebrafish (Reppert et al. 1995b). The MT2 receptor genes of all hamsters were found to contain stop codons, preventing the production of functional full-length MT2 receptors in these species (Weaver et al. 1996). The mRNA of human MT2 has been detected in retina, cerebellum, whole brain, and hippocampus, but not in the SCN, liver, or spleen (Weaver and Reppert 1996, Reppert et al. 1995a, Al-Ghoul et al. 1998). A truncated and most likely non-functional MT2 receptor isoform was found to be expressed in human pituitary (Slominski et al. 2003). Like MT1, MT2 receptor is also found in rat caudal arteries and testes, human myometrium plus adipose tissue, ovaries, and kidney (Drew et al. 1998, Niles et al. 1999, Brydon et al. 2001, Masana et al. 2002, Schlabritz-Loutsevitch et al. 2003, Soares et al. 2003).

Mel1c receptor has only been found in nonmammalian vertebrates; the full-length receptors have been cloned from chicken and *Xenopus laevis*, and a receptor fragment has been cloned from zebrafish (Ebisawa et al. 1994, Reppert et al. 1995b, Kokkola et al. 1997, Jockers et al. 1997). A total of five Mel1c isoforms have been cloned from *Xenopus laevis* (Ebisawa et al. 1994, Jockers
et al. 1997). Based on exhaustive analysis of GPCRs in the human genome, no human counterpart of Mel1c receptor was found to exist (Fredriksson et al. 2003).

A full length orphan receptor closely related to melatonin receptors has been cloned from human, mouse, and sheep, and a fragment of this receptor has been cloned from rat (Reppert et al. 1996a, Drew et al. 1998, Gubitz and Reppert 1999, Drew et al. 2001). This melatonin-related receptor, GPR50 (Gubitz and Reppert 1999), is approximately 45% identical to melatonin receptors but it does not bind $^{125}$I-Mel or related compounds. Its mRNA is expressed in hypothalamus, pituitary, and retina plus several peripheral tissues, suggesting that the encoded receptor and its natural, as yet unknown, ligand could be involved in neuroendocrine regulation and the coordination of physiological responses in the central nervous system and peripheral tissues (Reppert et al. 1996a, Drew et al. 1998, Drew et al. 2001).

In every cloned melatonin receptor gene, the coding region is composed of two exons and a long (> 2-13 kb) intron. The intron splice site is located in the beginning of TM II in the codons encoding for amino acids GN in every melatonin receptor and also in the melatonin receptor-related orphan receptor (Kokkola and Lahtinen 1998, Reppert et al. 1996a). The presence of an intron points to the possibility that alternative splicing of melatonin receptors could occur, creating receptors with altered structure and function. However, there is no proof that splice variants of melatonin receptors exist. One recent study indicates defects in splicing of M12 expressed in the human pituitary (Slominski et al. 2003). The deficient M12 receptor mRNA contains an insertion from the intron and a deletion from exon 2, creating a truncated and most likely non-functional receptor (Slominski et al. 2003).

Several genetic polymorphisms in the human MT1 receptor have been identified (Ebisawa et al. 1999). Two of these mutations resulted in changes in amino acid sequence of the receptor. One polymorphic mutation (Arg 54 → Trp) in the first intracellular loop of MT1 reduced the number of binding sites and slightly enhanced binding affinity of the receptor (Ebisawa et al. 1999). Two polymorphic mutations in the MT2 receptor changed the amino acid sequence of the receptor, but these mutations were without effect on the receptor binding characteristics (Ebisawa et al. 2000). None of the polymorphisms in MT1 and MT2 receptors were found to be associated with sleep disorders in the study populations involved (Ebisawa et al. 1999, Ebisawa et al. 2000). Polymorphisms of melatonin receptors have also been found to occur in sheep (Barrett et al. 1997, Messer et al. 1997, Pelletier et al. 2000, Notter et al. 2002), goat (Migaud et al. 2002), chicken (Kokkola et al. 1997), and Xenopus laevis (Brydon et al. 1999).

Melatonin receptors investigated up to now are all single copy genes. MT1 maps to human chromosome 4q35.1 whereas MT2 maps to human chromosome 11q21-22, implicating that MT1 and MT2 evolved by more complicated methods than a simple tandem duplication of an ancestral gene (Reppert et al. 1995a, Slaugenheupt et al. 1995). No genetic diseases that might involve mutated melatonin receptors have yet been mapped to these areas in human.
chromosomes (Reppert et al. 1995a). The melatonin-related receptor (GPR50) maps to human chromosome Xq28 (Gubits and Reppert 1999).

2.3.5. Melatonin receptor signal transduction

The nocturnal peak of melatonin secretion from the pineal gland persists for hours and its length changes with the season (Rettew 1991). In temperate latitudes the length of the night varies from just a few hours during the summer to 16 hours and even more during the winter. Melatonin receptors and associated regulated pathways must play a key role in conveying the duration of the melatonin signal to a biological process that regulates seasonal physiology (Barrett et al. 2003). Melatonin receptors are exposed daily to melatonin for prolonged periods of time and, as known from other receptor systems, regulation of receptors often occurs following prolonged agonist exposure (Lefkowitz 1998). The density and affinity of endogenous melatonin receptors changes throughout the 24-hour day, and also following melatonin exposure (Laitinen et al. 1989, Tenn and Niles 1993, Morgan et al. 1994, Vaneeck 1998). Prolonged exposure of recombinant MT1 and MT2 receptors to melatonin results in desensitization of the receptors (Witt-Enderby et al. 1998, MacKenzie et al. 2002).

Mice with targeted disruption (knock-out) of single or both melatonin receptor subtypes are viable and fertile and do not have abnormalities in circadian functions (Liu et al 1997, Jin et al. 2003). The knock-out studies have revealed that MT1 is necessary for the suppressive action of melatonin on SCN neuronal firing, as targeted disruption of the MT2 receptor did not alter this response to melatonin (Jin et al. 2003). MT1 was found to account for all melatonin receptor binding detected in mouse brain by in vitro autoradiography (Liu et al 1997). A natural knock-out of the MT2 receptor has occurred in golden, Djungarian, and Campbell’s hamsters which have retained normal seasonal reproductive and circadian responses to melatonin (Weaver et al. 1996). This indicates that MT1, which is the only functional melatonin receptor in hamsters, mediates these effects in these animals. In patch-clamp experiments of rat SCN neurons and HEK-293 cells transfected with MT1 receptors, melatonin potentiated GABA_A receptor function, whereas the activation of MT2 receptors had an opposite effect on GABA_A receptors (Wan et al. 1999).

Recombinant melatonin receptors couple to G proteins to inhibit cyclic AMP accumulation (von Gall et al. 2002). Under heterologous expression, the MT1 receptor inhibits cAMP synthesis through pertussis toxin sensitive G_s and G_o proteins and stimulates phospholipase C activity through G_{q,11} proteins. The activation of phospholipase C increases inositol phosphate accumulation and cytosolic calcium concentration (Godson and Reppert 1997; Brydon et al. 1999, Roka et al. 1999). The MT2 receptor also couples to inhibition of cAMP synthesis and stimulation of phosphoinositide hydrolysis (MacKenzie et al. 2002). Unlike
MT1, MT2 activation also inhibits cyclic GMP levels via the soluble guanylyl cyclase pathway (Brydon et al. 1999, Petit et al. 1999).

2.3.6. Melatonin receptor pharmacology

Two agonist radioligands have been used in melatonin receptor research. Truncated melatonin, which was used in earlier studies, has been replaced by 2-[125I]-iodomelatonin ([125I]-Mel, Vakkuri et al. 1984). The use of [125I]-Mel with its higher specific activity and higher affinity for melatonin receptors has facilitated closer identification and localization of melatonin binding sites (Kokkola and Laitinen 1998). All recombinant melatonin receptor subtypes bind [125I]-Mel with high affinity. The MT1 and Mel1c receptor subtypes both have affinities (Kd) for [125I]-Mel of ~20-60 pM and are pharmacologically identical. MT2 has a somewhat lower affinity for [125I]-Mel (Kd ~160 pM) (Reppert et al. 1996b). Melatonin receptor research is hampered by the lack of high-affinity antagonist radioligands. Due to its agonist nature, [125I]-Mel binds with high affinity only to melatonin receptors coupled to G proteins. If a receptor becomes uncoupled from its G protein, the agonist ligand will bind its receptor with low affinity (Witt-Enzerphy and Li 2000). Instead of a real decrease in receptor number, the decrease in [125I]-Mel binding observed in some situations can be caused by a loss of affinity for the radioligand due to receptor / G protein uncoupling.

The pharmacological profile for the "traditional" melatonin receptor ligands is indistinguishable in MT1 and Mel1c receptors, and MT2 receptors only differ in that 2-iodomelatonin, melatonin, and 6-chloromelatonin have equal affinity for this receptor (Reppert et al. 1996b, see Table 4.). There are a few ligands that display selectivity for MT2 receptors, but no MT1-selective compounds have been reported in the literature until very recently (Audinot et al. 2003). MT2-selective ligands belong to different structural classes, displaying different degrees of binding affinity and selectivity (Rivara et al. 2003). The MT2 receptor is characterized by a higher steric tolerance than MT1, and it probably has an additional pocket in the ligand binding site (Rivara et al. 2003). The pocket is positioned out of the plane of the aromatic nucleus of the ligand (indole ring in melatonin) and its occupancy is beneficial for the binding affinity of the ligand for the MT2 receptor. At the same time, the occupancy of this pocket in MT2 receptor lowers the intrinsic activity of the compounds (Rivara et al. 2003). Replacing the 5-methoxy group of melatonin with a bulkier substituent at this position favours MT1-selectivity, while MT2-selectivity is obtained by the presence of bulky substituents in the position 2 or 1. in the vicinity of the acylaminoethyl side chain (Audinot et al. 2003, Rivara et al. 2003). Agonists for both subtypes with a more pronounced selectivity ratio (1000 and beyond) have not been developed yet (Audinot et al. 2003).

A putative MT3 melatonin binding site (previously known as ML2) has a distinct pharmacological profile compared to other melatonin receptor subtypes (Molinari et al. 1996).
It binds $^{125}$I-Mel with nanomolar affinity. Instead of being a receptor, the MT3 site is quinone reductase 2, an enzyme involved in oxidoreductive detoxification (Nosjean et al. 2000, Nosjean et al. 2001).

**Table 4. Pharmacological characteristics of the mammalian melatonin binding sites**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>GPCR</th>
<th>Affinity for $^{125}$I-Mel</th>
<th>Rank order of ligand affinities</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT1</td>
<td>yes</td>
<td>20-60 pM</td>
<td>2-iodomelatonin &gt; melatonin &gt; 6-chloromelatonin &gt; 6-OH-melatonin &gt; luzindole &gt; NAS &gt;&gt; 5-MCA-NAT &gt;&gt; serotonin</td>
</tr>
<tr>
<td>MT2</td>
<td>yes</td>
<td>~160 pM</td>
<td>2-iodomelatonin = melatonin = 6-chloromelatonin &gt; 6-OH-melatonin &gt; luzindole &gt; NAS &gt; 5-MCA-NAT &gt;&gt; serotonin</td>
</tr>
<tr>
<td>MT3</td>
<td>no</td>
<td>1-4 nM</td>
<td>2-iodomelatonin = 6-chloromelatonin ≥ 5-MCA-NAT = NAS &gt; melatonin = 6-OH-melatonin &gt; luzindole &gt; serotonin</td>
</tr>
</tbody>
</table>

3. AIMS OF THE STUDY

The objective of the present study was to obtain more information about how melatonin binds and activates its G protein-coupled receptors. The practical work was carried out with the human MT1 receptor subtype. More specifically, the following aims had been defined:

- to construct a three-dimensional model of the melatonin binding pocket utilizing novel information about the primary structure of melatonin receptors and the structure of rhodopsin.

- to test the model of the melatonin binding pocket with site-directed mutagenesis and expression of the human MT1 receptor in yeast and mammalian expression systems.

- to study the role of particular conserved amino acids in the human MT1 receptor in constitutive activation and other functional properties.

- to study the role of conserved cysteines at the intracellular surface of the human MT1 receptor in receptor function.
4. MATERIALS AND METHODS

4.1. Database mining and molecular modelling

4.1.1. CoMFA (I)

Receptor protein sequences were extracted from the Swiss-Prot data bank (http://www.ebi.ac.uk/swissprot/index.html) and aligned using the Genetics Computer Group sequence analysis software (GCG, USA). The consensus amino acid sequence for the melatonin receptor was formed from melatonin receptor multiple sequence alignment using the GCG software. The structures of melatonin and melatonin analogues were calculated using the MOPAC program package (Schrödinger, USA) with Austin model 1 (AM1) parametrization (Stewart 1990), and the lowest energy conformations were used in the CoMFA. The inverse logarithm of the relative affinity of melatonin analogues in radioligand binding assays was used as the dependent variable in CoMFA. The three-dimensional quantitative structure-activity relationship analyses with CoMFA (Cramer et al. 1988) were done using the SYBYL modelling package (version 6.0, Tripos Inc., St. Louis, MO, USA). The consensus amino acid sequence for the melatonin receptor was aligned manually with a helical wheel model (Baldwin 1993) that was based on the projection structure of rhodopsin (Unger and Schertler 1995). The combined information from CoMFA maps and the helical wheel melatonin receptor model was used to predict the structure of the melatonin binding pocket.

4.1.2. Comparative modelling (IV)

For the model of the MT1 receptor protein (IV), the nucleotide sequence of the human melatonin receptor 1A (SWISS-PROT: ML1A_HUMAN [P48039]) was aligned with the sequence of bovine rhodopsin. The HOMOLOGY module of the InsightII modelling package (Accelrys, USA) was then used to assign the helix coordinates from the X-ray structure of bovine rhodopsin (Palczewski et al. 2000, Okada et al. 2002) to the receptor model and to search for the missing loops from a loop protein database. N- and C-termini were left out from the model. The energy of the resulting model was minimized in the SYBYL modelling package (version 6.9, Tripos Inc., St. Louis, MO, USA).

4.1.3. Amino acid numbering schemes

In study I, the numbering scheme was identical to the original helical wheel model (Baldwin 1993), where all TM helices consist of 26 amino acids, with TM helices numbered with Roman numerals and number I denoting the first amino acid in the helix. In MT1 mutagenesis studies (II, III, IV), amino acid numbering counts the residues starting from the
first residue in the natural human MT1 receptor or in the natural human melatonin-related receptor (GPR50). In studies III and IV, the numbering scheme where the most conserved amino acid in each helix is assigned the position index "50" (Ballesteros and Weinstein 1995) is shown for comparison.

4.2. DNA constructs

The human MT1 receptor cDNA (Reppert et al. 1994, GenBank accession number NM_005958) was subcloned into the cloning vector pcDNA3 (Invitrogen, USA). The mutations were generated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, USA). The location and identity of the mutated amino acids can be seen in Figure 6. For expression in the yeast Saccharomyces cerevisiae, the receptors were subcloned into the yeast expression plasmid pDTPGK (YEpDT-PGK, Kang et al. 1990). To allow for the detection of receptor protein in immunological assays, a triple hemagglutinin (HA) epitope tag was subcloned after the initiating Met codon of the receptors in pcDNA3. The human melatonin-related receptor (GPR50, GenBank accession number NM_004224) was also subcloned into pcDNA3, and mutated and tagged using the same protocol as with the MT1 receptor.

![Figure 6. Schematic illustration of the human WT MT1 receptor showing the seven transmembrane helices (I-VII). Melatonin receptor 'fingerprint' amino acid motifs are presented with the one-letter amino acid code. The 'fingerprints' include an N-R-Y motif in TM III, an N-A-x-x-Y motif in TM VII, and a disulphide bond between two cysteines in the second and the third extracellular loop. Amino acids mutated in studies forming this thesis are identified and shown as black circles.](image-url)
4.3. Cell culture, transformations and transfections

The yeast *Saccharomyces cerevisiae* strain SDY102 (MATa leu2 3,112 trp1 ure3 52 prc1 prb1 pep1 Δfar1::URA3 Δhis3::APT2 fas1::FUS1 HIS3 LEU2::FUS1 lacZ Δtec2::URA3 Δest2::URA3) was grown in yeast minimal medium with appropriate supplements. Yeast transformations were performed using the lithium acetate method (Gietz et al. 1992).

COS-7 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) and CHO cells in Ham's F-12 nutrient mixture. The media were supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin and the cells were cultured as monolayers at 37°C in a humidified atmosphere of 5% CO₂/95% air. For transient transfections, COS-7 cells were transfected with the DEAE-dextran method (Cullen 1987). Stable transfections of CHO cells were performed with Lipofectamine 2000 transfection reagent (Gibco, USA), and G-418 was used for selecting stable cell clones.

4.4. Preparation of cell membranes and lysates

COS-7 monolayers were harvested 72 h after transfections, and CHO cells were harvested from subconfluent cultures. For membrane preparation, the cell monolayers were washed with and harvested by scraping in PBS containing 5 mM EDTA, and the membrane fraction was isolated with differential centrifugation (Kurkinen et al. 1997). For the preparation of CHO cell lysates, the cells were harvested by trypsinization and the cells were pelleted by centrifugation for 10 min at 250 × g at room temperature. The cell pellets were washed with PBS and centrifuged as above twice, after which the dry pellets were snap-frozen on dry ice. The frozen pellets were thawed for 1 min in a water bath at room temperature, after which snap-freezing was repeated. The lysates were stored at -75°C until use. For ¹²⁵I-Mel binding assays, CHO cell monolayers were washed with and harvested by scraping in PBS containing 5 mM EDTA. The cell suspension was aliquoted and pelleted by centrifugation for 5 min at 1600 × g at 4°C. The cell pellets were resuspended in ¹²⁵I-Mel binding buffer and the resulting cell suspensions were stored at -75°C. Protein concentrations of the cell preparations were determined with the Bio-Rad protein assay (Bio-Rad, USA), using bovine serum albumin as a standard.

4.5. Immunological assays

4.5.1. Receptor ELISA

This indirect immunoassay was performed with slight modifications to a previously reported protocol (Schöneberg et al. 1995) to measure the expression levels of HA-tagged receptors. Cells growing as monolayers in 96-well plates were fixed with 4% paraformaldehyde,
permeabilized as necessary, and incubated with 10 µg/ml of mouse anti-HA monoclonal antibody (12CA5, Roche, Germany) followed by incubation with a 1:5000 dilution of peroxidase conjugated sheep anti mouse IgG antibody (Roche, Germany). The amount of HA tagged receptors was quantitated with a plate reader at 490 nm after colour development with o-phenylenediamine as substrate.

4.5.2. Immunofluorescence microscopy

Confocal immunofluorescence microscopy was employed to study the subcellular distribution of HA-tagged receptors (Schöneberg et al. 1995). The cells were grown on glass coverslips or chamber slides. After fixing with 4% paraformaldehyde, the cells were permeabilized, if necessary, and incubated with 10 µg/ml of mouse anti-HA monoclonal antibody (12CA5, Roche, Germany). The secondary antibody for COS-7 cells was fluorescein-conjugated sheep anti-mouse IgG (Roche, Germany) diluted 1:40, and for CHO cells Alexa Fluor® 488 -conjugated goat anti-mouse IgG (Molecular Probes, USA) diluted 1:200. The images were obtained with a confocal laser-scanning microscope.

4.5.3. Western blot

CHO cell lysates were resuspended in sample buffer (62.5 mM Tris-HCl (pH 6.8), 0.9% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.005% bromophenol blue) and boiled for 5 minutes. Samples of 50,000 cells (equivalent of approximately 5 µg of protein) were separated with 10% SDS-PAGE, transferred to nitrocellulose membrane and incubated with a 1:1000 dilution of mouse anti-HA monoclonal primary antibody (12CA5, Roche, Germany). After incubation with a 1:10,000 dilution of horseradish peroxidase -conjugated goat antiaquouse IgG (Stressgen, Canada), the antibody-protein complexes were visualized by enhanced chemiluminescence (Western Lightning, Perkin Elmer, USA). The membranes were stripped with 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50°C, and then reprobed with mouse anti-β-actin antibody (Sigma, USA). Autoradiographs were scanned and quantitated, and the amount of HA-tagged proteins was normalized to β-actin protein levels.

4.6. Ligand binding

Saturation binding experiments were performed in binding buffer (50 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 1 mM EDTA) with 2.5 - 5 µg of protein in a total incubation volume of 250 µl. In competition experiments, 10 pM ¹²⁵I-Mel was used with or without competing drugs, and in saturation binding experiments the concentration range of ¹²⁵I-Mel was 7.5-300 pM. Nonspecific binding was determined in the presence of 1 µM melatonin. The reactions were incubated at 25°C for 90 min with constant shaking and terminated by the addition of 4 ml of
ice-cold wash buffer (50 mM Tris-Cl (pH 7.4)), followed by rapid filtration through Whatman GF/B glass fiber filters. After two more 4 ml washes, filters were placed in polypropylene tubes and the radioactivity was measured using a Wallac Rackgamma counter.

4.7. Functional assays

4.7.1. Yeast functional assay

The activation of human melatonin receptors in the yeast strain SDY102 transformed with melatonin receptor cDNA constructs results in the increase of β-galactosidase activity (*FUS1-lacZ* reporter gene) through the activation of the modified yeast mating pathway. The β-galactosidase activity was measured using a colorimetric assay with CPRG dye (chlorophenol red-β-d-galactopyranoside, Boehringer Mannheim, Germany). Yeast cells were incubated in 96-well plates in buffered minimal medium together with 100 µg/ml CPRG and various concentrations of melatonin receptor ligands. After an incubation period of 16 - 48 h at +30°C, the red colour was quantitated using a plate reader at 570 nm.

4.7.2. [35S]GTPγS binding assay

In these assays, 5 µg of cell membranes in a final assay volume of 400 µl were used. COS-7 cell membranes were kept on ice until the initiation of the binding reaction whereas CHO cell membranes were always preincubated for 30 min at room temperature in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 5 mM MgCl2, 10 µM GDP, and 0.5 U/ml ADA, under constant shaking and protected from light. For S-nitrosylation of the CHO cell membranes, nitrosoglutathione (GSNO) or nitrosocysteine (CysNO) were included in the preincubation with the final concentrations of 0.5 mM. The final concentrations of the components in the binding reactions were 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 5 mM MgCl2, 0.5 % BSA, 10 µM GDP, 0.5 U/ml ADA, and 150 pM [35S]GTPγS. Nonspecific binding was defined using 10 µM GTPγS. Reaction tubes were incubated for 90 min at 25°C under constant shaking. Reactions were quickly terminated by the addition of 4 ml ice-cold wash buffer (50 mM Tris-HCl, 5 mM MgCl2, pH 7.4) followed by rapid filtration through Whatman GF/B glass fiber filters and two additional 4 ml washes with the buffer. Radioactivity was measured using a Wallac Rackbeta liquid scintillation counter.

4.8. Data analyses

Ligand binding data were analyzed with GraphPad Prism software (GraphPad, USA) using non-linear regression analysis. Functional data were analyzed with GraphPad Prism using non-linear fitting for sigmoid dose-response curves. Statistical analyses were made with one-
way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. When comparison was made between only two groups, unpaired T-test was used.
5. RESULTS

5.1. Rhodopsin-based model of melatonin binding pocket

The CoMFA analysis of the binding affinity of melatonin analogues revealed three regions surrounding the ligand in which a decrease in negative density of the ligand increased affinity. These regions were near the substituent at position 6 of melatonin, over the bridge of two methylenes and the exocyclic nitrogen, and near the hydrocarbon substitution of the carbonyl group attached to the exocyclic nitrogen. CoMFA also indicated two regions in which the increase in negative density increased affinity. These regions were located near the carbonyl oxygen and near the methoxy group (I / Figure 1a). Increase in bulkiness near the methoxy group in position 5 and near the substituent in position 2 were also found to increase affinity of the ligands (I / Figure 1b).

The energetically minimized conformation of melatonin was found to be a tilted L-form (I / Figure 4a). There were some criteria that had to be fulfilled for melatonin binding at its receptor. The 5-methoxy group of melatonin had to be specifically recognized, since it is essential for high affinity for the melatonin receptor. The oxygen of the N-acetyl group of melatonin was also recognized specifically and this recognition took place at a distance of about 10.8 Å (1.08 nm) from the recognition site of the 5-methoxy group. An aromatic interaction between the receptor and the indole moiety of melatonin stabilized the docking of melatonin at its receptor. Because of the tilted L-conformation of melatonin, the 5-methoxy and N-acetyl groups were recognized in a plane that was clearly outside the plane of the aromatic interaction.

Based on the helical wheel model of the melatonin receptor, a model of the melatonin binding pocket was constructed, in which specific recognition of melatonin and its analogues was suggested to occur through amino acids in the TM V, VI, and VII. These amino acids were conserved in all melatonin receptors and they were all found to point towards the hydrophilic interior of the melatonin receptor. According to the model, the 5 methoxy group of melatonin would interact with TM V. The oxygen would form a hydrogen bond with a histidine (H V:10, H195, His546) in TM V and the 5-methoxy methyl group would have an aliphatic interaction with a valine (V V:7, V192, Val543), which is located approximately one helical turn above the histidine (I / Figure 5). The N-acetyl group of melatonin would point towards TM VII, where a conserved serine (S VII:6, S280, Ser228) would allow specific hydrogen bonding with the carbonyl oxygen, and an alanine (A VII:10, A284, Ala742), which is located below the serine, could be an additional anchor for the methyl group attached to the carbonyl group. An aromatic interaction could take place between the indole ring and the phenylalanine (F VI:9, F244, Phe641) in TM VI, which is at a proper distance and is conserved in melatonin receptors. Furthermore, an interaction between a methionine (M III:7, M107, Met117) in TM III and the nitrogen of the amide moiety in the N-acetyl group of melatonin was suggested to be possible (I / Figure 5).
5.2. Mutations based on the model

A total of seven mutant human MT1 melatonin receptors were constructed to address specific questions about the validity of the rhodopsin-based melatonin receptor model (I). The WT MT1 and the mutant receptors were expressed in the yeast *Saccharomyces cerevisiae* and in mammalian COS-7 cells. In the yeast, the receptors were tested for their ability to induce reporter gene *FUS1-lacZ* expression (β-galactosidase activity) in the colorimetric CPRG assay using four melatonin receptor agonists (melatonin, 2-iodomelatonin, 6-hydroxymelatonin, N-acetylserotonin). The rank order of agonist potencies was in good agreement with that previously reported for the MT1 receptor (II / Table 1, Dubocovich et al. 1997, see also Table 3). In COS-7 cells, the quantity, subcellular localization, ligand binding, and G protein activation of the receptors were assessed.

A methionine M107 (Met$^{332}$) in TM III was mutated into threonine (M107T) and into aspartate (M107D). The M107T mutant receptor could not be distinguished from the WT MT1 receptor in any aspect; the functional responses in the yeast and in mammalian cells were similar, so were the ligand binding characteristics and expression pattern in mammalian cells (II / Figure 2, III). The non-conservative M107D mutation had dramatic effects on ligand binding and receptor function. Melatonin was not able to evoke functional responses through the mutant receptor in the yeast or mammalian cells, and only the highest concentrations of 2-iodomelatonin were active in the yeast functional assays (II / Figure 2, III). The ligand binding characteristics in mammalian cells could not be determined due to the poor $^{125}$I-Mel binding capacity (III / Table 1). The results from receptor ELISA showed that the expression level of the M107D mutant receptor protein was decreased, compared to the WT MT1, but no obvious changes in receptor localization were noticed in the immunofluorescence microscopy (III / Figures 2 - 3).

A histidine H195 (His$^{546}$) in TM V was mutated into alanine (H195A). Double mutants of H195 together with valine V192 (V192T+H195A) and serine S280 (H195A+S280A) were also constructed. In the yeast, the functional responses of the mutants H195A and H195A+S280A were potentiated 3-6 -fold (II / Figure 2). N-acetylserotonin was converted from a partial agonist into a full agonist with these mutations, but otherwise the mutations had no effect on maximum functional responses (E$_{max}$, II / Figure 2). In the mammalian expression system, the functional responses of the mutants H195A and H195A+S280A were not potentiated, in contrast to the results obtained in the yeast. With H195A+S280A, there was even a trend towards decreased potency in mammalian cells, with a more prominent trend with the single mutant H195A (III / Table 3). Both H195A and H195A+S280A had decreased affinity for $^{125}$I Mel (III / Table 2). The number of binding sites (B$_{max}$) was slightly but non significantly increased in COS-7 cells expressing the H195A mutant transiently, and the increase was significant with H195A+S280A (III / Table 1). The data from receptor ELISA and
immunofluorescence microscopy also indicated a slight increase in the quantity of H195A+S280A receptor protein on the cell membrane in these cells (III / Figures 2-3). The double mutant V192T+H195A was absolutely silent in the yeast and mammalian functional assays, and no 125I Mel binding was detectable with this mutant (II / Figure 2, III / Table 1, Table 3). According to receptor ELISA and immunofluorescence microscopy, the cell membrane expression of the V192T+H195A receptor protein was significantly decreased in transiently transfected COS-7 cells (III / Figures 2-3).

A serine S280 (Ser\textsuperscript{3,38}) in TM VII was mutated into alanine (S280A). A double mutant S280F+A284G was also created. The single mutation S280A did not affect any signalling characteristics in the yeast (II / Figure 2). In mammalian cells, ligand binding characteristics and agonist potencies in functional assays did not change with this mutation, but the maximum functional response (E\textsubscript{max}) was significantly decreased (III / Tables 1-3). Three separate transfections were performed in order to find out whether the decrease in the maximum functional response with this receptor mutant could be caused by inconsistencies in the transfection protocols, but the maximum functional response remained low in all batches of the cells. The double mutant S280F+A284G could not be activated with melatonin and only the highest concentrations of 2-iodomelatonin were active in the yeast functional assays (II / Figure 2). In the mammalian expression system, a modest functional response was obtained with S280F+A284G. The potency of melatonin was decreased by more than three orders of magnitude with this mutation and the maximum functional response (E\textsubscript{max}) was significantly decreased, compared to the WT MT1 (III / Table 3). The mutations changed the subcellular localization of the S280F+A284G mutant receptor; the receptor was concentrated in intracellular organelles. The overall S280F+A284G receptor protein level measured with receptor ELISA was not significantly changed (III / Figures 2-3).

5.3. Melatonin-related receptor

The human melatonin-related orphan receptor (GPR50) and a site-directed mutant with substitution of an alanine 779 (Ala\textsuperscript{7,38}) into serine (A779S) in TM VII were also expressed in mammalian COS-7 cells. Based on receptor ELISA and immunofluorescence microscopy, the WT melatonin-related receptor was expressed both on the cell surface and inside the cell, in low but detectable levels. Its localization was found to be comparable to the WT MT1 receptors. No specific 125I-L-Mel binding was detected in cells expressing either WT or A779S melatonin-related receptor.

5.4. Constitutive activity of MT1 receptors

Two regions of the human MT1 melatonin receptor were mutated in search for constitutive activation of the receptor. The mutant receptors were expressed in the yeast Saccharomyces...
corvisiae and mammalian COS-7 cells. In the yeast, the receptors were tested in a colorimetric CPRG assay, and in COS-7 cells the effects of mutations on the quantity, localization, binding, and function of the receptors were analyzed (II, III).

An asparagine 124 (Asn^{124}) in the end of TM III was mutated into alanine (N124A). In the yeast, the N124A mutation dramatically reduced agonist potencies and resulted in very steep dose-response curves (II / Figure 4). In mammalian cells, this receptor gave no functional response and had very poor $^{125}$I-Mel binding ability, which prevented the determination of accurate binding characteristics (III / Table 1). In receptor ELISA, the quantity of the N124A receptor protein on the cell membrane was significantly reduced, compared to the WT MT1, with immunofluorescence microscopy showing the same trend (III / Figures 2-3). No constitutive activity was obtained with the mutation, as evidenced by the lack of an increase in basal signalling activity and the lack of potentiation of functional responses in both the yeast and mammalian systems.

A proline 253 (Pro^{253}) in TM VI was mutated into alanine (P253A). Melatonin was inactive in the yeast and mammalian functional assays with this receptor as well, and only the highest concentrations of 2-iodomelatonin were active in the yeast functional assays (II / Figure 4). This receptor had a very low maximum ligand binding capacity ($B_{\text{max}}$) in mammalian cells and its quantity on the cell membrane was significantly reduced, compared to the W1 MT1 (III / Table 1, Figure 2). Immunofluorescence microscopy revealed that, unlike WT M11, the P253A mutant receptor seemed to be retained in the endoplasmic reticulum (III / Figure 3). No constitutive activity was obtained with this mutation, either.

No enhancement of constitutive activity of the MT1 receptor was obtained with site-directed mutagenesis (II, III). On the other hand, the WT MT1 receptor was found to be constitutively active in a stable CHO cell line with close to physiological MT1 receptor density (WT$_{\text{high}}$, $B_{\text{max}} \sim 160$ fmol/mg protein, IV). The constitutive activation was absent in a cell line with only slightly lower MT1 receptor density (WT$_{\text{low}}$, $B_{\text{max}} \sim 90$ fmol/mg protein). The melatonin receptor antagonist / inverse agonist luzindole significantly and dose-dependently inhibited basal G protein activation in WT$_{\text{high}}$ cell membranes, but not in other MT1 cell lines (IV / Figure 5). Basal G protein activation in WT$_{\text{high}}$ cells was significantly higher than in WT$_{\text{low}}$ cells, also indicating higher spontaneous activity of the MT1 receptor in the WT$_{\text{high}}$ cell line (IV).

5.5. Mutations in the second intracellular loop

Two conserved cysteines in the interphase between the end of TM III and the second intracellular loop of the MT1 receptor were mutated into serines, either individually or as a pair. The cysteine into serine mutation was as conservative as possible (-SH into -OH), since
it did not have a big influence on bulkiness or hydrogen bonding properties of the amino acid side chain. The cysteines were interesting because of their location in close proximity to the receptor region that most likely is important for G protein activation. Furthermore, they were also potential targets of S nitrosylation, which might have a role in regulating GPCR function. Stable expression of the WT MT1 and cysteine mutants in mammalian CHO cells was chosen instead of transient transfection to obtain better functional responses. In computer modelling of the WT MT1 receptor, the cysteines were found to be located in the intracellular (cytoplasmic) surface of the receptor, not buried within the TM area or within the receptor interior (IV / Figure 8). According to the model, it seemed very unlikely that a disulphide bond could exist between C127 and C130 or that either one of these two cysteines could form a disulphide bond with other cysteines in the MT1 receptor molecule.

The mutation of cysteine 127 (Cys^{327}) into serine (C127S) probably rendered most of the receptor population into a desensitized state, while it did not affect the ligand binding affinity and agonist potency of the remaining, possibly G protein-associated pool of receptors (IV / Table 1, Figure 4). Based on the immunological studies (receptor ELISA, Western blot and immunofluorescence microscopy), the cell line expressing the mutant receptor C127S contained very high amounts of receptor protein (IV / Figures 2-3), but the number of binding sites identified with ^125I-Mel binding (B_{max}) and the maximum functional response (E_{max}) of this cell line were not in proportion to the massive amount of receptor protein in the cells. The subcellular localization was not changed with this mutation (IV / Figure 3).

When cysteine 130 (Cys^{325}) was mutated into serine (C130S), the potency of melatonin was significantly decreased (IV / Table 1). The maximum functional response (E_{max}) in the C130S cell line was similar to the WT_{low} cell line (IV / Table 1). The affinity (K_{d}) for ^125I-Mel was not affected by the C130S mutation (IV / Figure 4). The number of binding sites (B_{max}) in the cell line expressing the mutant receptor C130S was significantly lower than in the WT MT1 cell lines (IV / Figure 4). The localization of C130S receptor protein was similar to the WT MT1 receptor and its expression level in this cell line was slightly higher than the expression level of the WT MT1 receptor in the WT_{low} cell line, which gave similar maximum functional responses (IV / Figures 2-3).

Cysteine S-nitrosylation with either GSNO or CysNO did not significantly change the potency of melatonin (EC_{50}) or the maximum functional response (E_{max}) in the cell lines expressing WT, C127S, or C130S MT1 receptor (Table 5). S-nitrosylation with both GSNO and CysNO has been found to specifically modify G protein activation of some other GPCRs (T. Kokkola et al., unpublished observations).

The double mutation C127S+C130S inhibited normal MT1 receptor binding and activation. No functional response was obtained with any of the cell lines expressing the double mutant receptor (IV / Table 1). A cell line expressing high amounts of the C127S+C130S receptor

protein was chosen for further analyses. In this cell line, the subcellular localization of the receptors was similar to the WT receptors (IV / Figures 2-3) but only residual $^{125}$I-Mel binding was detected (IV / Figure 4). Furthermore, no saturation of the specific binding was obtained with the concentrations of the radioligand used in the binding assays, indicating that the affinity ($K_d$) for $^{125}$I-Mel was dramatically decreased in the C127S i C130S cell line.

**Table 5.** The effects of cysteine S-nitrosylation with GSNO or CysNO on the functional responses evoked with melatonin in MT1 receptor cell lines. The same data was used in IV / Figure 7.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>GSNO</th>
<th>CysNO</th>
<th>Control</th>
<th>GSNO</th>
<th>CysNO</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^{+iv}$</td>
<td>-9.52 ± 0.00</td>
<td>-9.43 ± 0.10</td>
<td>-9.40 ± 0.11</td>
<td>483 ± 46</td>
<td>556 ± 20</td>
<td>486 ± 57</td>
</tr>
<tr>
<td>WT$^{jiv}$</td>
<td>-9.29 ± 0.06</td>
<td>-9.40 ± 0.17</td>
<td>-9.27 ± 0.11</td>
<td>215 ± 4</td>
<td>215 ± 19</td>
<td>196 ± 7</td>
</tr>
<tr>
<td>C127S</td>
<td>-9.10 ± 0.07</td>
<td>-9.05 ± 0.14</td>
<td>-9.01 ± 0.21</td>
<td>238 ± 20</td>
<td>214 ± 13</td>
<td>198 ± 4</td>
</tr>
<tr>
<td>C130S</td>
<td>-8.46 ± 0.03</td>
<td>-8.61 ± 0.05</td>
<td>-8.49 ± 0.07</td>
<td>240 ± 13</td>
<td>201 ± 15</td>
<td>218 ± 12</td>
</tr>
</tbody>
</table>

Values are mean ± SE from three to five independent experiments performed in duplicate. $E_{max}$ is expressed as percentage over basal with nonspecific binding subtracted.
6. DISCUSSION

6.1. Model of melatonin recognition

The model for melatonin recognition at its G protein-coupled receptor, described in (I), was the first melatonin receptor model that used a rhodopsin-based arrangement of the seven TM helices. Previously, bacteriorhodopsin was used as a template for GPCR modelling, because no information about the three-dimensional structure of GPCRs was available (Trumpp-Kallmeyer et al. 1992). The use of bacteriorhodopsin to predict the structure of GPCRs was questionable, because bacteriorhodopsin shows none of the distinctive features of the GPCR protein family and, instead of being a receptor, it is a photon-driven proton pump (Lanyi and Luecke 2001). The existence of seven transmembrane domains, most likely occupying an α-helical structure, was predicted from hydrophobicity plots of the GPRC primary amino acid sequences long before the three-dimensional rhodopsin structure was known (Donnelly et al. 1989). All members of the GPCR superfamily were (and still are) thought to have the same basic structure in the membrane-embedded part, mainly because of the sequence similarities and their common ability to activate G proteins to initiate signal transduction (Trumpp-Kallmeyer et al. 1992, Baldwin et al. 1997). Significant achievements in elucidation of the structure of GPCRs were the projection map of rhodopsin (Schertler et al. 1993) and the three-dimensional structure of rhodopsin from tilted two-dimensional crystals (Unger and Schertler 1995). Based on the projection map of rhodopsin, a helical wheel model of the probable arrangement of the seven TM helices was constructed (Baldwin 1993). The consensus sequence of melatonin receptors could be aligned with the helical wheel model to construct a melatonin receptor model, which suggested the recognition of melatonin by specific amino acid residues in a binding pocket formed by the TM helices (I). The amino acids suggested to interact with melatonin pointed towards the binding pocket and were highly conserved in melatonin receptors, but were not present in other GPCRs.

The energetically minimized structure of melatonin and its analogues was calculated in (I) to adopt a tilted L conformation with the N acetyl side chain extended, and this geometry was found to be lower in energy than the one used in a previously published, bacteriorhodopsin-based melatonin receptor model (Sugden et al. 1995). Other research groups have suggested that, when binding to the receptor, melatonin is more likely to form a folded structure, in which the aminoethyl side chain of melatonin and its analogues is folded towards the indole ring in a syn conformation (Sicsic et al. 1997, Marot et al. 1998). The distance between the N-acetyl group and the 5-methoxy groups is critical in binding to the receptor (Harris et al. 2002). The results from CoMFA (I) agreed well with other more recent studies, in that an increase in bulkiness near the methoxy group in position 5 and near the substituent in position 2 would increase the affinity of a melatoninergic ligand (Mor et al. 1998, Rivara et al. 2003).
Table 6. Summary of the results of mutagenesis and polymorphism studies in MT1 receptors

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Location</th>
<th>Mutations</th>
<th>Binding</th>
<th>Activation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>R54 (Arg&lt;sup&gt;1.68&lt;/sup&gt;)</td>
<td>IC I</td>
<td>R54W</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>NT</td>
<td>2)</td>
</tr>
<tr>
<td>S103 (Ser&lt;sup&gt;2.42&lt;/sup&gt;)</td>
<td>TM III</td>
<td>S103A</td>
<td>$B_{max} \sim K_d \sim$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>5)</td>
</tr>
<tr>
<td>M107 (Met&lt;sup&gt;1.32&lt;/sup&gt;)</td>
<td>TM III</td>
<td>M107T</td>
<td>$B_{max} \sim K_d \sim$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>II, III, 5)</td>
</tr>
<tr>
<td>M107D</td>
<td></td>
<td></td>
<td>$B_{max} \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>H, III</td>
</tr>
<tr>
<td>S110 (Ser&lt;sup&gt;3.35&lt;/sup&gt;)</td>
<td>TM III</td>
<td>S110A</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>5)</td>
</tr>
<tr>
<td>S114 (Ser&lt;sup&gt;3.39&lt;/sup&gt;)</td>
<td>TM III</td>
<td>S114A</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>5)</td>
</tr>
<tr>
<td>N124 (Asn&lt;sup&gt;3.69&lt;/sup&gt;)</td>
<td>TM III</td>
<td>N124A</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>No (mamm.)</td>
<td>III, 6)</td>
</tr>
<tr>
<td>C12/ (Cys&lt;sup&gt;3.22&lt;/sup&gt;)</td>
<td>IC 2</td>
<td>C12/S</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>IV</td>
</tr>
<tr>
<td>C127S+C130S</td>
<td></td>
<td></td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>No</td>
<td>IV</td>
</tr>
<tr>
<td>C130 (Cys&lt;sup&gt;3.22&lt;/sup&gt;)</td>
<td>IC 2</td>
<td>C130S</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>IV</td>
</tr>
<tr>
<td>C127S+C130S</td>
<td></td>
<td></td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>No</td>
<td>IV</td>
</tr>
<tr>
<td>A157 (Ala&lt;sup&gt;5.25&lt;/sup&gt;)</td>
<td>TM IV</td>
<td>A157V</td>
<td>$B_{max} \sim K_d \sim$</td>
<td>NT</td>
<td>2)</td>
</tr>
<tr>
<td>V192 (Val&lt;sup&gt;5.25&lt;/sup&gt;)</td>
<td>TM V</td>
<td>V208A*</td>
<td>$B_{max} \sim K_d \sim$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>1)</td>
</tr>
<tr>
<td>V208L+</td>
<td></td>
<td></td>
<td>$B_{max} \sim K_d \sim$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>1)</td>
</tr>
<tr>
<td>V1927+H195A</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>II, III</td>
</tr>
<tr>
<td>H195 (His&lt;sup&gt;5.46&lt;/sup&gt;)</td>
<td>TM V</td>
<td>H195A</td>
<td>$B_{max} \sim K_d \sim$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ (mamm.)</td>
<td>III</td>
</tr>
<tr>
<td>H195A+S280A</td>
<td>$B_{max} \uparrow K_d \uparrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ (mamm.)</td>
<td>III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V1927+H195A</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>II, III</td>
</tr>
<tr>
<td>H211F*</td>
<td>$B_{max} \sim K_d \sim$</td>
<td>No</td>
<td>No</td>
<td>1)</td>
<td></td>
</tr>
<tr>
<td>H211L+</td>
<td>$B_{max} \sim K_d \sim$</td>
<td>No</td>
<td>No</td>
<td>1)</td>
<td></td>
</tr>
<tr>
<td>C250 (Cys&lt;sup&gt;6.47&lt;/sup&gt;)</td>
<td>TM VI</td>
<td>C250A</td>
<td>$B_{max} \sim K_d \sim$</td>
<td>No</td>
<td>4)</td>
</tr>
<tr>
<td>A252 (Ala&lt;sup&gt;6.69&lt;/sup&gt;)</td>
<td>TM VI</td>
<td>A252C</td>
<td>$B_{max} \sim K_d \sim$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>4), 5)</td>
</tr>
<tr>
<td>A252C+G258T</td>
<td>No</td>
<td>No</td>
<td>NT</td>
<td>4)</td>
<td></td>
</tr>
<tr>
<td>P253 (Pro&lt;sup&gt;6.50&lt;/sup&gt;)</td>
<td>TM VI</td>
<td>P253A</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>II, III</td>
</tr>
<tr>
<td>G258 (Glu&lt;sup&gt;6.55&lt;/sup&gt;)</td>
<td>TM VI</td>
<td>G258T</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>4), 5)</td>
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<tr>
<td>A252C+G258T</td>
<td>No</td>
<td>No</td>
<td>NT</td>
<td>4)</td>
<td></td>
</tr>
<tr>
<td>S280 (Ser&lt;sup&gt;7.38&lt;/sup&gt;)</td>
<td>TM VII</td>
<td>S280A</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>II, III</td>
</tr>
<tr>
<td>S280F+A284G</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~</td>
<td>II, III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H195A+S280A</td>
<td>$B_{max} \uparrow K_d \uparrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ (mamm.)</td>
<td>III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S280I+A284G</td>
<td>$B_{max} \downarrow K_d \downarrow$</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ (mamm.)</td>
<td>III</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Studies were conducted with human MT1 receptor except for mutants marked with *, which are ovine MT1 receptor mutants. IC = intracellular loop, TM = transmembrane domain, ~ = no significant changes, ↓ = decrease in value, ↑ = increase in value, ↓↓ / ↑↑ = decrease / increase of more than one order of magnitude, NT = not tested, No = no ligand binding or receptor activation was detected, mamm. = mammalian cells, yeast = yeast cells. References: 1) Conway et al. 1997, 2) Ebisawa et al. 1999, 3) Conway et al. 2000, 4) Gubitz and Reppert 2000, 5) Conway et al. 2001, and 6) Nelson et al. 2001.
6.2. Melatonin receptor mutagenesis studies

Due to the relatively recent cloning of the first member of the melatonin receptor family, the number of mutagenesis and polymorphism studies of melatonin receptors is not very large yet. The results presented in this thesis make up an essential part of what is known about the effects of mutations upon the binding and functional characteristics of melatonin receptors. Table 6 presents a summary of studies conducted with MT1 receptors so far. The effects of mutations will be discussed in more detail in the following chapters. In radioligand binding assays with the agonist radioligand $^{125}$I-Mel, only receptors that are coupled to G proteins are detected. Since there are no antagonist radioligands available for melatonin receptors, the binding assays do not reveal the total number of binding sites and the $B_{max}$ values from $^{125}$I-Mel binding assays have to be interpreted with caution.

6.2.1. Roles of TM V and TM VII

In the model of melatonin binding pocket (I), histidine H195 (His$^{5,46}$) in the TM V of melatonin receptors was suggested to form a hydrogen bond with the oxygen in the 5-methoxy group of melatonin, an interaction that would be strengthened by valine V192 (Val$^{5,43}$), offering an aliphatic milieu for the methyl group attached to the 5-oxygen. This cooperation between H195 and V192 could explain why ligands like N-acetylsertotonin, in which the 5-methoxy group has been substituted by a 5-hydroxy group, are dramatically less active than ligands with a 5-methoxy group in melatonin receptors. This could also explain why a bulky group over the 5-oxygen, as in 5-benzyloxy-N-acetyltryptamine, declines binding affinity. V192 and H195 were replaced with amino acids conserved in mammalian serotonin (5-HT) receptor subtypes 1, 5, and 7 to construct a double mutant V192T+H195A. Corresponding residues are generally thought to interact with the substituents on the aromatic ring of the ligands for biogenic amine neurotransmitter receptors, such as adrenergic and serotonergic receptors (Trumpp-Kallmeyer et al. 1992). A single mutant H195A and a TM V - TM VII double mutant H195A+S280A were also constructed. In H195A+S280A, both of the most important ligand recognition sites in the model of the melatonin binding pocket (I) were mutated into alanines.

The results from mutagenesis studies of H195 (His$^{5,46}$) in the human MT1 receptor and corresponding histidine residue in the ovine MT1 receptor indicated that this amino acid is indeed involved in ligand binding and activation of the receptor. The results from the expression of H195A mutant in the yeast and in mammalian cells were, however, conflicting. In the yeast, the H195A mutation potentiated functional responses (II), whilst in COS-7 cells a non significant trend in the opposite direction (decrease in potency) was observed (III). The affinity for $^{125}$I Mel was also decreased in the mammalian expression system, and the number of binding sites ($B_{max}$) was slightly increased (III). The corresponding histidine (H211, His$^{5,46}$) has also been mutated into phenylalanine (H211F) and leucine (H211L) in the ovine MT1 receptor.
(Conway et al. 1997). In this study, binding assays with COS-7 cells transiently expressing H211F and H211L mutant receptors showed that the mutations decreased receptor affinity for $^{125}$I-Mel but had no effect on the number of binding sites ($B_{max}$). Functional assays of H211F and H211L mutants were performed in transiently transfected HEK 293 cells, and no melatonin mediated effects were detected. Receptor expression level in HEK-293 cells was very low and the authors were not able to determine it by either ligand binding or immunocytochemistry. In any case, it seemed unlikely that the lack of functional response would have been caused by a selective lack of expression in HEK-293 cells, since the mutations did not affect the receptor number in COS-7 cells (Conway et al. 1997). In the human MT2 melatonin receptor, a corresponding histidine into alanine (H208A) mutant expressed in mammalian HEK-293 cells had significantly reduced binding affinities for $^{125}$I-Mel and melatonin, proving that this residue is important for ligand binding also in the other mammalian melatonin receptor subtype (Gerdin et al. 2003).

In mammalian expression systems, mutagenesis of the conserved histidine in TMV of MT1 receptors decreased receptor affinity for melatonin analogues with a 5-methoxy group. For melatonin analogues lacking the 5-methoxy group (N-acetylserotonin, N-[2-(1-naphthyl)ethyl]acetamide), no decrease in affinity was observed (Conway et al. 1997). Concluding from these observations, this histidine seems to be important for the correct recognition of the 5-methoxy group of melatoneric ligands. The effect of mutagenesis on the functional response were dependent upon the amino acid that replaced histidine in this position. Alanine in this position was tolerated quite well (II, III) but phenylalanine or leucine completely inhibited receptor activation (Conway et al. 1997). Phenylalanine or leucine in this position might block receptor activation due to steric hindrance by their bulky hydrophobic side chains, but the small alanine would not have the same obstructing effect. The finding that the H195A mutation actually potentiated functional responses in the yeast suggests that the histidine does not directly participate in melatonin recognition, but more likely lies close to the melatonin binding pocket and has a role in maintaining receptor conformation. The mutation H195A might be a cause of a conformational change in the receptor that is more propitious for the activation of the only yeast endogenous heterotrimeric G protein (Dohlman and Thorner 2001) than for the activation of mammalian G proteins. COS-7 cells express at least G12, G13, and G5 subtypes of G proteins (Ammer and Christ 2002).

Valine V192 of the human MT1 receptor was mutated into threonine together with the H195A mutation, producing the double mutant V192T+H195A. In contrast to the mild effects of the single H195A mutation, the double mutant showed no specific $^{125}$I-Mel binding and the functional response was absent both in the yeast and COS-7 cells (II, III). Receptor ELISA and confocal microscopy showed that the double mutant was expressed, with low amount of receptor protein on the cell membrane and higher expression inside the cell. Mutagenesis of the corresponding valine (V298, Val$^{145}$) in the ovine MT1 receptor into alanine (V208A) or leucine (V208L) produced a decrease both in receptor affinity and agonist potency (Conway et
al. 1997). The decreases in affinities were in the same rank order as the decreases in potency with both valine mutants, with leucine having greater effects (3.5-fold) than alanine (-2-fold). These results suggest that the conserved valine in TM V of MT1 receptors appears to be at or close to the melatonin receptor binding pocket, but it is not absolutely required for receptor function (Conway et al. 1997). The MT1 receptor can still be functional with one of the aliphatic amino acids valine, alanine, or leucine at this position, but a hydrophilic threonine disrupts the structure of the binding pocket. The MT1 receptor with threonine in this position also probably suffers from trafficking defects, and is not normally transported to the cell membrane.

The model of the melatonin binding pocket suggested hydrogen bonding of the carbonyl oxygen in the N-acetyl group of melatonin with a conserved serine (S280) (Ser-38) in TM VII of melatonin receptors. Alanine A284 (Ala47) could strengthen this interaction by offering an aliphatic milieu for the methyl group attached to the carbonyl group. The mutation S280A, which eliminated the assumed interaction between melatonin and the hydroxyl group of serine, had no effect on ligand binding characteristics, functional responses in the yeast or the potency of melatonin in COS-7 cells, but seemed to reduce the maximal functional response (Emax) in COS-7 cells (II, III). The corresponding mutation in serine S293 of the human MT2 melatonin receptor expressed in mammalian HEK-293 cells (S293A) was also without effect on ligand binding characteristics (Gerdt et al. 2003). The testing of mutant M11 and M12 receptors has thus revealed that melatonin binding does not require interaction with this serine (Ser-30) in melatonin receptors. It also proved that the model of melatonin receptor recognition (I) had not identified the interaction site of the N-acetyl group of melatonin correctly.

As the single mutant S280A behaved very similarly to the WT MT1 receptor, it was not surprising that the double mutant H195A+S280A behaved very similarly to the single mutant H195A (II, III). The results from expression of the mutant H195A+S280A in the yeast and COS-7 cells were again conflicting in such a way that in the yeast the functional responses were potentiated, whereas in COS-7 cells there was a trend towards weakened potency. The second mutation further decreased the affinity of the receptor for 125I-Mel.

A double mutation in TM VII, S280F+A284G, converted two MT1 residues to residues conserved in serotonin receptors. This mutation was found to be extremely harmful for receptor function in the yeast expression system (II), as it totally prevented melatonin signalling, equaling to more than 100,000-fold increase in the EC50, compared to the WT receptor. In mammalian cells, the signalling capacity of this mutant was profoundly compromised (III), but melatonin was still able to activate the receptor with an EC50 of about 400 nM (3500-fold increase in the EC50), despite the low expression level of this mutant. This is further evidence that the receptor conformation that optimally activates G proteins has to be different in the yeast and mammalian cells. The subcellular localization of this double mutant
differed from the WT MT1 but the amount of receptor protein in the cells had not significantly changed. The receptor probably was not able to accommodate the bulky phenylalanine residue in the position 280 without conformational changes that affected receptor trafficking. The conserved mutation of alanine A284 to glycine was not likely the major cause of these effects, since the bovine MT1 receptor contains a glycine in this position (Messer et al. 1997). No proof of the binding or functional capacity of the bovine receptor has been presented yet, but there is no reason to believe that the cloned sequence would not represent the functional bovine MT1 receptor and that a glycine in this position would not be tolerated in melatonin receptors.

### 6.2.2. Other melatonin recognition domains

The possible interaction between methionine M107 (Met^{33}) in TM III and the nitrogen of the amide moiety in the N-acetyl group of melatonin was investigated by mutating it into threonine (M107T). Threonine was selected because it is found in the corresponding location in all cloned melatonin-related receptors (GPR50), which do not bind melatonin or other related compounds. The mutant M107T could not be distinguished from the WT MT1 receptor in any aspect; the functional responses in the yeast and in mammalian cells were similar, so were the ligand binding characteristics and expression pattern in mammalian cells (II, III). Our results agreed with a study where WT and M107T FLAG-tagged human MT1 receptors were transiently expressed in COS-7 cells (Conway et al. 2001). Methionine M107 is not likely to participate in melatonin recognition or melatonin receptor activation, and the lack of melatonin binding in the melatonin-related receptor is thus not caused by this particular sequence difference. The M107 was also mutated into aspartic acid (M107D). Aspartic acid in this position is found in biogenic amine receptors, for example in serotonergic receptors, where it interacts with the protonated ammonium group of the ligands (Strader et al. 1987, Ho et al. 1992). M107D receptor was not responsive to melatonin in the yeast or COS-7 cells (II, III). Some receptor activation could be obtained with high concentrations of 2-iodomelatonin in the yeast, but the EC_{50} had increased more than 80,000-fold, compared to the WT receptor. The ^{125}I-Mel binding capacity of M107D was very low, and the level of receptor protein in the COS-7 cells transfected with this receptor construct was significantly decreased. The change from neutral methionine into aspartic acid was probably fatal to the receptor structure, resulting in low expression level and severely impairing receptor function.

The model also suggested that an aromatic interaction could take place between the indole ring of melatonin and the phenylalanine F257 (Phe^{641}) in TM VI. This residue has been targeted by site directed mutagenesis in MT2 receptor only. The substitution of phenylalanine F257 (Phe^{641}) with alanine (F257A) had no effect on ^{125}I Mel or melatonin binding characteristics of the human MT2 melatonin receptor (Gerdin et al. 2003). While this phenylalanine residue was not
important for melatonin binding, it appeared to have a role in binding of other ligands with selectivity for the MT2 melatonin receptor through aromatic interactions (Gerdin et al. 2003).

Studies with chimeric receptor constructs of the human MT1 receptor and the human melatonin related receptor (GPR50) have revealed that TM IV and TM VI play critical roles in ligand binding to the MT1 melatonin receptor. When these TM domains in MT1 receptor were replaced with corresponding domains of the melatonin-related receptor, any detectable $^{125}$I-Mel binding was destroyed (Conway et al. 2000, Gubitz and Reppert 2000). Specific $^{125}$I-Mel binding was not totally abolished if other TM domains were exchanged. Individual amino acids in TM VI of the human MT1 receptor were targeted with site-directed mutagenesis. Of the amino acids in TM VI, residues at positions 252 (6.49) and 258 (6.55) were obviously non-homologous between melatonin and melatonin-related receptors. To test the significance of these differences on receptor function, human MT1 melatonin receptor mutants A252C (Ala$^{6.49} \rightarrow$ Cys) and G258T (Gly$^{6.55} \rightarrow$ Thr) were constructed. A mutant C250A (Cys$^{6.47} \rightarrow$ Ala) served as a control to demonstrate that any random mutation in TM VI of the human MT1 receptor would not have an inhibitory effect on melatonin binding. The mutants were tested in $^{125}$I-Mel binding assays (C250A, A252C, G258T; Conway et al. 2000, Gubitz and Reppert 2000) and functional assays (A252C, G258T; Conway et al. 2000). The G258T mutation severely compromised both high-affinity ligand binding and ligand-mediated signal transduction in the MT1 receptor, but A252C or C250A mutations did not significantly affect these functions (Conway et al. 2000, Gubitz and Reppert 2000). Glycine G250 (Gly$^{6.55}$) thus seems to be an important conserved residue in TM VI of melatonin receptors.

In TM III of the human MT1 receptor, some conserved amino acids have also been mutated (Conway et al. 2001). Mutation S103A (Ser$^{5.25} \rightarrow$ Ala) did not affect MT1 receptor function. S110A (Ser$^{5.35} \rightarrow$ Ala) and S114A (Ser$^{5.39} \rightarrow$ Ala) mutations reduced the number of binding sites ($B_{\text{max}}$) and the affinity for $^{125}$I-Mel, and also reduced affinities for other agonists and partial agonists. By contrast, luzindole was found to have similar binding affinities to the WT, S110A, and S114A receptors. The potency of melatonin to stimulate signal transduction was also compromised with S110A and S114A mutations. These data indicated that S110 and S114 are critical for the formation of the high-affinity ligand binding site for agonists and partial agonists but not for the antagonist luzindole (Conway et al. 2001). In the MT2 receptor, S123A (Ser$^{5.35} \rightarrow$ Ala) and S127A (Ser$^{5.39} \rightarrow$ Ala) were not critical for ligand binding, indicating that the binding pockets of the MT1 and MT2 receptors are different in this regard (Gerdin et al. 2003).

In the human MT2 melatonin receptor, the effects of mutagenesis of some other conserved amino acids have also been tested. The substitution of asparagine N175 (Asn$^{6.46}$) in TM IV with alanine (N175A) was without effect on the affinity for $^{125}$I-Mel, but it decreased the affinity for melatonin (Gerdin et al. 2003). The role of N175 in MT2 receptor function is thus still unclear. In TM VI, a conserved tryptophan W264 (Trp$^{6.46}$) was mutated into alanine (W264A). The mutation significantly increased the affinity for $^{125}$I-Mel, but had no effect on the affinity for
melatonin in the MT2 receptor. This tryptophan is probably not essential for melatonin binding, but was found to be important for the binding of some other ligands with selectivity for the MT2 melatonin receptor through aromatic interactions (Gerdin et al. 2003). Mutagenesis of the conserved cysteines C113 and C190 in the first and the second extracellular loop of the human MT2 receptor totally abolished ligand binding (Mscch et al. 2002). These two extracellular cysteines are probably linked by a disulphide bond, which is important in maintaining the MT2 melatonin receptor in correct conformation. Alanine substitutions of C219 in TM V, C263 in TM VI, or C302 in TM VII did not alter the affinities of the receptors for 125I-Mel, indicating that these cysteines are not directly participating in ligand recognition in the human MT2 receptor (Mscch et al. 2002).

Some DNA polymorphisms of the human MT1 and MT2 receptors have been found to predict amino acid changes. In the human MT1 receptor, mutation R54W (Arg\textsuperscript{54}→Trp) in the first intracellular loop significantly reduced the number of binding sites (B\textsubscript{max}) and slightly enhanced binding affinity of the receptor (Ebisawa et al. 1999). The mutant A157V (Ala\textsuperscript{157}→Val) in TM IV behaved similarly to the WT MT1. In the human MT2 receptor, DNA polymorphisms produced mutations G24E in the N-terminus and L66F (Lys\textsuperscript{166}→Phe) in the first intracellular loop (Ebisawa et al. 2000). The ligand binding characteristics of these MT2 receptor mutants were similar to the WT MT2. These MT1 or MT2 receptor DNA polymorphisms were not found to be associated with sleep disorders in the study populations involved.

To conclude what is currently known about ligand recognition domains in melatonin receptors, valine Val\textsuperscript{52-53} and histidine His\textsuperscript{55-56} in TM V, and glycine Gly\textsuperscript{62,63} in TM VI seem to be important conserved residues for melatonin binding. The two conserved serines in TM III (Ser\textsuperscript{33} and Ser\textsuperscript{39}) are important for agonist binding in the MT1 receptor, but not in the MT2 receptor subtype. The results from several mutagenesis studies can be utilized to construct improved models of melatonin recognition in its GPCRs.

### 6.2.3. G protein coupling of melatonin receptors

Two receptor domains were mutated as an attempt to generate constitutively active MT1 receptors (II, III). Asparagine N124 (Asn\textsuperscript{349}) is part of the melatonin receptor family consensus sequence N-R-Y, which distinguishes melatonin receptors from other rhodopsin-like GPCRs, most of which have a D-R-Y or E-R-Y motif at the corresponding location. Mutations in aspartate of this triplet motif have been found to be important in G protein activation and to result in constitutive activity in the gonadotropin-releasing hormone receptor (Arora et al. 1997), in the α\textsubscript{1B} and α\textsubscript{2B} adrenergic receptors (Scheer et al. 1995, Ge et al. 2003), among other receptors (Parnot et al. 2002). No constitutive activation of the human MT1 receptor was obtained with the N124A mutation. In the yeast, the N124A receptor had dramatically reduced agonist potencies and a very steep dose-response curve (II). In COS-7
cells, the mutant bound $^{125}\text{I-Mel}$ very weakly and gave no functional response (III). The cell membrane expression of the N124A receptor protein seemed to be decreased. The same mutant expressed stably in murine AtT20 cells failed to bind $^{125}\text{I-Mel}$ and was improperly trafficked (Nelson et al. 2001). In the same study, the MT1 mutants N124L and N124K behaved like the mutant N124A, while the mutants N124D and N124E showed high-affinity $^{125}\text{I-Mel}$ binding, normal trafficking, but deficient functional responses. These results emphasize the importance of N124 in melatonin receptor function.

Proline P253 (Pro$^{6,50}$) in TM VI of the MT1 receptor was mutated into alanine, yielding the mutant P253A. Instead of leading to constitutive activity, this mutation destroyed the signalling capacity of this receptor, both in the yeast and mammalian system (II, III). The cell membrane expression of P253A was significantly decreased in transiently transfected COS-7 cells, and it was probably retained in the Golgi. The $^{125}\text{I-Mel}$ binding capacity of this receptor mutant was also very weak. Constitutive activation of the yeast $\alpha$-factor and $\alpha$-factor receptors has been obtained by the replacement of the corresponding proline in TM VI with any other amino acid and it has been speculated that the same mechanism would control the activity of other GPCRs (Konopka et al. 1996, Stefan et al. 1998). The speculation is contradicted by the results of the current study, and the reports that substitutions affecting Pro$^{6,50}$ in rhodopsin, m5 or m6 muscarinic acetylcholine receptors and C5a receptors have not been found to promote constitutive activation of these receptors (Kaushal and Khorana 1994, Wess et al. 1993, Kolakowski et al. 1995, Spalding et al. 1998). These receptors belong to a different GPCR family from the yeast mating factor receptors (which are fungal pheromone receptors), and it is possible that Pro$^{6,50}$ has a different role in the rhodopsin-like receptors.

The conserved cysteines C127 (Cys$^{5,37}$) and C130 (Cys$^{5,37}$) are in the interphase between TM III and the second intracellular loop of the human MT1 receptor, in the region participating in G protein activation. The cysteines were mutated into serines, either individually or as a pair, and CHO cell lines stably expressing the mutant and WT MT1 receptors were constructed (IV). Cysteine to serine mutation was chosen because of its conservative nature; changing -SH (sulphydryl, in cysteine) into -OH (hydroxyl, in serine) did not change the bulkiness or hydrogen bonding properties of the amino acid side chain. Moreover, such mutations are commonly used to study the effects of cysteine S-nitrosylation on receptor function. Cysteine S-nitrosylation is emerging as an important post-translational protein modification (Hess et al. 2001, Ahern et al. 2002). Reactive cysteines have an acid-base motif spatially close to the target sulphydryl group (Hess et al. 2001). In the MT1 receptor model, no acid-base motif for S-nitrosylation was found spatially close to the cysteines C127 or C130 (IV). S-nitrosylation with either GSNO or CysNO did not affect signalling of the WT or C127S and C130S mutant MT1 receptors (IV). CysNO was tested because it is a small molecule and could possibly reach close to a cysteine that would be inaccessible for the larger GSNO. S-nitrosylation with the same concentrations of both GSNO and CysNO has been found to modify G protein activation of some other GPCRs (T. Kokkola et al., unpublished observations), whereas
human MT1 melatonin receptor function is not regulated by S-nitrosylation, not at least under heterologous expression conditions employed in this study.

The C127S mutant cell line contained very high amounts of receptor protein. The number of binding sites (B_max) and the maximum efficacy (E_max) of this cell line were not in proportion to the massive amount of receptor protein in the cells. Ligand binding and functional characteristics of C127S cell line were not significantly different from a cell line expressing WT MT1 receptor, in which receptor protein seemed to be present in much lower level. There is a possibility that the C127S mutation promoted receptor desensitization, which lowered the apparent number of binding sites (B_max) in agonist radioligand binding assays. In the MT2 receptor, corresponding cysteine into serine mutation (C140S) produced a small but significant decrease in the affinity for melatonin and a more prominent decrease in the number of binding sites (Mseeh et al. 2002). The mutation also partially protected against the NEM-induced decrease in {superscript}125{I}-Mel binding affinity. Cysteine alkylation by NEM was deduced to create steric hindrance between receptor and G protein, which did not occur if the cysteine was mutated into serine. Functional responses of C127S cell line were measured with {superscript}35{S}GTP{gamma}S binding assays, which measure the activation of pertussis toxin sensitive G proteins (G{subscript}i/o proteins). In HEK-293 mammalian cells, the MT1 receptor has been found to couple to G{subscript}i2 and G{subscript}i3 but not to G{subscript}i1 or G{subscript}o (Brydon et al. 1999). The C127/S mutation in MT1 could have inhibited receptor interaction with maybe just one subtype of G proteins. If the interaction with one G protein subtype had been missing, the uncoupled receptors would not have shown high-affinity binding. This would not have changed the affinity for {superscript}125{I}-Mel in the receptor population still coupled to other G proteins, but had lowered the apparent number of binding sites. The functional responses in {superscript}35{S}GTP{gamma}S binding assays also would have been compromised by the lack of the interaction with one G protein subtype.

In the C130S cell line, the potency of melatonin to activate G proteins was significantly decreased. The potency of 2-iodomelatonin was slightly decreased as well, although the effect was not significant. The affinity for {superscript}125{I}-Mel was not affected by the mutation, but the apparent number of binding sites (B_max) was decreased in this cell line. In the MT2 receptor, the corresponding cysteine into serine mutation (C143S) produced a small but significant decrease in the affinity for melatonin and an increase in the number of binding sites (Mseeh et al. 2002). The mutation was found to fully protect against the NEM-induced decrease in the number of binding sites, suggesting that this cysteine could be located at or close to the ligand binding pocket (Mseeh et al. 2002). Another possibility is that C130 is distant from the ligand binding site, but its alkylation evokes a conformational change in receptor structure and causes the observed NEM-induced decrease in the number of binding sites (Mseeh et al. 2002). In bovine rhodopsin, the corresponding cysteine (C140) was found to be crucial for efficient G protein activation, as C140T mutation inhibited G protein activation of bovine rhodopsin by 50% (Yamashita et al. 2000).
Contrary to the rather mild effects of the single mutations, the C127S+C130S double mutation severely impaired ligand binding and receptor function. It seems that if both of the cysteines were mutated into serine, the combined effect of mutations destroyed receptor / G protein interaction. The double mutation did not prevent normal receptor trafficking, since the receptor protein was present also on the cell membrane. Modelling of the C127S+C130S mutant receptor revealed that there is no reason to expect that the receptor conformation would change with the mutations. There were no major differences between the WT and C127S+C130S mutant receptor structures, and no signs of conformational changes of the ligand binding pocket were detected. In simulations, both receptors turned out to be stable within simulation time and no breakdown of the arrangement of the seven TM helices could be observed. As the modelling of the MT1 receptor molecule showed that the cysteines were not likely to form disulphide bonds with each other or other cysteines, the effects of the double mutation were most probably not caused by the disruption of intramolecular disulphide bonds. The formation of a disulphide bond between C127 or C130 receptor and some other protein molecule could in theory be possible, but the modelling studies cannot currently provide any evidence for that possibility. The change from more lipophilic cysteines to hydrophilic serines affected the hydrophobicity of the end of TM III, and the increase in hydrophilicity was highly potentiated with the double mutation. It is thus feasible to assume that the mutations could have destroyed the lipophilic interaction required for contact between the receptor and G protein.

In contrast to the WT CHO cell lines, the high receptor expression levels of the mutant CHO cell lines were not correlated with high number of binding sites in agonist radioligand \(^{125}\text{I-Mel}\) binding assays. Melatonin receptor research is hampered by the lack of high-affinity antagonist radioligands. The low \(B_{\text{max}}\) values of C127S, C130S, and C127S+C130S cell lines can reflect the fact that, instead of a real decrease in receptor number, the decrease in \(^{125}\text{I-Mel}\) binding can be caused by a loss of affinity for the radioligand due to receptor / G protein uncoupling (Witt-Enderby and Li 2000). The functional dose-response curves for the WT and mutant cell lines were constructed with melatonin and 2-iodomelatonin, which are the two most common agonists used in melatonin receptor research. The structures of these two ligands are almost identical and future studies should test the effect of cysteine mutations on the dose-response curves of other melatonergic ligands with different chemical structures.

The receptor expression level was found to influence the degree of constitutive activation of WT MT1 receptors. Luzindole functioned as an inverse agonist in the WT MT1 cell line with higher receptor expression level (WT\textsubscript{high}), indicating that the MT1 receptor in this cell line was constitutively active. The basal G protein activity was also higher in the constitutively active cell line. This cell line also had higher number of binding sites (\(B_{\text{max}}\)) and maximum functional response (\(E_{\text{max}}\)), while the affinity (\(K_d\)) and potency (\(EC_{50}\)) of the ligands were not significantly different in WT MT1 cell lines expressing different levels of the receptor. Agreeing with results from other receptors, the constitutively active MT1 receptor showed a
Trend towards increased affinity and potency (Samama et al. 1993, Ballesteros et al. 2001). The higher number of binding sites of WT<sub>high</sub> was the likely cause of constitutive activation of MT1 in this cell line, since constitutive activation of receptors is often obtained by receptor overexpression (de Ligt et al. 2000). On the other hand, it cannot be said that MT1 was actually overexpressed in this cell line, since the number of binding sites was on the physiological level (~160 fmol/mg protein). Constitutive activity is usually detected when recombinant receptors are expressed in picomolar level. One example of constitutive activity with lower expression level has been observed in CHO cell line expressing histamine H<sub>2</sub> receptors. Constitutive activity was apparent in a cell line with H<sub>2</sub> receptor B<sub>max</sub> of ~290 fmol/mg protein but not in a cell line with H<sub>2</sub> receptor B<sub>max</sub> of ~100 fmol/mg protein (Smnt et al. 1996). This lends support to the results of the current study, in that the 2 - 3-fold difference in the WT MT1 receptor expression level could promote the detection of constitutive activity.

Taken together, the region in the end of TM III of the human MT1 receptor containing amino acids asparagine N124, cysteine C127, and cysteine C130 is important for the coupling of the receptor to the G proteins. The cysteines, projecting to the intracellular cavity, probably form a part of the contact surface between the melatonin receptor, G proteins, and/or other associated signalling partners, and they are more important for the contact with some G protein subtypes than others. These amino acids are conserved in all members of the melatonin receptor family and it is likely that they also play an important role in the function of other melatonin receptor subtypes.

6.3. Future directions

The better understanding of how melatonin receptor is activated and which are the regions mediating G protein coupling awaits for future research. Melatonin is involved in a wide range of physiological functions and the functional role of each subtype has to be characterized in order to design subtype-selective ligands. Melatonin is currently used to treat disturbed circadian rhythms. Other potential therapeutic uses of melatonin include cancer therapy (Vijayalaxmi et al. 2007), treatment of seasonal affective disorder (SAD, Ippämäki et al. 2003), enhancement of immune function (Maestroni 1998), prevention of vascular dementia, and coordination of metabolism and intestinal motility (Delargrange et al. 2003). Models of melatonin receptors need to be upgraded and all the information about novel melatonergic ligands and their affinities need to be taken into consideration when building new models.
7. SUMMARY

The first rhodopsin-based model of melatonin receptor binding pocket was constructed. This model has served as a starting point for melatonin receptor mutagenesis studies by several research groups.

Melatonin receptor function was explored with a novel yeast based bioassay. The human MT1 melatonin receptor was shown to be able to directly couple to the yeast Saccharomyces cerevisiae endogenous G proteins and to activate yeast mating pathway. A simple functional colorimetric assay was developed in which a change in the colour of the yeast culture medium is the measure of receptor activation. Some differences in the effects of site-directed mutagenesis of the human MT1 melatonin receptor were found to exist between yeast and mammalian expression systems. These findings suggest that caution should be used in extrapolating results generated in yeast-based systems to the mammalian situation.

Site-directed mutagenesis studies revealed that a conserved histidine (H195, His546) in TM V is involved in ligand binding and function of the human MT1 melatonin receptor. This histidine was chosen to be the subject of mutagenesis studies because it is one of the important melatonin recognition sites of the rhodopsin-based receptor model described in this thesis. Two other melatonin recognition sites of the model were not found to be important for ligand binding or receptor activation in the MT1 receptor. The empirical studies thus proved that the theoretical model was not able to correctly identify the amino acids that form the binding pocket for melatonin.

Constitutive activation of the human MT1 melatonin receptor was not obtained with mutations in asparagine N124 (Asn140) or proline P253 (Pro558). Proline 253 was found to be important in maintaining the receptor in correct conformation, since mutating this amino acid severely impaired ligand binding and receptor signalling, and it also affected receptor trafficking. Mutations in N124 were also harmful for receptor function, receptor function was affected more in the mammalian expression system than in the yeast. The expression level of the wild-type MT1 receptor in stably transfected cell lines was found to correlate with the level of constitutive activity of the receptor.

Two conserved cysteines (C127, Cys58 and C130, Cys555) in the interphase between TM III and the second intracellular loop of the human MT1 melatonin receptor, immediately following the melatonin receptor fingerprint amino acid motif N-R-Y, were found to be involved in G protein coupling of the receptor. The cysteines are probably integral parts of the contact surface between the melatonin receptor and associated signalling partners.

The results from studies like this can provide us with a better understanding of the molecular basis of the biological functions regulated by melatonin and help building improved models of melatonin receptors.
8. REFERENCES


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References


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